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*RESEARCH ARTICLE*

# Radiolabeling, Characterization, and *In Vitro* Cell Culture Studies of Donepezil-Loaded Niosome Formulations For Brain Imaging

Emre OZGENC[*°], Hande AYDIN[**], Zeynep SENYIGIT[***], Evren ATLIHAN GUNDOGDU[****]

***Radiolabeling, Characterization, And In Vitro Cell Culture Studies of Donepezil-Loaded Niosome Formulations For Brain Imaging***

***SUMMARY***

*Dementia has posed a significant public health challenge for aging populations in developed countries for many years. Alzheimer's Disease (AD) is the most prevalent cause of dementia, marked by cognitive and behavioral irregularities resulting from cholinergic dysfunction, a progressive and irreversible neurodegenerative disorder. Restoring cholinergic neurotransmission can alleviate impaired cognitive and behavioral symptoms in AD patients. Donepezil (DNP) is an acetylcholinesterase inhibitor utilized to treat mild to moderate AD symptoms. Niosomes, non-ionic surfactant vesicles, present various benefits as drug delivery systems, including stability, non-immunogenicity, permeation potential, and controlled release ability. In this research, we prepared, characterized, radiolabeled with 99mTc, and evaluated DNP-loaded niosome formulations as radiopharmaceuticals for brain imaging agents. The findings suggest that this innovative radiopharmaceutical ([99mTc]Tc-formulation) has the potential to serve as a promising alternative imaging agent for neurological disorders.*

***Key Words:*** *Donepezil, Tc-99m, niosome, cytotoxicity, radiopharmaceutical*

***Beyin Görüntüleme İçin Radyolojik İşaretlenmiş Donepezil Yüklü Niozom Formülasyonlarının Karakterizasyonu ve In Vitro Hücre Kültürü Çalışmaları***

***ÖZ***

*Demans, uzun yıllardan beri gelişmiş ülkelerdeki yaşlanan nüfus için önemli bir halk sağlığı sorunu teşkil etmektedir. Alzheimer Hastalığı (AH), ilerleyici ve geri dönüşü olmayan bir nörodejeneratif hastalık olan kolinerjik fonksiyon bozukluğundan kaynaklanan bilişsel ve davranışsal düzensizliklerle karakterize edilen demansın en yaygın nedenidir. Kolinerjik nörotransmisyonun yeniden sağlanması, AD hastalarında bozulmuş bilişsel ve davranışsal semptomları hafifletebilir. Donepezil (DNP), hafif ila orta dereceli Alzheimer semptomlarını tedavi etmek için kullanılan bir asetilkolinesteraz inhibitörüdür. Niozomlar, stabilite, non-immunojenite, permeasyon potansiyeli ve kontrollü salım yeteneği gibi bir dizi avantaj sunan non-iyonik yüzey aktif madde vezikülleridir ve ilaç taşıma sistemleri olarak kullanılabilirler. Bu araştırmada, DNP yüklü niozom formülasyonları hazırlandı ve karakterize edildi. Daha sonra 99mTc ile radyoişaretlendi ve beyin görüntüleme ajanları olarak kullanılmak üzere değerlendirildi. Elde dilen bulgular sonucunda, bu yenilikçi radyofarmasötiğin ([99mTc]Tc-formülasyonun) nörolojik bozukluklar için umut vadeden alternatif bir görüntüleme ajanı olarak hizmet etme potansiyeline sahip olduğunu görüldü.*

***Anahtar Kelimeler:*** *Donepezil, Tc-99m, niozom, sitotoksisite, radyofarmasötik*

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## INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia, accounting for 70-80% of diagnosed cases of neurodegenerative brain disease (Pratik et al., 2021). It has emerged as a growing global health concern, quickly becoming one of the most expensive, lethal, and burdening diseases of this century (Van der Flier et al., 2023). The number of people worldwide living with dementia is predicted to reach 74.7 million in 2030 and 131.5 million in 2050 (Prince, 2015). The pathology of AD is characterized by the accumulation of misfolded proteins, inflammatory changes, and oxidative stresses, resulting in the loss of synaptic contacts and neuronal cell death (Ahmed et al., 2016). AD causes memory loss and deterioration of other cognitive abilities that interfere with daily life (Breijyeh & Karaman, 2020). The early signs of the disease include a decline in non-memory aspects of cognition, such as finding the right words, forgetting recent events, trouble understanding visual images and spatial relationships, and impaired reasoning or judgment. As the disease progresses, symptoms such as increased confusion, behavior changes, impaired mobility, hallucinations, and seizures become more severe. Although the disease's progression may differ for each individual, most people live between four and eight years after diagnosis (Lane et al., 2017; Rasmussen & Langerman, 2019). While there is no definitive cure for Alzheimer's disease, certain medications can temporarily slow the worsening of dementia symptoms. The United States Food and Drug Administration (FDA) has approved two types of drugs to treat the symptoms of Alzheimer's disease: Acetylcholinesterase (AChE) inhibitors [i.e., Donepezil (DNP), Rivastigmine, Galantamine] and N-methyl-D-aspartate (NMDA) antagonists (i.e., Memantine). DNP is a new class of AChE inhibitor having an N-benzyl piperidine and an indanone moiety that shows longer and more selective action for the treatment of mild to moderate AD (Sugimoto, 2001; Nayak et al., 2020). It is effective against the three major domains of AD symptoms: functional ability, behavior, and cognition, and can be administered orally or transdermally (Knowles, 2006). Early and accurate diagnosis of AD is fundamental for subsequent patient management. Early diagnosis enables patients to seek early intervention with symptomatic treatment, lifestyle changes to maintain quality of life, and risk-reduction strategies that can clinically reduce cognitive, functional, and behavioral decline (Porsteinsson et al., 2021). AD is diagnosed with physical and neurological exams, blood tests, mental status and neuropsychological testing, and brain imaging. Researchers are working on new brain imaging techniques to diagnose better and track the progress of AD. Brain imaging is a complex field that faces several challenges. With the increasing number of available imaging modalities, specialized agents are becoming more prevalent. These agents are designed to address the limitations of current imaging techniques and provide more accurate and detailed information about the brain. As a result, efforts towards developing and utilizing such agents are growing rapidly. Despite these advancements, there are still significant challenges to be overcome in daily clinical practice (Ali et al., 2016; Subramaniam, 2017). Brain SPECT agents are a type of diagnostic imaging agent that are used to examine the brain for various diseases. These agents include $^{99m}$TcO4-, [$^{99m}$Tc] DTPA, $^{201}$TI, and [$^{67}$Ga]citrate. They are not taken up by normal brain cells but can penetrate tumor cells due to the altered blood-brain barrier. This makes them useful for identifying brain diseases. On the other hand, SPECT perfusion agents such as [$^{123}$I] IMP, [$^{99m}$Tc]HMPAO, and [$^{99m}$Tc]ECD diffuse into the normal brain. These agents are fat-soluble and are effective in detecting various cerebrovascular diseases, including stroke, Parkinson's disease, Huntington's disease, epilepsy, dementia, and psychiatric disorders. In summary, brain SPECT agents and SPECT perfusion agents are both important tools for diagnosing brain diseases. Brain SPECT agents are useful for detecting diseases in the altered blood-brain barrier, while SPECT perfusion agents effectively detect cerebrovascular diseases in the normal brain (Ono et al., 2014; Yomo & Oguchi, 2017). However, newer

radiopharmaceuticals are needed. Niosomes are fascinating colloidal systems comprising synthetic surfactants that are non-ionic. These surfactants possess the unique ability to self-assemble and form bilayer structures that enclose one or more aqueous compartments within. This remarkable feature makes them ideal for targeted drug delivery applications, where precise and controlled release of therapeutic agents is required (Bragagni et al., 2014). They exhibit a wide range of desirable properties, including tunable vesicle size, high loading capacity, sustained drug release, targeted drug delivery, osmotic stability, ease of fusogenicity, and intracellular drug release (Keerthana et al., 2022). Niosomal formulations have prolonged circulation in the bloodstream, allowing for enhanced targeted action due to their non-ionic surfactants. They enable the targeted delivery of various types of drugs, thanks to their versatile structure (Umbarkar, 2021). Niosomes have also been suggested as a tool to deliver drugs to the brain due to the high level of cerebral glucose uptake (Bragagni et al., 2012). Niosomal formulations are among the most suitable systems for transporting the drug to the desired area. Research on radiolabeling studies with niosomal formulations for use in the diagnosis of diseases is becoming widespread. Developing an effective and feasible method for radiolabeling niosomal formulations will allow these drug delivery systems to be used with nuclear medicine imaging methods. In this study, we prepared and characterized DNP-loaded niosome formulations. These formulations were radiolabeled with $^{99m}$Tc and evaluated as radiopharmaceuticals for brain imaging agents.

## MATERIAL AND METHODS

The laboratory procured Cholesterol (Chol), Span 85, and Chloroform from Sigma-Aldrich in the United States, while the Nuclear Medicine Department of Ege University provided the Molybdenum-99 ($^{99}$Mo)/$^{99m}$Tc generator used to elute [$^{99m}$Tc] $TcO_4^-$. For cell culture experiments, Gibco Invitrogen in Grand Island, NY, supplied the necessary reagents and supplies. HT-22 was chosen and obtained from the American Type Culture Collection (ATCC) for cell culture studies. All other reagents were of analytical grade unless otherwise stated and obtained from commercial sources.

### Preparation of niosome formulations

The process of creating niosome formulations involved utilizing the thin-film hydration technique (TFH), (Arunothayanun et al., 2000; Nayak et al., 2020). The first step was dissolving Span 85 and cholesterol in 10 mL of chloroform within a round-bottom flask. The next step involved evaporating the chloroform under vacuum at 60°C using a rotary evaporator, which resulted in a thin film (Buchi Rotavapor®, B-100). Afterward, the thin film was hydrated for 45 minutes with PBS (pH 7.4) under rotation at 65°C (Akbarzadeh et al., 2021). Once hydrated, the formulation was sonicated using Bandelin Sonopuls® HD 2070. The formulations were then stored in the refrigerator at 4°C until use. For DNP-loaded formulations, 15 mg of the active agent was dissolved in chloroform along with Span 85 and cholesterol. More information about the composition and structure of the niosome formulations was shown in (Table 1).

**Table 1.** The composition of niosome formulations

| Formulation Code | Span 85: Cholesterol Ratio | Cholesterol (mg/mL) | DNP (mg/mL) |
|---|---|---|---|
| F1 | 1 | 15 | - |
| F2 | 1 | 30 | - |
| F3 | 1 | 45 | - |
| F4 | 0.5 | 15 | - |
| F5 | 0.5 | 30 | - |
| F6 | 0.5 | 45 | - |
| F3+DNP | 1 | 45 | 1.5 |

**Characterization of niosome formulations**

**Particle size, polydispersity index, and zeta potential**

At room temperature, the Malvern Zetasizer (Malvern Nano ZS, Malvern, UK) was used to determine the mean diameters, polydispersity index values, and Zeta potential of niosomes. The measurements were performed in triplicates, and the results were presented as the mean value ± SD.

**Viscosity studies**

The viscosity of formulations was measured using a sine-wave viscometer (Vibro SV-10) at a measurement range of 10–10.000 mPa·s. The viscosity was measured by placing the sample fluid between the sensor plates of the viscometer and measuring it at 25°C.

**Injectability studies**

The injectability of the niosome formulation was measured using the TA-XT Plus in compression mode and an injectability probe (Universal Syringe Rig) equipped with a 30 kg load cell. To conduct the test, a 2 mL syringe filled with the formulation was placed in the metallic support of the probe. A constant force of 0.5 N was applied to push the plunger. The force-time curves were used to calculate the work and force required to expel the formulation from the syringe. The area under the resulting curve was used to determine the work of expulsion (Rungseevijitprapa & Bodmeier, 2009). The study was performed at room temperature (25°C), and measurements were taken in triplicates. The results were presented as the mean value ± standard deviation.

**Encapsulation efficiency (EE)**

The encapsulation efficiency of the formulation was determined by using a dialysis bag (Spectrum Ltd., Shanghai, China) with 8000–12,000 Da of molecular weight. For that, 1 mL of formulation was put into dialysis bags. The samples were ultra-centrifuged at 5000 rpm at room temperature and filtered using a cellulose nitrate membrane. The concentration of donepezil was analyzed by spectrophotometry.

**Cell culture studies**

For cytotoxicity studies, the HT-22 cells were utilized. The cells were grown in a cell medium of DMEM, which was supplemented with 10% fetal bovine serum. To prepare the cell monolayers, 1 × 105 cells were seeded onto 96-well plates. The cell culture was maintained at 37°C, 90% humidity, and 5% $CO_2$.

***In vitro* cytotoxicity studies**

The purpose of the study was to determine the effect of different concentrations of DNP solutions on the cytotoxicity of HT-22 cells, which are similar to hippocampal neuronal cells. The HT-22 cells were cultured in DMEM, which was supplemented with 10% fetal bovine serum, 0.5 mg/mL glutamine/penicillin-streptomycin, and then incubated at 37°C with 5% $CO_2$. The experiment involved using the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay to analyze the cytotoxicity of DNP solutions with varying concentrations (0.05 mg/mL, 0.1 mg/mL, 0.5 mg/mL, 1.5 mg/mL, 2 mg/mL, 5 mg/mL) on HT-22 cells. To seed the cells, 1 × $10^6$ cells/well in 1 mL DMEM were added to 96-well flat-bottom plates. DNP was solubilized in DMEM (1 mg/mL) and then incubated at different concentrations (0.05 mg/mL, 0.1 mg/mL, 0.5 mg/mL, 1.5 mg/mL, 2 mg/mL, 5 mg/mL). The DNP solutions were added to the well plates and incubated for 24, 48, and 72 hours. After the incubation period, 100 µL of MTT solution (5 mg/mL) was added to each well and incubated at 37°C. Following that, the culture medium was removed, and 200 µL of dimethyl sulfoxide was added to dissolve the MTT formazan crystals. The absorbance was read at 570 nm using a microplate reader. Finally, the IC50 (the concentration that inhibits 50% of cell viability) was calculated using the "GraphPad Prism" and the "ne-site total binding" algorithm programs (Gundogdu et al., 2012).

**Radiolabeling studies**

To determine the best radiolabeling conditions, we conducted experiments with varying amounts of stannous chloride as the reducing agent. The formula-

tions were radiolabeled by reacting with [$^{99m}$Tc]$TcO_4^-$ at room temperature (25°C) and pH 7.0. We added different amounts of the reducing agent solution (50, 250, and 1000 μg $mL^{-1}$ stannous chloride in distilled water) to the formulations dispersed in 0.5 mL of 0.9% sodium chloride solution (SF). We then carried out the radiolabeling process using freshly eluted 37 MBq/0.1 mL [$^{99m}$Tc]$TcO_4^-$ and shook the resulting radiolabeled formulations for 60 seconds before incubating them for 15 minutes. To analyze the radiolabeling efficiency of the formulations, we used Radio Thin Layer Chromatography (RTLC) (Gundogdu et al., 2015; Karpuz et al., 2021). Additionally, we radiolabeled the blank formulation using the same procedure and used it as a control.

**Quality Control by Radio Thin Layer Chromatography (RTLC)**

We conducted RTLC to verify the effectiveness of the labeling process of the formulations. The experiment involved using 2 μL of the labeled formulations and acetone (Sigma-Aldrich) as the mobile phase on Whatman paper no. 1. The RTLC was performed for 15 minutes. Afterward, we analyzed the radioactivity of the strips using a TLC scanner (Bioscan AR 2000).

***In vitro* stability studies**

An evaluation was conducted to determine the stability of [$^{99m}$Tc]Tc-formulations in cell medium (McCoy's 5A supplemented with 10% fetal bovine serum). To conduct the experiment, 3.7 MBq of [$^{99m}$Tc]Tc-formulations were mixed with 0.4mL SF and incubated at 37°C. Radiolabeling efficiency studies were conducted during the experiment to determine the stability of the mixture.

**RESULTS AND DISCUSSION**

The results of this study highlight the potential of niosomes as a valuable tool for treating and imaging brain diseases, including AD. The ability of niosomes to cross the blood-brain barrier and deliver drugs to the central nervous system is significant in improving therapeutic outcomes. Niosomes can enhance the solubility and stability of pharmaceutical molecules, allowing for better drug delivery and controlled release. This is particularly important in the case of brain diseases where the blood-brain barrier is a significant obstacle to drug delivery. DNP-loaded niosome formulations have been developed as potential nanocarriers for nuclear imaging techniques. The study confirms that the radiolabeled formulations have favorable radiolabeling efficiency and stability, showing promise as radiolabeled nanocarriers for use in nuclear imaging and further studies.

**Preparation and characterization of niosome formulations**

Niosomes are a type of vesicular drug delivery system mainly composed of non-ionic alkyl or polyglycerol etheric surfactant and cholesterol in an aqueous medium. These drug delivery systems are formulated with non-ionic amphiphiles in specific aqueous solutions using self-assembly technology to create the vesicles.

Niosomes have emerged as a safe and effective option for drug delivery, primarily due to their composition of biocompatible, biodegradable, non-toxic, and non-immunogenic materials. This makes them ideal for use in numerous clinical applications. Niosomes offer several advantages, such as stability, low cost, ease of formulation, and scale-up potential. They can effectively encapsulate hydrophilic, lipophilic, and amphiphilic drugs, allowing for controlled and sustained release of drugs. Furthermore, niosomes have a higher bioavailability than conventional dosage forms and can protect drugs from biological enzymes and acids (Verma et al., 2023). They also show great promise in targeting drugs to the central nervous system and overcoming the blood-brain barrier (Gharbavi et al., 2018). Overall, niosomes represent an excellent option for drug delivery in the clinical setting, with the potential for significant therapeutic benefits. Niosomes have emerged as a promising drug delivery system for treating neurological disorders, such as epilepsy, seizures, trauma, Parkinson's disease, multiple sclerosis, dementia, and AD. They can potentially

reduce AD pathogenesis and offer a more stable and beneficial drug delivery system for the brain.

Various methods have been used to produce niosomes, such as sonication, ether injection, microfluidization, TFH, and bubble technique. The TFH technique is a straightforward and practical approach for niosome preparation. In this method, surfactants and cholesterol are dissolved in an organic solvent within a round-bottom flask. Subsequently, the organic solvent is removed through rotary evaporation and vacuum to form a thin layer on the inner surface of the flask. Then, a solution, such as water or PBS, is introduced, and the dried layer is rehydrated above the surfactant's transition temperature to form niosomes (Zinatloo-Ajabshir & Zinatloo-Ajabshir, 2019). Niosomes can be either uni-lamellar or multi-lamellar vesicles composed of non-ionic surfactants, cholesterol, and ionic surfactants used to prevent formulation aggregation. We prepared niosome vesicles with non-ionic surfactant and cholesterol. Surfactant (Span 85) and cholesterol ratios were selected as 0.5:1 and 1:1, and the lipid-to-DNP ratio was 10-30.

Cholesterol is a vital component in maintaining the stability and rigidity of niosomes. However, an excessive amount of cholesterol can lead to reduced fluidity in vesicles, impeding the penetration and permeability of drugs (Umbarkar, 2021). By adjusting the ratio of cholesterol to surfactant, one can effectively study the influence of cholesterol on factors such as nanoparticle size, zeta potential, size distribution, and entrapment efficiency (Agarwal et al., 2004). Within (Table 2), the effects of varying ratios of cholesterol to surfactant on the size and zeta potential values of niosomes are displayed. By adjusting this ratio, niosomes with a range of particle sizes can be generated. Notably, the F3 formulation exhibited the lowest particle size and polydispersity index value compared to the other niosomes. This finding suggests that F3 is the ideal option for additional investigation.

The PDI measures the degree of uniformity in particle size. It is calculated as the ratio of the standard deviation to the mean particle size. A PDI value of 0.3 or less indicates a high degree of uniformity in particle size. This metric is an important consideration in various fields, such as pharmaceuticals, material sciences, and biotechnology, where particle size uniformity is crucial to product performance and efficacy. The PDI value of niosome formulations was measured as 0.304 ± 0.040, indicating a uniform particle size distribution (Shadab et al., 2014; Asmari et al., 2016; Yasir et al., 2017). The characterization of the DNP-loaded niosome formulation demonstrated a uniform particle size of 199.30±4.86 nm, which is considered ideal for niosome formulations. It was observed that the incorporation of the active ingredient (DNP) into the niosome nucleus did not significantly change the particle size but led to an increase in particle distribution relative to blank niosome particles. The increase in PDI value was significant ($p < 0.05$) compared to blank niosomes, however, the PDI value is still around 0.3 which shows a high degree of uniformity.

The zeta potential magnitude indicates the colloidal system's potential stability. When all suspended particles have a significantly positive or negative zeta potential, they repel each other, preventing particle aggregation. Conversely, if the particles have low zeta potential values, there is no force to impede their tendency to aggregate. The importance of the zeta potential is that its values can be correlated with the electrophoretic movement of the particles and the stability of the formulation. Typically, particles with zeta potentials above +30 mV or below -30 mV are generally considered stable. The zeta potential value provides information about the charge on the niosomes. A sufficiently high zeta potential value within the range of +30 mV to -30 mV contributes to stability by preventing particle aggregation (Shadab et al., 2014; Asmari et al., 2016; Yasir et al., 2017). The zeta potential value of F1-F6 are presented in Table 2 and formulations F1-F6 exhibited a negative charge ranging from (-31.8) to (-33.8). The zeta potential values of F3 and F3+DNP were found to be -32.6 ±

0.862 mV and -15.4 ± 0.656 mV, respectively. It can be seen that DNP incorporation into niosomes caused a significant increase in zeta potential that can be attributed to the preferential adsorption of counter ions or hydrogen ions on the niosome shell with the addition of an active substance. (Behbahani et al., 2017). Also, results showed that Zeta potential values didn't change significantly by changing cholesterol to surfactant ratios. The optimized niosome formulation demonstrated a uniform particle size of 199.30±4.86 nm, a PDI value of 0.304 ± 0.040, and a zeta potential value of -15.4 ± 0.656 mV, indicating good stability.

**Table 2.** Particle size, polydispersity index, and zeta potential values of formulations

| Formulation Code | Particle size (d.nm) ± SD | Polydispersity Index ± SD | Zeta Potential (mV) ± SD |
|---|---|---|---|
| **F1** | 318.10 ± 3.960 | 0.210 ± 0.008 | -33.6 ± 2.166 |
| **F2** | 259.10 ± 3.470 | 0.202 ± 0.041 | -33.8 ± 2.307 |
| **F3** | 198.70 ± 9.407 | 0.148 ± 0.016 | -32.6 ± 0.862 |
| **F4** | 216.90 ± 9.756 | 0.183 ± 0.096 | -31.8 ± 1.686 |
| **F5** | 227.30 ± 7.778 | 0.159 ± 0.060 | -32.6± 1.069 |
| **F6** | 235.80 ±5.798 | 0.149 ± 0.044 | -34.2 ± 2.574 |
| **F3+DNP** | 199.30± 4.860 | 0.304 ± 0.040 | -15.4 ± 0.656 |

**pH Measurements**

It is crucial to maintain the appropriate pH value in parenteral formulation to ensure its safety and efficacy. For intravenous (i.v.) and intramuscular injections, the target pH should be as close as possible to the physiological pH, within a range of pH 2-11. For subcutaneous injection, the acceptable range is pH 4-9 due to potential irritation issues. The pH value of the F3 niosome formulation has been measured as 6.94 ± 0.07, which falls within the safe range for i.v. administration. Therefore, it can be concluded that the F3 niosome formulation is safe to use for i.v. administration.

**Viscosity studies**

Viscosity plays a crucial role in ensuring the smooth flow and distribution of drugs during the injection process. Therefore, appropriate viscosity is essential for parenteral formulations. The viscosity of the DNP-loaded niosome formulation and serum physiological (SP) were determined to be 0.820 ± 0.226 cP and 0.850 ± 0.212 cP, respectively. The results showed that there was no statistically significant difference between the viscosity of the niosome formulation and SP. This indicates that the niosome formulation exhibits similar viscosity characteristics to SP, which is an important aspect to ensure the smooth flow and distribution of the drug during the injection process.

**Injectability study**

Injectability is an important aspect to consider when optimizing the usability and patient comfort of parenteral formulations. It refers to the ability of a formulation to be successfully administered by a syringe. To test the injectability of the niosome formulation, it was compared with serum physiological (SP) at 25°C. The work required to expel niosome formulation from the syringe was determined to be 7.772 ± 1.167 N.sec, and it was found to be statistically similar to the injectability of SP, as seen in (Table 3) and (Figure 1). The results of the injectability studies were found to be in accordance with the results of the viscosity studies. This indicates that the injectability values of the niosome formulation are suitable for parenteral administration, ensuring successful and comfortable receipt of the drug.

**Table 3.** The injectability value of formulations

| Formulation | Stiction (N) | Plateau Force (N) | Work (N.sec) |
|---|---|---|---|
| **F3 + DNP** | 3.84 ±0.11 | 2.58±0.40 | 7.772±1.167 |
| **Serum Physiological** | 4.22±1.09 | 2.06±0.56 | 7.145±0.986 |

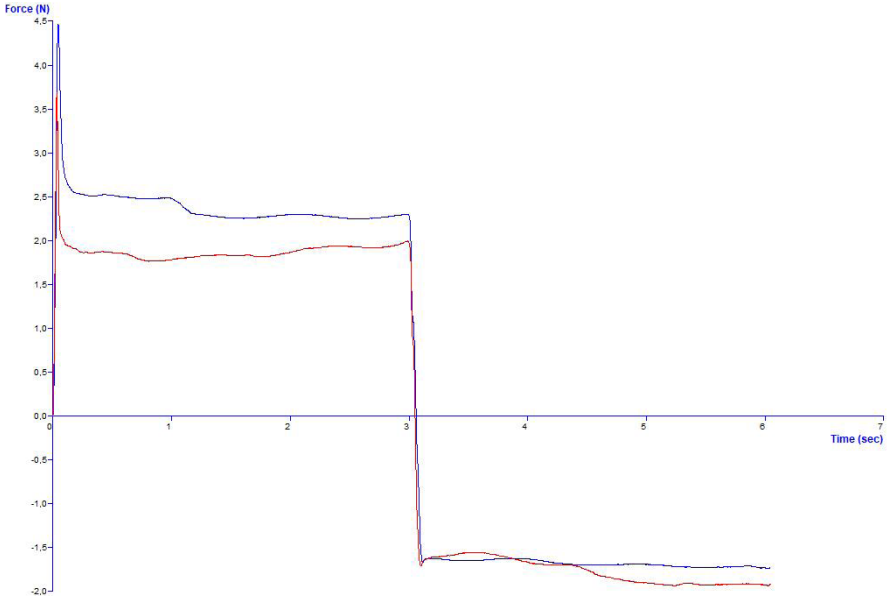


**Figure 1.** Force time profiles from injectability testing using TPA (Blue line: F3+DNP, Red line: SP)

**Encapsulation efficiency (EE)**

The calibration curve of the active ingredient DNP is shown in Figure 2.

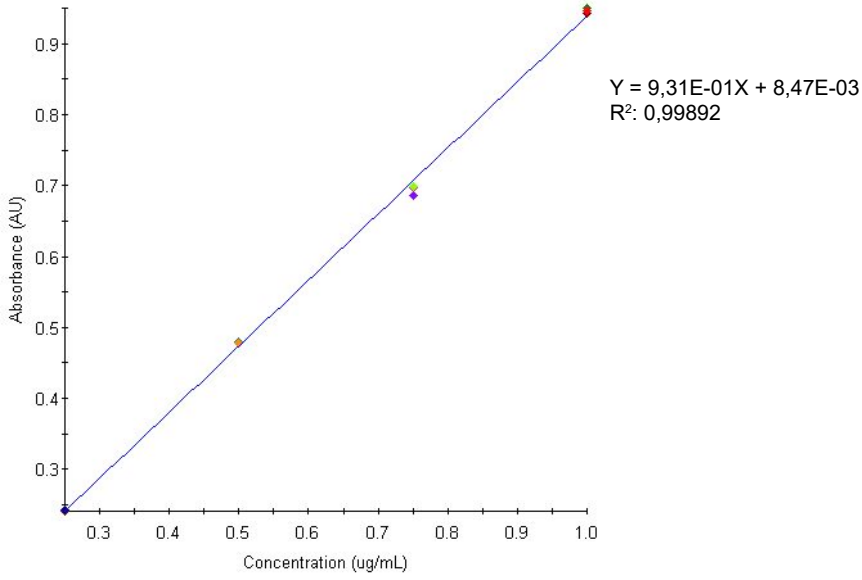


**Figure 2.** Calibration curve of donepezil

The encapsulation efficiency of donepezil in the optimized formulation (F3) was found to be 80%. The precise management of a drug delivery system hinges upon the EE of the formulation. The desired EE values are crucial in avoiding dose-dependent side effects, which ultimately enhance patient adherence to treatment (Amasya et al., 2019). According to this study, the developed formulation successfully attained the intended EE value.

**Radiolabeling studies**

[$^{99m}$Tc]$TcO_4^-$ is a highly useful radioactive tracer that is generated by the 99Mo/99mTc generator. However, in its stable form, it exists as a pertechnetate in the +7 oxidation state, which makes it unable to bind to any compound directly. To facilitate its binding to a pharmaceutical compound, reducing agents are employed to convert it to a more reactive +4/+5 oxidation state (Elitez et al., 2018; Wu et al., 2020). Among the commonly used reducing agents for [$^{99m}$Tc]$TcO_4^-$ radiopharmaceuticals are stannous salts. For this study, stannous chloride was utilized as a reducing agent to label the niosome formulation with [$^{99m}$Tc]$TcO_4^-$ directly. The study examined the impact of different amounts of stannous chloride (50, 250, and 1000 µg $mL^{-1}$) on the radiolabeling process. The results, which demonstrate the radiolabeling efficiency of the niosome formulation, are presented in (Figure 3).

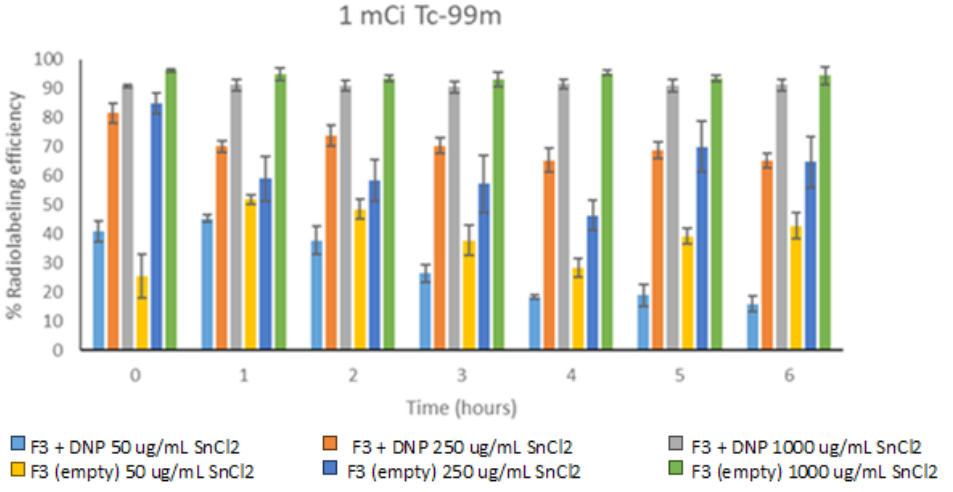


**Figure 3.** Radiolabeling efficiency % of formulations in different stannous chloride concentrations

**In vitro stability studies**

Cold kits are dissolved in SF and labeled with [$^{99m}$Tc]$TcO_4^-$ in nuclear medicine before being administered to patients. Likewise, in cell culture studies, radiolabeled formulations are incubated with a cell medium for a designated time. Hence, it's crucial to examine the stability of radiolabeled formulations in the cell medium. (Figure 4) illustrates the outcomes of this examination.

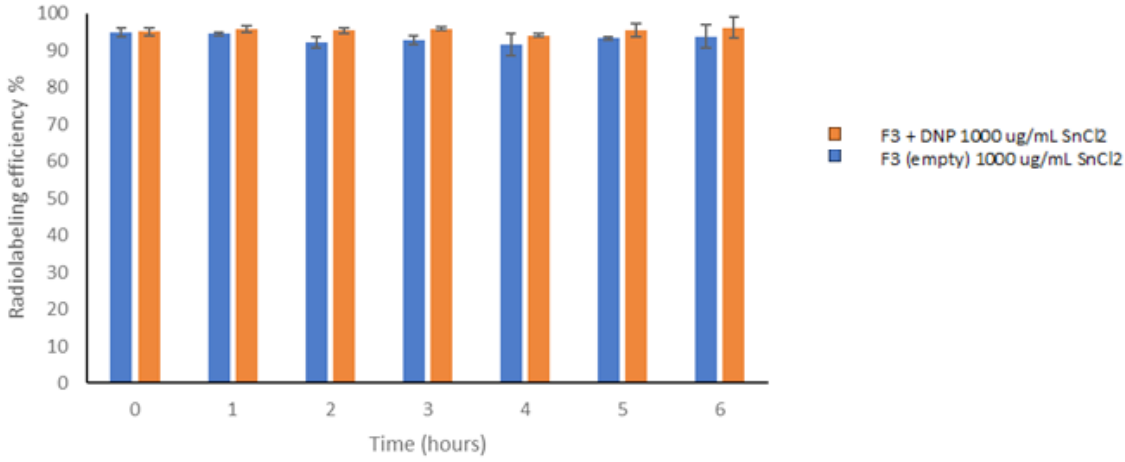


**Figure 4.** *In vitro* stability of radiolabeled formulations

Based on the findings displayed in Figure 3, the radiolabeling efficiency of the formulations remained consistent for up to 6 hours after the radiolabeling process. These results strongly suggest that the in vitro stability and labeling efficiency are well above 90% while in a cell medium. With this level of stability, the [$^{99m}$Tc]Tc-formulations can serve as an imaging agent for a minimum of 6 hours after preparation.

***In vitro* cytotoxicity studies**

The objective of this study was to examine the cytotoxicity of DNP solutions with varying concentrations (0.05 mg/mL, 0.1 mg/mL, 0.5 mg/mL, 1.5 mg/mL, 2 mg/mL, and 5 mg/mL) on HT-22 cells. As shown in (Figure 5), the DNP solution with a concentration of 1.5 mg/mL displayed cell viability of over 95%, while the other concentrations indicated over 85% cell viability after 72 hours of treatment. This suggests that the DNP solution with a concentration of 1.5 mg/mL is less toxic than the other concentrations. Using GraphPad Prism, the IC50 value of DNP for HT-22 cells was calculated and determined to be 24.54 ± 1.44 µM (Figure 6). These results suggest that DNP concentrations equal to or less than 1.5 mg/mL and below the IC50 value applied to HT-22 cells are unlikely to cause toxicity. Therefore, it can be considered safe and effective as a drug delivery system, making it a suitable option for administration.

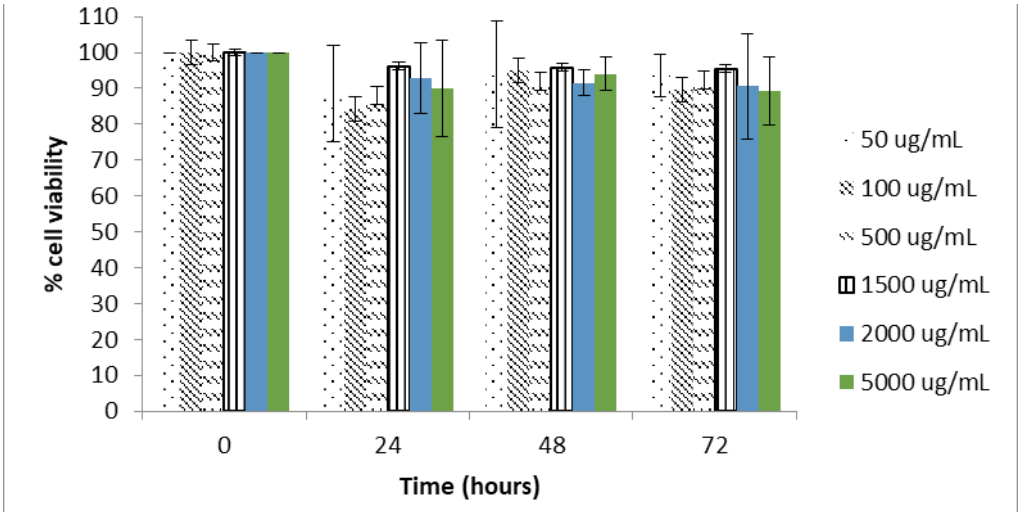


**Figure 5.** Cell viability % of DNP solution at different concentrations

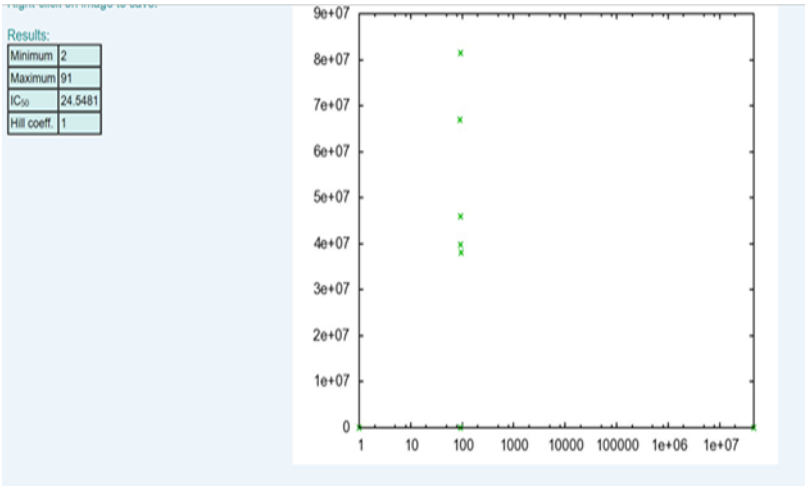


**Figure 6.** IC50 value of DNP in DMEM (IC50 (24,54 μM)

## CONCLUSION

The purpose of this investigation was to create a novel radiopharmaceutical capable of imaging various regions of the brain. To accomplish this, DNP HCl niosomes were fabricated using the TFH technique, and different surfactant/cholesterol ratios (0.5, 1) were evaluated. The cholesterol-to-surfactant ratio was identified as a crucial factor in determining the size, zeta potential, and size distribution of the niosomes. Following multiple trials, the F3 niosome was selected as the optimal formulation and radiolabeled with $^{99m}$Tc. The labeled compounds remained stable for up to 6 hours at room temperature and during experimentation in cell media. The highest radiolabeling efficiency was achieved when the formulations included 1000 μg of stannous chloride and 37 MBq [$^{99m}$Tc] $NaTcO_4^-$ at pH 7.0 with a 15-minute incubation time.

In summary, the newly developed radiopharmaceutical, [$^{99m}$Tc]Tc-formulation, displays potential as an alternative imaging agent. Additionally, the findings propose that niosomes could be a promising factor for the treatment and imaging of brain diseases, particularly Alzheimer's disease. Further research and development in this field are necessary to examine the full potential of niosomes and the utilization of [$^{99m}$Tc] Tc-formulation as an effective and targeted delivery system and imaging agent for neurological disorders.

## AUTHOR CONTRIBUTION STATEMENT

Conception (EO, HA, ZS, EAG), Design (EO, HA, ZS, EAG), Data collection (EO, HA, ZS, EAG), writing manuscripts (EO, HA, ZS, EAG), supervision (EO, HA, ZS, EAG)

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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*RESEARCH ARTICLE*

# Theoretical Rationale for the Combined Use of Gabapentin and Fingolimod for the Treatment of Multiple Sclerosis Using *In Silico* Methods

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***Theoretical Rationale for the Combined Use of Gabapentin and Fingolimod for the Treatment of Multiple Sclerosis Using In Silico Methods***

***SUMMARY***

*Chronic neuropathic pain in multiple sclerosis is found in 25-90 % of patients. Optimal pharmacotherapy should include both disease-modifying agents and medications that affect neuropathic pain. Taking into account that the development of multiple sclerosis is based on a chronic demyelinating inflammatory process (foci of demyelination), the rational treatment of relapsing-remitting multiple sclerosis should include both disease-modifying therapy and symptomatic therapy, in particular, medications affecting neuropathic pain. The study showed that under physiological conditions, ionic interaction between fingolimod and gabapentin is possible with the formation of salts capable of reversible dissociation without changing the molecule's structure, and with the formation of the corresponding protonated forms. The acid-base properties of the compounds were analyzed using the ACD/pKaDB and ChemAxon programs. At the pharmacokinetic level, no interaction is expected between finngolimod and gabapentin as they use different transport systems, and different metabolic enzymes. Fingolimod and gabapentin differ significantly in the extent of plasma proteins binding, which excludes their interaction during absorption, distribution, metabolism, and excretion. Therefore, the synergistic combination of fingolimod and gabapentin can be a promising therapeutic alternative for the effective treatment of multiple sclerosis. Its positive additive effects are expected to relieve symptoms of the disease, reduce the intensity of inflammatory processes in the central nervous system, produce a neuroprotective effect, contribute to remyelination due to the action of fingolimod, and relieve symptoms of neuropathic pain under the influence of gabapentin.*

***Key Words:*** *Multiple sclerosis, combination therapy, fingolimod, gabapentin.*

***Multipl Skleroz Tedavisinde Gabapentin ve Fingolimodun Birlikte Kullanım Olasılığının In Silico Metotlarla Teorik Olarak Doğrulanması***

***ÖZ***

*Multipl sklerozda kronik nöropatik ağrı hastaların %25-90'ında bulunur. Optimal farmakoterapi, hem hastalığı değiştirici ajanları hem de nöropatik ağrıyı etkileyen ilaçları içermelidir. Multipl skleroz gelişiminin kronik demiyelinizan inflamatuvar sürece (demiyelinizasyon odakları) dayandığı göz önüne alındığında, tekrarlayan-düzelen multipl sklerozun rasyonel tedavisi, hem hastalığı modifye edici tedaviyi hem de semptomatik tedaviyi, özellikle nöropatik ağrıyı etkileyen ilaçları içermelidir. Çalışma, fizyolojik koşullar altında fingolimod ve gabapentin arasındaki iyonik etkileşimin, molekülün yapısını değiştirmeden geri dönüşümlü ayrışma yeteneğine sahip tuzların oluşumu ve karşılık gelen protonlanmış formların oluşumu ile mümkün olduğunu gösterdi. Bileşiklerin asit-baz özelliklerinin analizi ACD/pKaDB ve ChemAxon programları kullanılarak yapıldı. Sonuçlarımız, fingolimod ve gabapentin için fizyolojik koşullar altında, molekülün yapısını değiştirmeden ters ayrışma yeteneğine sahip tuzların oluşumu ve karşılık gelen protonlanmış formların oluşumu ile iyonik etkileşimin mümkün olduğunu ortaya koydu. Farmakokinetik düzeyde, fingolimod ve gabapentin arasında herhangi bir etkileşim beklenmemektedir; çünkü bunlar ortak taşıma sistemlerini ve metabolik enzimleri kullanmazlar, ayrıca emilim, dağılım, metabolizma ve eliminasyon sırasındaki etkileşimleri hariç tutan değişen derecelerde protein bağlamaya sahiptirler. Bu nedenle fingolimod ve gabapentinin sinerjistik kombinasyonu, multipl sklerozun etkili tedavisi için umut verici bir terapötik alternatif olabilir. Olumlu katkı etkilerinin hastalığın semptomlarını hafifletmesi, merkezi sinir sistemindeki inflamatuvar süreçlerin yoğunluğunu azaltması, nöroprotektif etki oluşturması, fingolimodun etkisine bağlı olarak remiyelinizasyona katkıda bulunması ve gabapentin etkisi altında nöropatik ağrı semptomlarını hafifletmesi beklenmektedir.*

***Anahtar Kelimeler:*** *Multipl skleroz, kombinasyon tedavisi, fingolimod, gabapentin.*

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## INTRODUCTION

Multiple sclerosis (MS) is the the most prevalent chronic inflammatory disease of the central nervous system (CNS), and is one of the leading causes of disability, especially among young adults of working age. Up to 80 % of all MS patients have a relapsing-remitting MS phenotype (Walton et al., 2020). Neuropathic pain may develop in many pathologies, including metabolic disorders (Nefedov & Kalbus, 2022), mechanical damage to the peripheral nervous system (Burlaka, Belenichev, Nefedov, Aliyeva, & Bukhtiyarova, 2020), inlammation, and autoimmune processes (Duffy, Lees, Perera, & Moalem-Taylor, 2018), and its therapy is not always effective because it must affect several parts of this pathological process. Lesions of the peripheral and central nervous systems are leading factors in the development of neuropathic pain in patients with MS. Inflammation is one of the most essential factors in the development of neuropathy, and its complex pharmacotherapy should include an anti-inflammatory component (Duffy et al., 2018; Nefedov & Kalbus, 2022). Central sensitization in MS is based on increased activation of N-methyl-D-aspartate (NMDA) receptors. Glutamate plays a critical role in the toxic neuronal and myelin damage in MS. Glutamate is formed in large quantities in the brain under the influence of proinfammatory cytokines (interleukin-1β, TNF-α) and encephalitogenic T-lymphocytes, which are induced in the central nervous system in MS and anti-NMDAR encephalitis (Gulec et al., 2020; Sinari et al., 2020). The concentration of glutamate in the cerebrospinal fluid increases, and the activity of enzymes responsible for the degradation of glutamate decreases during the period of exacerbation of MS (Huang et al., 2020). Suppression of inhibitory reactions mediated by glycine and Gamma-aminobutyric acid (GABA) plays an essential role in the mechanisms of formation of aggregates of hyperactive neurons in the structures of the CNS (Cawley et al., 2015). Chronic neuropathic pain in MS is found in 25-90 % of patients; therefore, rational pharmacotherapy of MS should include both disease-modifying agents and medications that affect neuropathic pain (Nefodov et al., 2018).

Essential antinociceptive therapy for pain in MS includes analgesics, anti-inflammatory medications, long-acting prostaglandins; and membrane stabilizing medications, which reduce neuronal excitability and ephaptic transmission (Racke, Frohman, & Frohman, 2022). *In vivo* studies demonstrated that GABA-A and GABA-B agonists and modulators ıncreased preservation of myelinated sensitive fibers, and diminished axonal damage in the CNS. Further, decreased mononuclear inflammatory infiltration, pro-inflammatory cytokines reduction, and reduced levels of reactive oxygen species were also reported (Stamoula et al., 2023). Thus, GABA modulators, especially Gabapentin, can be considered promising agents for combination therapy of pain in MS.

Gabapentin is efficacious in numerous clinical studies, case reports, and chart reviews in a variety of neuropathic pain syndromes of central origin (Nicholson, 2001).

Among the wide range of disease-modifying medications for treating MS, oral forms are often preferred. In particular, Fingolimod, the first oral disease-modifying drug, is commonly used to treat active MS. The mechanism of action of Fingolimod is to affect the function of leukocytes through the sphingosine-1-phosphate signaling system (Bennett et al., 2004). Fingolimod is effective in experimental autoimmune encephalomyelitis, an animal model of MS; and it was subsequently investigated in two phase III clinical trials in relapsing-remitting MS. These studies demonstrated that Fingolimod is a safe and effective medication (Ayzenberg, Hoepner, & Kleiter, 2016). It was also found that in a model of convulsive seizures, Fingolimod produced a positive effect on the GABA system and increased the concentration of GABA in the CNS (Abd El-Kader, Moursi, Khaleefa, Noureldin, & Shoala, 2021). All this suggests the possibility of a rational combination of gabapentin and fingolimod.

Given the potential benefits of combination therapy with gabapentin and fingolimod for patients with MS, the purpose of the study was to assess the possibility of combined use, effectiveness, and safety of gabapentin and fingolimod in the treatment of relapsing-remitting MS.

## MATERIAL AND METHODS

Potential chemical reactions of the compounds were predicted based on the presence and reactivity of the functional groups that are part of their structure.

The acid-base properties of the drugs were analyzed using ACD/pKaDB program (ACD/pKaDB Web site, 2001), and ChemAxon software (ChemAxon Web site, 2010). Molecular weight, lipophilicity (logP and logD), and solubility were calculated by additive methods of these programs. Biological targets (receptors, enzymes, and transporters) were obtained from the relevant sources and databases DrugBank, PubChem (PubChem, 2001). Available data on the probability of interaction of the compounds with one or another isoform of cytochrome, transporters, or pharmacological targets were calculated by the admetSAR program and are freely available on the website https://www.drugbank.ca/.

## RESULTS AND DISCUSSION

The ability of fingolimod and gabapentin to interact with the specific enzyme systems involved in the transport and metabolism of drugs was predicted with the help of the admetSAR program on the website https://www.drugbank.ca/ (Table 1.) Accurate data on the involvement of specific enzyme systems in the metabolism of fingolimod were also included in the analysis (Lipinski, Lombardo, Dominy, & Feeney, 2001).

In the structures of fingolimod and gabapentin, potential reaction centers were identified, namely functional groups for further analysis of the possibility of physicochemical interactions (Figure 1.), as well as protolytic forms depending on pH with the corresponding physicochemical parameters (ionization constant, logP and logD, solubility, etc.) (Figures 2 and 3; Tables 2 and 3.). The balance between the benefits of combined pharmacological action and the potential risk of side effects and incompatibilities determines the appropriateness and possibility of combination therapy.

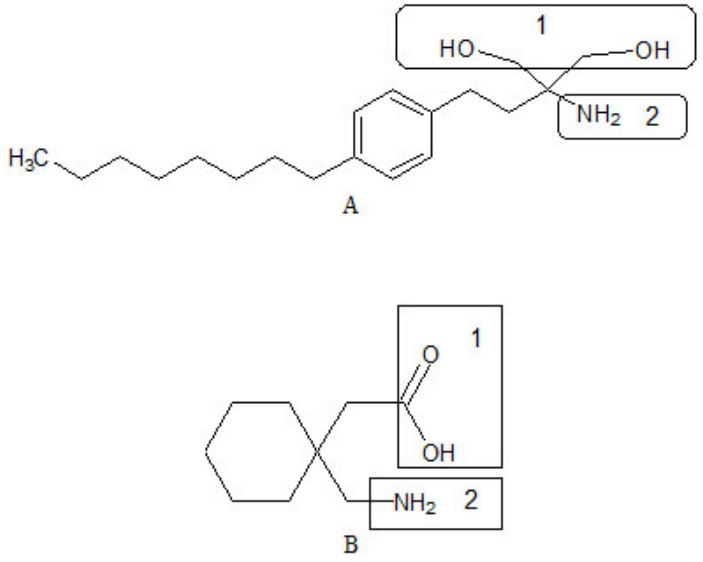



**Figure 1.** Structural formulas of fingolimod (A) and gabapentin (B) with specific functional groups.

**Table 1.** Predicted properties of fingolimod and gabapentin for interaction with specific enzyme systems.

| Property | Fingolimod | | Gabapentin | |
|---|---|---|---|---|
| | Value | Probability | Value | Probability |
| Absorption in the gastrointestinal tract | + | 0.9884 | + | 0,941 |
| Permeability through GEB | + | 0.5779 | + | 0.9382 |
| Substrate Pgp | Substrate | 0.6975 | Not a substrate | 0.6557 |
| Inhibitor Pgp I | Not an inhibitor | 0.9505 | Not an inhibitor | 0.9789 |
| CYP450 2C9 substrate | Not a substrate | 0.8251 | Not an inhibitor | 0.7982 |
| CYP450 2D6 substrate | Not a substrate | 0.6702 | Not a substrate | 0.893 |
| CYP450 3A4 substrate | Not a substrate | 0.7685 | Not a substrate | 0.8124 |
| CYP450 1A2 substrate | Inhibitor | 0.5519 | Not a substrate | 0.7612 |
| CYP450 2C9 inhibitor | Not an inhibitor | 0.8526 | Not an inhibitor | 0.9409 |
| CYP450 2D6 inhibitor | Inhibitor | 0.6567 | Not an inhibitor | 0.9273 |
| CYP450 2C19 inhibitor | Not an inhibitor | 0.8152 | Not an inhibitor | 0.9418 |
| CYP450 3A4 inhibitor | Not an inhibitor | 0.7348 | Not an inhibitor | 0.9547 |

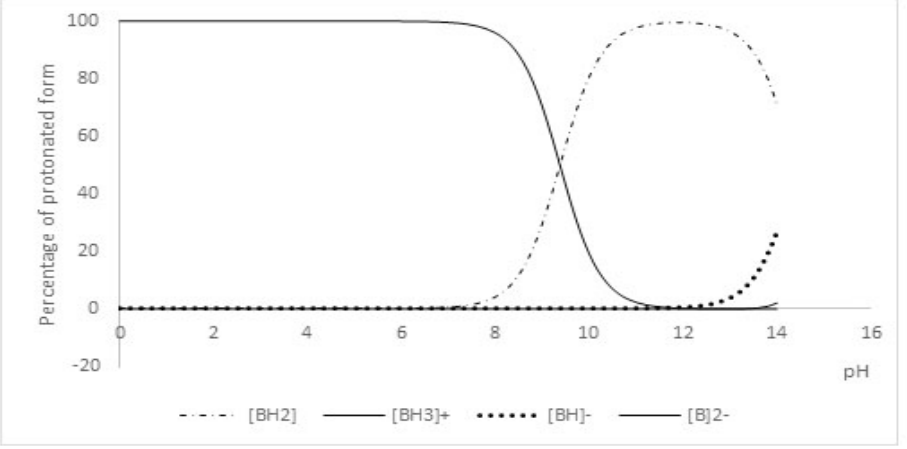
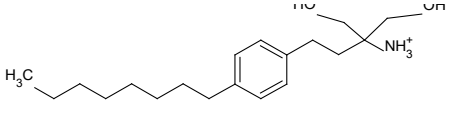
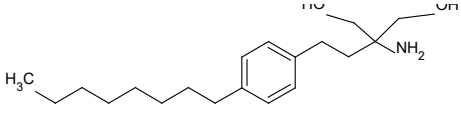
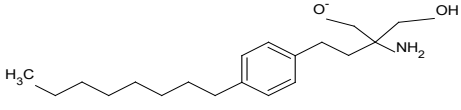
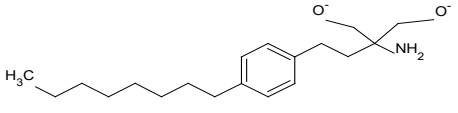



**Figure 2.** Percentage correlation between protonated forms of fingolimod depending on pH.

**Table 2.** Protolytic forms of fingolimod and calculated physicochemical parameters.

| Protolytic form | Physicochemical parameters | | | | |
|---|---|---|---|---|---|
| | pKa | LogP (LogD) | Solubility, g/L | Proton donors | Proton acceptors |
|  $[BH_3]^+$ | 8.7±0.3 | 2.15±1.0 | 8.68 | 3 | 0 |
|  $[BH_2]$ | – | 5.25±0.61 | 0.015 | 2 | 1 |
|  $[BH]^-$ | 12.2±0.2 | 1.15±1.0 | 0.012 | 1 | 2 |
|  $[B]^{2-}$ | 13.3±0.2 | 0.8±0.3 | 0.11 | 0 | 3 |

Note: * – calculated for non-ionized form.

The theoretical basis for substantiating this medication combination is to analyze possible interactions at different levels: the pharmaceutical, pharmacokinetic, and pharmacological. Interactions at the pharmaceutical level determine the possibility of creating a stable and effective combination medication. The pharmacokinetic level takes into account the characteristics of absorption, distribution, metabolism, and excretion of administered medications to propose optimal pharmacotherapy regimens. Potential positive and negative effects of medication interaction at the levels of receptors, enzymes, and systems are studied at the pharmacological level. All of the aspects mentioned above determine the need for a step-by-step analysis of possible interaction between fingolimod and gabapentin.

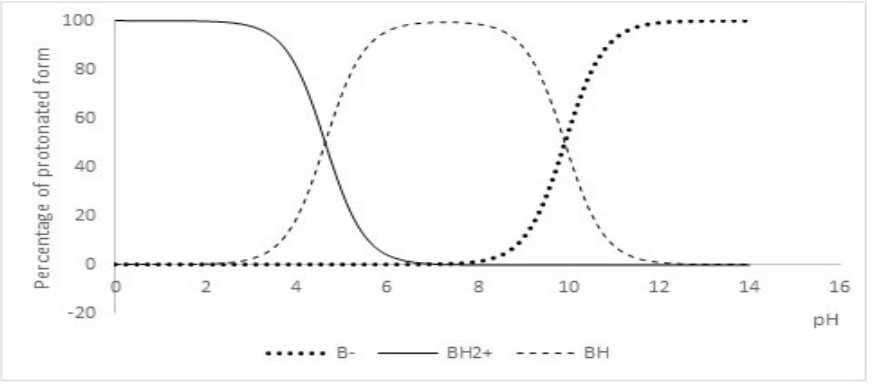


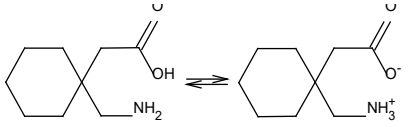
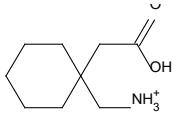
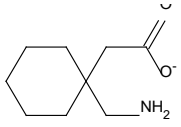

**Figure 3.** Percentage correlation between protonated forms of gabapentin depending on pH.

**Interactions at the pharmaceutical level**

Pharmaceutical interaction is due to a set of properties of compounds, and their possibility can be characterized based on the presence and reactivity of functional groups and physicochemical parameters (theoretically calculated, or empirically determined), such as ionization constants, tendency to salt and complex formation, solubility and others. The molecule of fingolimod has two types of functional groups: primary alcohol hydroxy groups (1) and primary amino group (2) (Figure 1A). Under physiological conditions, only amino groups can be reversibly ionized, and can form salts, whereas hydroxy groups form alkoxy anions only at highly alkaline pH.

Salt formation, while hydroxy groups form alkoxy anions only at strongly alkaline pH. Since the balance between protonated forms depending on pH is determined by the values of dissociation constants, the corresponding indicators for different forms of fingolimod (Table 2.) and the percentage of these forms depending on pH (Figure 2.) were calculated.

**Table 3.** Protolytic forms of gabapentin and calculated physicochemical parameters.

| Protolytic form | Physicochemical parameters | | | | |
|---|---|---|---|---|---|
| | pKa | logP (logD) | Solubility, g/L | Proton donors | Proton acceptors |
|  [BH] | – | 1.19* | 90.8 | 1* | 2* |
|  $[BH_2]^+$ | 3.68±0.1 | -1.52 | 16.23 | 2 | 1 |
|  $[B]^-$ | 10.3±0.3 | -1.6 | 16.33 | 1 | 2 |

At significantly high pH, anions $[BH]^-$ (pKa = 12.2 ± 0.2) and $[B]^2$ (pKa = 13.3 ± 0.2) can theoretically be formed. Still, this process is not significant because these conditions do not occur in the body, or dosage forms. The gabapentin molecule (Figure 1B.) combines both acidic (1) and alkaline (2) functional groups, due to which the compound exhibits amphoteric properties and can also exist as a double-charged internal bipolar ion (zwitterion). The isoelectric point of gabapentin is 7.14 (Drug Future Chem Data Web site, 2022), and at physiological pH, it exists almost entirely in the form of zwitterion [BH] (Figure 3.). The percentage of cation $[BH_2]^+$ (pKa = 3,68 ± 0,1) is highest at pH below 3 (Figure 3.), which may have some value in the acidic environment of the stomach. In contrast, the formation of the anion $[B]^-$, which is present in significant quantities only at pH >10 (pKa = 10.3 ± 0.3), is not substantial. In general, based on the ionization constants of fingolimod and gabapentin, it can be expected that under physiological conditions, only ionic interaction between compounds is possible with the formation of salts capable of reversible

dissociation without changing the structure of the molecule, with the formation of corresponding protonated forms.

According to the protonated state, the characteristics of compounds, such as lipophilicity (logP or logD – lipophilicity at pH 7.4) and solubility in water that are related to the number of proton donors and acceptors, change and affect the ability of compounds to overcome histohematic barriers. Thus, fingolimod in non-ionized form has a high value of lipophilicity (logP 5.25±0.61) and the lowest solubility in water. In contrast, its ionized forms ($[BH_3]^+$, $[BH]^-$ and $[B]^{2-}$) are more water-soluble (Table 2.). A similar trend was observed for gabapentin (Table 3.). It should be noted that the value of lipophilicity (logP 1.19) is calculated for the non-ionized form, while the natural solubility in water is due to zwitterion. The reversible ionization ability is due to the presence in the molecule of donors and acceptors, which number naturally varies depending on the ionization (protonation/deprotonation) of the medication (Tables 2 and 3.).

**Interaction at the pharmacokinetic level**

At the pharmacokinetic level, drug interaction can occur at the stages of absorption, metabolism, distribution, or excretion, and occur primarily through the use of the same transport or biotransformation mechanisms. Absorption of various drugs from the gastrointestinal tract occurs with involving many mechanisms, such as passive and facilitated diffusion, and active transport. Medications with low molecular weight (up to 300 Da), uncharged medications (or those capable of reversible ionization – organic bases and acids), and structures with optimal lipophilicity are mainly transferred through biological barriers (Leo, Hansch, & Elkins, 1971). In addition, the number of hydrogen bond acceptors or donors should not be too large (up to five) so as not to create difficulties in transitioning from hydrophilic to lipophilic phases when crossing biomembranes (Lipinski et al., 2001). Active transport, on the contrary, ensures the transport through the cell membrane of those compounds that do not meet these requirements. Active transport requires energy consumption, special transport systems, and some structural similarity to endogenous substrates transported in this way.

Active transport systems are represented in the gastrointestinal tract by various transporters, which primary function is to increase the absorption of compounds to be included in the processes of energy or plastic metabolism. Interactions with these systems can be expected if the investigated compounds are similar to endogenous compounds or have specific functional groups that determine the possibility of their interaction with such systems. Thus, organic anion transporters (OAT1, OAT3, OAT4, OATP1A2) can participate in the absorption of organic acids (in the form of anions); organic cation transporters (OCT1, OCT2, family SLC-transporters) promote the transfer of organic positively-charged ions, and also take part in the transfer processes of non-specific transporters of reversible transport (mainly Pgp). They can also reduce the concentration of the compounds in some tissues (brain), or accelerate the excretion of foreign compounds of a particular chemical structure. These transporters are expressed not only in the intestinal wall, but also in other organs (kidneys) and tissues (hematoencephalic, hematotesticular barriers), where their functioning strives to maintain homeostasis. Although facilitated diffusion does not expend energy on the transfer of compounds, it attracts specific transport systems and experiences saturation effects. Both fingolimod and gabapentin are low molecular weight compounds (307.4 and 171.23 Da, respectively), the number of proton donors and acceptors in them does not exceed five (Tables 2 and 3.). In addition, lipophilicity values of their ionized forms existing at intestinal pH prove the theoretical possibility of their absorption by simple diffusion, in which the compounds do not have mutual influence on the mass transfer of each other. Gabapentin has an active transport mechanism – L-amino acid transport (Berry, Beran, Plunkeft,

Clarke, & Hung, 2003; Stewart, Kugler, Thompson, & Bockbrader 1993), which limits its absorption, but that applies only to its use in high doses (1,200-4,800 mg/day). Fingolimod, present as a cation and structurally similar to endogenous compounds, could theoretically use OCTs, but it is described to be absorbed slowly but almost completely (Zollinger et al., 2011). It is likely that fingolimod, which is prone to reversible ionization, forms a highly lipophilic non-ionized form that dissolves well, accumulates in the lipophilic regions of cell membranes, and enters the systemic circulation for a long time. Therefore, the mutual influence of these agents used at therapeutic doses on the absorption processes is not expected.

Drug interactions at the pharmacokinetic level include competition for transport systems at the level of distribution between tissues, or for enzyme systems that metabolize compounds. To consider this, the ability and likelihood of whether fingolimod and gabapentin are substrates or inhibitors of shared enzyme systems were predicted (Table 1.). The findings indicate a high probability of no interaction between fingolimod and gabapentin at the levels of shared enzyme systems. Thus, with the predicted ability to overcome hemato-intestinal and blood-brain barriers (high absorption and entry into the brain), they are not substrates or inhibitors of the Pgp transporter and the most common systems. In addition, gabapentin is practically not metabolized *in vivo*, whereas fingolimod undergoes intensive metabolism using the CYP 4F2 system, which metabolizes some medications (Lipinski et al., 2001).

Analysis of pharmacokinetic parameters of fingolimod and gabapentin requires special attention (Table 4.). First, the medications differ significantly, by 2-3 orders of magnitude, in dosages recommended to achieve a therapeutic effect that may be a problem when creating a combined drug (uneven dosage per unit, analytical quality control, etc.). Secondly, the medications have significant differences in such pharmacokinetic parameters as absorption and elimination. Fingolimod is a compound with a long time of absorption and elimination ($t_{1/2}$ 163 ± 56.3 hours), whereas gabapentin is characterized by both rapid absorption and relatively rapid elimination from the body. Thirdly, although fingolimod and gabapentin have relatively similar clearance values, there is a big difference in their volumes of distribution (1,200 ± 260 L/kg for fingolimod and 0.8 L/kg for gabapentin) that may result in their long elimination period. Finally, due to differences in physicochemical properties (including lipophilicity), fingolimod and gabapentin bind to plasma proteins to varying degrees, which precludes their interaction during the distribution phase (transport in albumin-bound form). Given the above, the interaction between fingolimod and gabapentin at the metabolic system level is unexpected. It is also possible to exclude their mutual influence on distribution and transportation in a state bound to plasma albumin, although significant differences in pharmacokinetic parameters preclude their use in the combined dosage form.

**Table 4.** Some pharmacokinetic parameters of fingolimod and gabapentin.

| Parameter | Fingolimod (David et al., 2012; David et al., 2018) | Gabapentin (Tjandrawinata et al., 2014; Goa & Sorkin, 1993) |
|---|---|---|
| The usual dose, mg | 0.125-5 | 800-1200 |
| Absorption constant, $hour^{-1}$ | 0.043 | 0.86 |
| Elimination constant, $hour^{-1}$ | 0.0042 | 0.14 |
| Time to reach maximum concentration, Tmax, hours | 12 (6-36) | 2,5 |
| Elimination half-life, hours | 163 ± 56.3 | 5-9 |
| Volume of distribution, L/kg | 1200 ± 260 (PubChem, 2001) | 0.8 |
| Degree of binding to blood plasma proteins, % | 99.7 (PubChem, 2001) | < 3 |
| Clearance, L/hour | 6.3 ± 2.3 (GILENYA, 2001) | 13.5 |

**Interaction at the pharmacological level**

Pharmacological interaction, as an indicator of the final total effect of a combination of drugs, is a necessary point to assess their effectiveness and safety. Pharmacological interactions can be direct (realized at the levels of target molecules, secondary messengers and mediator systems), or indirect (realized at the levels of target cells, organs and functional systems). In the body, fingolimod is converted to an active metabolite fingolimod phosphate by the enzyme sphingosine kinase (EC 2.7.1.91). Fingolimod and fingolimod phosphate have a high (< 0.2 nM) affinity for sphingosine-1-phosphate receptors subtype 1 (S1PR1). The therapeutic activity of fingolimod requires phosphorylation *in vivo* by sphingosine kinases to form the active moiety fingolimod phosphate (Brinkmann et al., 2010). Fingolimod phosphate binds to lymphocytic receptors S1PR1, causing internalization and degradation of the receptors. Functional antagonism at the receptors S1PR1 mediates the therapeutic effects of fingolimod, such as reducing inflammation and supporting structural restoration of the CNS parenchyma in patients with multiple sclerosis (Brinkmann et al., 2010; Matloubian et al., 2004). Initially, fingolimod acts as a potent S1PR1 agonist, primarily binding to and activating S1PR1. However, this effect is temporary, as excessive stimulation of S1PR1 effectively attracts β-arrestins to the receptor complex, promoting receptor internalization, and detaching the receptor from G protein and signaling pathways. This β-arrestin-controlled endocytic regulation reduces signaling through S1PR1, whereas chronic exposure to fingolimod causes a decrease in the amount of S1PR1 on the cell surface and long-term modulation of S1PR1 signaling. Downregulation of S1PR on lymph node T cells affects lymphocytes so that they do not respond to the output signal preventing infiltration of T cells (including proinflammatory Th17 cells) into the CNS, thereby reducing the risk of developing inflammation. Recent data also suggest that fingolimod may promote neuronal survival through microglial production of brain-derived neurotrophic factors.

The most commonly reported side effects of fingolimod are a temporary decrease in heart rate, and delayed atrioventricular conduction at the beginning of the treatment. Occurring bradycardia may be mediated by activation of the intra-rectifying potassium channel or G-protein activated by the intra-rectifying $K^+$ channel (IKACh/GIRK), and vasoconstriction is likely mediated by Rho-kinase-dependent (EC 2.7.11.1), and calcium-dependent mechanisms. The use of fingolimod in patients with MS is associated with an increased risk of infections, especially lower respiratory infections; herpes virus infections, herpes simplex virus infections, and mycosis due to a weakened immune system.

Gabapentin is a structural analog of GABA, a mediator that performs an inhibitory function. Its mechanism of action differs from the mechanism of action of other drugs that interact with GABA receptors. Gabapentin was found not to interact with $GABA_A$ or $GABA_B$ receptors of GABA uptake carriers of brain, as well as with benzodiazepine, glutamate, glycine or NMDA receptors (Taylor, 1997). The exact mechanism of action of gabapentin is still being determined. It is established that gabapentin binds to voltage-sensitive calcium channels, in particular the α2δ-1 subunit of $Ca^{2+}$ channels, which may provide its analgesic effect. In vitro, gabapentin modulates the action of glutamic acid decarboxylase (GAD), an enzyme that synthesizes GABA. Results with human and rat brain nuclear magnetic resonance (NMR) spectroscopy show that gabapentin increases GABA synthesis. Gabapentin enhances non-synaptic GABA responses from neuronal tissues and reduces the release of several monoamine neurotransmitters (Shrivastava, Triller, & Sieghart, 2011). Adverse reactions when taking gabapentin are rare, and even when taking the drug at a dose of 49 g/day, their manifestation does not have serious consequences. Symptoms of overdose included dizziness, double vision, slurred speech, drowsiness, loss of consciousness, lethargy, and mild diarrhea (Goldenberg, 2010).

Given the mechanisms of realization of the target pharmacological effects of fingolimod and gabapentin, the summative (additive) action on the symptoms of MS is expected: a reduction of the intensity of inflammatory processes in the central nervous system, neuro-

protective effect, and remyelination due to fingolimod, combined with the relief of symptoms of neuropathic pain under the influence of gabapentin. The absence of common receptor and physiological systems in the mechanisms of their action mediates the absence of risk of exacerbations of adverse reactions (Khan & Smith, 2014; Sternberg et al., 2018; Racke, Frohman, & Frohman, 2022). Analysis of the physicochemical, pharmacokinetic, and pharmacodynamic properties of of fingolimod and gabapentin suggests that this drug combination can be successfully used in the treatment of multiple sclerosis. At the same time, the urgent question is about creating a dosage form based on a fixed combination of fingolimod and gabapentine.

## CONCLUSION

Our results suggest that under physiological conditions for fingolimod and gabapentin, ionic interaction is possible with the formation of salts capable of reverse dissociation without changing the molecular structure with the formation of the corresponding protonated forms. At the pharmacokinetic level, no interaction is expected between fingolimod and gabapentine, as they do not use shared transport systems, metabolic enzymes, and have different degrees of protein binding that prevent their interactions during absorption, distribution, metabolism, and elimination. Therefore, the synergistic combination of fingolimod and gabapentine can be a promising therapeutic alternative for the effective treatment of MS. Their positive summative (additive) effect would be expected to relieve the symptoms of MS, reducing the intensity of inflammatory processes in the central nervous system, producing neuroprotective action, and contributing to remyelination due to the action of fingolimod, and ease the symptoms of neuropathic pain under the influence of gabapentine. The urgent question is about creating a dosage form based on a fixed combination of fingolimod and gabapentine.

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## AUTHOR CONTRIBUTION STATEMENT

Developing the concept and doing a majority of the literature search (VL, ON and IB), designing the analyses methodology for estimation of the reactivity of functional groups (IB and OK). Carrying out some of the analysis and interpretation (ON), carrying out data collection and processing (ON, IS and NV). Suggesting the use of synergistic combination of fingolimod and gabapentin for the treatment of MS (ON and IF). The manuscript preparation, editing and review (IB and IS).

## CONFLICT OF INTEREST

All the authors of this article declare that there is no conflict of interest.

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*RESEARCH ARTICLE*

# Evaluation of Patients' Willingness to Use and Pay for Pharmaceutical Care Services in Türkiye

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***Evaluation of Patients' Willingness to Use and Pay for Pharmaceutical Care Services in Türkiye***

***SUMMARY***

*Pharmacists' consultancy roles, covering many essential tasks such as preventing diseases, monitoring medication, and increasing patient compliance, significantly improve healthcare. Patients' views on pharmaceutical care services should be evaluated to improve these services. Thus, the main objective of this study is to assess patients' willingness to use and pay for pharmacy services provided in community pharmacies. A measurement tool was developed according to relevant literature, including 24 statements, and applied to the patients in Van, Türkiye. Obtained data (n=445) were first subjected to descriptive statistical analysis, and then the exploratory factor analysis (EFA) was applied. Lastly, independent samples t-test and ANOVA tests were conducted using factor scores to test the hypotheses. As a result of the EFA, a three-factor solution was obtained. The factors were labeled as advanced pharmacy services, core pharmacy services, and providing information for usage. Cronbach's Alpha values of the factors are 0.859, 0.883, and 0.691, respectively. It was determined that the participants were more willing to benefit from the services discussed under the core pharmacy services factor. These services were followed by the services in the factors of providing information for usage and advanced pharmacy services. Contrary to this situation, it is seen that the participants are more willing to pay for the services included in the advanced pharmacy services. This paper has put forth that patients were generally willing to use pharmaceutical services but not willing to pay for them.*

***Key Words:*** *Community Pharmacist, Patient, Pharmaceutical care, Willingness to Pay.*

***Turkiye'de Hastaların Farmasötik Bakım Hizmetlerini Kullanma ve Ödeme Yapma Gönüllülüklerinin Değerlendirilmesi***

***ÖZ***

*Eczacıların hastalıkları önleme, ilaç takibi, hasta uyumunu artırma gibi birçok temel görevi kapsayan danışmanlık rolleri, sağlık hizmetlerini önemli ölçüde geliştirmektedir. Bu hizmetlerin iyileştirilmesi için hastaların farmasötik bakım hizmetlerine yönelik görüşleri ele alınmalıdır. Bu nedenle, bu çalışmanın temel amacı hastaların serbest eczanelerde sunulan farmasötik bakım hizmetlerini kullanma ve bu hizmetlere ödeme yapma gönüllülüklerini değerlendirmektir. İlgili literatüre uygun olarak 24 ifadeyi içeren bir ölçüm aracı geliştirilmiş ve Van'daki hastalara uygulanmıştır. Elde edilen veriler (n=445) öncelikle betimsel istatistiksel analize tabi tutulmuş, daha sonra açıklayıcı faktör analizi (EFA) uygulanmıştır. Son olarak hipotezleri test etmek amacıyla faktör skorları kullanılarak bağımsız örneklem t testi ve ANOVA testleri yapılmıştır. AFA sonucunda üç faktörlü bir çözüm elde edilmiştir. Faktörler ileri eczane hizmetleri, temel eczane hizmetleri ve kullanım ile ilgili bilgi verme olarak adlandırılmıştır. Faktörlerin Cronbach Alfa değerleri 0,859, 0,883 ve 0,691'dir. Katılımcıların temel eczane hizmetleri faktörü altında ele alınan hizmetlerden yararlanmaya daha gönüllü oldukları tespit edilmiştir, Bu hizmetleri kullanım ile ilgili bilgi verme ve ileri eczane hizmetleri faktörlerindeki hizmetler izlemiştir. Bu durumun aksine katılımcıların ileri eczane hizmetleri kapsamındaki hizmetler için ödeme yapmaya daha gönüllü oldukları görülmüştür. Bu makale, hastaların genel olarak farmasötik hizmetlerini kullanmaya gönüllü olduklarını ancak bu hizmetler için ödeme yapmaya gönüllü olmadıklarını ortaya koymuştur.*

***Anahtar Kelimeler:*** *Serbest eczacı, Hasta, Farmasötik Bakım, Ödeme Gönüllülüğü*

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## INTRODUCTION

Pharmacists' consultancy roles, covering many essential tasks such as preventing diseases, monitoring medication, and increasing patient compliance, significantly improve healthcare. In this context, community pharmacists are described as the closest and most accessible healthcare professionals. Pharmacists are responsible for ensuring that patients receive the information they need about the drug treatment to ensure rational drug usage (Berger, 2009). This responsibility is also emphasized in many national and international legal regulations. Sancar et al. (2013) stated that most community pharmacists (97.3%) want to provide pharmaceutical care services, and 83.5% of them think that this service is one of the duties of pharmacists. Tecen-Yucel et al. (2023) found that pharmacists have positive attitudes toward providing pharmaceutical care; in contrast, they do not very intend to provide these services. From a different point of view, there are some studies evaluating pharmaceutical care services on patients' health status in Türkiye. The results of these studies put forth that pharmacist consultancy has a positive effect on patient outcomes (Pehlivanli et al., 2021; Ayhan and Sancar, 2023).

On the other hand, patients' attitudes, views, and willingness for pharmaceutical care services should be evaluated to improve these services. In the literature, some studies investigate pharmaceutical care services from the patients' perspectives for different chronic diseases. Brown et al. (2017) revealed that patients are most willing to benefit from "how to use prescribed drugs" and "to provide more appropriate generic drug" services. Waszyk-Nowaczyk et al. (2023) evaluated diabetic patients' needs and opinions on implementing pharmaceutical care services. They put forth that patients are especially willing to use 'new drug' services, including getting information for newly prescribed medicines and medication reviews by pharmacists. According to Jaber et al. (2019), most participants exhibited a positive attitude toward pharmaceutical care services in Jordan, and 96% believed pharmacists should be more committed to the consulting role. Gül et al. (2023) evaluated patient satisfaction with services provided in community pharmacies in Türkiye and put forth that patients' satisfaction levels were above medium.

Hill and Dowse (2007) stated reimbursement and compensation as the most critical barriers to providing pharmaceutical care services by pharmacists. Thus, evaluating individuals' willingness to pay for these services is another critical issue. Willingness to pay (WTP) is the maximum amount of money individuals can contribute to compensate for a change in benefits or receive extra services or treatment (Suh, 2000). Payment volunteerism or willingness to pay is essential in evaluating health benefits and medication programs. Measuring WTP for medicines and pharmaceutical services can be assessed by asking individuals how much they will be willing to pay for products or certain health services (Soodi et al., 2023). The American Pharmaceutical Association National Survey (1983) states that between 13% and 57% of individuals are willing to pay, depending on the pharmacy's service types. Gore and Madhavan (1994) found that patients are more willing to pay for pharmacists' prescription advice than for over-the-counter (OTC) drug consultancy. Still, the general tendency to pay is not very favorable. According to Larson (2000), 56% of consumers were willing to pay for pharmaceutical care, and $50 was considered reasonable for a one-time consultation, first visit, and 1-year follow-up. Additionally, AlShayban et al. (2020) stated that individuals' level of satisfaction with the services they received from the pharmacy was directly proportional to their willingness to pay.

Pharmaceutical care services are still in their infancy in some developing countries. Türkiye is one of them, and pharmacists' consultation services are being provided free of charge. Although there is an increasing trend in providing pharmaceutical care services, studies in this area are limited. In this regard, this study's motivation comes from conducting a study that considers the willingness to benefit from

and pay for pharmacy services to fill the literature gap. To the best of the authors' knowledge, this is the first study that comprehensively investigates the mentioned issues in Türkiye.

## MATERIAL AND METHODS

### Measurement tool

This study aims to evaluate patients' willingness to use and pay for pharmacy services. For this aim, a measurement tool was developed according to relevant literature.

The measurement tool includes six demographic questions and 24 expressions prepared with a 4-point Likert scale (1-lowest, 4-highest) in Turkish to evaluate patients' willingness to use pharmaceutical care services. Patients are also asked whether they are willing to pay for these services. The statements in the questionnaire were prepared by the research team based on the relevant literature (Suh, 2000; Sancar et al., 2013; Brown et al., 2017).

### Sample size and data collection

This study's population consists of individuals between 18-65 who apply to community pharmacies at least once a year in Van, Türkiye. It was impossible to determine the number of units in the main population. The minimum sample size was calculated as 385; on 0.05 significance level, z = 1.96, d (sensitivity)= 0.05, and p and q values being 0.5. To increase the reliability, it was tried to reach the maximum number of individuals that could be reached.

The study was carried out with permission number 73040253-044-E.439 of Van Governorship Provincial Health Directorate and 2019/1626 Number of Van-Bitlis-Hakkari Chamber of Pharmacists. In line with the World Medical Association Declaration of Helsinki, Ethical Principles for Medical Research on Human Volunteers, researchers administered questionnaires face-to-face between 15.03.2020 and 15.06.2020. After getting informed consent from patients, the questionnaire was applied at randomly selected ten community pharmacies in the city center of Van.

### The hypothesis of the study

$H_1$: Gender affects patients' willingness to use the services offered in pharmacies.

$H_2$: Age affects patients' willingness to use the services offered in pharmacies.

$H_3$: Education level affects patients' willingness to use the services offered in pharmacies.

$H_4$: Income affects patients' willingness to use the services offered in pharmacies.

$H_5$: Having a chronic disease affects patients' willingness to use the services offered in pharmacies.

### Data analysis

The data obtained were first subjected to descriptive statistical analysis with the IBM SPSS Statistics 22.0 package program, and then the exploratory factor analysis (EFA) was applied. Lastly, independent samples *t*-test and ANOVA tests were conducted using factor scores to test the hypothesis.

## RESULTS AND DISCUSSION

In this study, data was obtained from 445 individuals. The demographic characteristics of the participants are given in Table 1.

**Table 1.** Characteristics of the participants

| Characteristics of the participants | Frequency (%) |
|---|---|
| *Gender* | |
| Female | 62.50 |
| Male | 37.50 |
| *Age* | |
| <25 | 36.00 |
| 25-50 | 57.50 |
| >50 | 6.50 |
| *Education level* | |
| Primary school | 6.70 |
| High school | 24.50 |
| Undergraduate | 60.20 |
| Graduate | 8.50 |
| *Income* | |
| <2500 TL | 58.20 |
| 2500-5000 TL | 30.60 |
| >5000 TL | 11.20 |
| *Having a chronic disease* | |
| Yes | 15.30 |
| No | 84.70 |

The mean and standard deviation values of patients' responses to the statements to evaluate their willingness to use the services offered in the pharmacy are presented in Table 2. In addition, the study evaluated whether the patients were willing to pay for these services, and obtained data are also given in Table 2.

**Table 2.** Frequencies of patients' willingness to pay for services provided by pharmacists

| Services provided from pharmacies | Mean | Std deviation | Willingness to pay | |
|---|---|---|---|---|
| | | | Yes | No |
| Evaluation of the suitability of drugs | 3.26 | 0.864 | 48 | 397 |
| Evaluation of the efficacy of drugs | 3.26 | 0.925 | 48 | 397 |
| Evaluation of the safety of drugs | 3.34 | 0.846 | 48 | 397 |
| Evaluation of drug-drug interactions | 3.21 | 0.871 | 45 | 400 |
| Evaluation of drug-nutrient interactions | 3.27 | 0.846 | 47 | 398 |
| Evaluation of drug-related side effects | 3.26 | 0.889 | 49 | 406 |
| Informing about how to use my medical device | 3.20 | 0.854 | 50 | 395 |
| Informing about how to use my medicines | 3.30 | 0.829 | 49 | 396 |
| Providing solutions to complaints about the medicines I use | 3.12 | 0.938 | 53 | 392 |
| Informing about how to use medical supplies | 2.97 | 0.865 | 53 | 392 |
| Counseling for immunization | 2.88 | 0.896 | 50 | 395 |
| Counseling for nutritional supplements | 3.01 | 0.905 | 55 | 390 |
| Counseling for weight loss products | 2.89 | 0.985 | 64 | 381 |
| Counseling for smoking cessation | 2.81 | 0.962 | 73 | 372 |
| Counseling for mother-baby health | 2.93 | 0.948 | 75 | 370 |
| Counseling for cosmetics | 2.95 | 0.989 | 84 | 361 |
| Counseling for family planning | 2.71 | 0.985 | 63 | 382 |
| Counseling for alternative and traditional treatment methods | 2.86 | 0.865 | 48 | 397 |

In light of the findings in Table 2, the participants generally want to benefit from the determined services. While the service the participants wanted to benefit from the most was drug safety evaluation, the least preferred service was the consultancy service for family planning. It is seen that the vast majority of patients are not willing to pay for the services provided. 18.9% of the participants stated that they might be willing to pay for counseling on cosmetics, followed by counseling on mother-baby health and smoking cessation, with 16.9% and 16.4%.

In the second step of the analysis, EFA was conducted using principal component analysis with Varimax rotation. Factors were created according to the Kaiser criterion and the scree plot. Items were removed due to low factor loadings, which are under 0.50. As a result, a three-factor solution was obtained, which explained 57.3 % of the variance. The Kaiser-Meyer-Olkin (KMO) value was calculated as 0.904. The obtained factors were labeled as *advanced pharmacy services (APS), core pharmacy services (CPS)*, and *providing information for usage (PIU);* Cronbach's Alpha values of the factors are 0.859, 0.883, and 0.691, respectively. This value shows a high level of reliability of the factors according to the existing literature. The factor loadings of the obtained factors and the expressions in the factors are given in Table 3.

**Table 3.** Factors and factor loadings

| Items | Factors | | |
|---|---|---|---|
| | APS | CPS | PIU |
| Counseling for smoking cessation | 0.737 | | |
| Counseling for cosmetics | 0.736 | | |
| Counseling for weight loss products | 0.732 | | |
| Counseling for family planning | 0.723 | | |
| Counseling for mother-baby health | 0.711 | | |
| Counseling for nutritional supplements | 0.618 | | |
| Counseling for alternative and traditional treatment methods | 0.518 | | |
| Counseling for immunization | 0.486 | | |
| Evaluation of the suitability of drugs | | 0.824 | |
| Evaluation of the efficacy of drugs | | 0.800 | |
| Evaluation of the safety of drugs | | 0.770 | |
| Evaluation of drug-drug interactions | | 0.765 | |
| Evaluation of drug-nutrient interactions | | 0.690 | |
| Evaluation of drug-related side effects | | 0.633 | |
| Informing about how to use my medical device | | | 0.767 |
| Informing about how to use my medicines | | | 0.713 |
| Providing solutions to complaints about the medicines I use | | | 0.537 |
| Informing about how to use medical supplies | | | 0.511 |

As a result of the EFA, six services were included under the *CPS*. The average response rates for these services varied between 3.21 and 3.34. As a result of the drug and disease monitor study conducted by community pharmacists on diabetes patients by Abduelkarem and Sackville (2008), it was revealed that this program increases the quality of service received from pharmacies and the quality of life of the patient, reduces the drug treatment and diabetes complications of the patient. Accordingly, it has been observed that the patients are willing to use them. Furthermore, Adekunle et al. (2023) stated that nearly half of the patients were willing to accept medication management services provided in pharmacies.

According to Table 2 and Table 3, 4 services were evaluated under the *PIU*, and it was revealed that the participants were generally willing to benefit from these services. Parallel to Jaber et al. (2019), most participants were willing for the pharmacist to provide a pharmaceutical care service that could reduce medication-related problems. Addressing the issue specifically for patients using ACEIs, Sancar et al. (2011) revealed that individuals are willing to consult pharmacies to find solutions to their complaints about dry cough and get results. In another study, Adekunle et al. (2023) point out that patients were "definitely willing" to accept drug information (68.3%) from pharmacies. Similarly, Brown et al. (2017) revealed one of the services that patients are most willing to "provide services on how to use prescribed drugs."

As a result of the EFA, eight services were considered under the *APS*, and it was revealed that the participants wanted to benefit from these services at least. Contrary to the findings of this study, Gore and Madhavan (1994) stated that patients are willing to benefit from the non-prescription product consultancy offered by pharmacists. Brewer et al. (2018) demonstrated that most patients are willing to be screened for HCV in community pharmacies and that pharmacists have the opportunity to address this. Einarson et al. (1988) found that the participants were positive about their cholesterol and potassium blood levels being monitored by pharmacists. Condino et al. (2015) showed that smoking cessation programs can be effectively implemented in community pharmacies in Portugal.

Lastly, independent samples *t*-tests and ANOVA tests were used to evaluate the effects of demographic characteristics on factor scores. As a result of the *t*-test, gender did not significantly affect the 95% confidence interval, while individuals with chronic diseases had a higher mean in all factors. In other words, individuals with chronic diseases were statistically more willing to benefit from pharmacy services. This study also compared the effects of age, education, and income levels on willingness to benefit from pharmacy services. A one-way ANOVA revealed a significant effect of age on *core pharmacy services*, $p < 0.05$. Tukey post hoc test showed that the patients under the age of 25 scored significantly less than those between the ages of 25-50 and over 50 years old ($p < 0.05$). So, with the increase in age, the willingness to benefit from services has also increased. There was no statistically significant difference between patients' educational levels ($p < 0.05$).

Moreover, a one-way ANOVA was performed to compare the effect of income levels on willingness to benefit from services. As a result, the two groups had a statistically significant difference in *advanced pharmacy services*. Tukey post hoc test revealed that patients with income under 2500 TL scored significantly higher than those between 2500-5000 TL ($p < 0.05$).

When the five main hypotheses discussed in this study are evaluated, it is seen that the first hypothesis of the study, "$H_1$: Gender affects patients' willingness to benefit from the services offered in pharmacies." is rejected. The study determined that gender did not make a statistically significant difference across service groups. Contrary to this study, Brown et al. (2017) revealed that women accepted medication management services offered by pharmacists at a higher rate.

The study's second hypothesis discussed the effect of patients' ages on their willingness to benefit from pharmacy services. The study determined that age affected *CPS* but did not affect other services. It has been shown that increasing age increases the level of willingness in pharmaceutical consultancy services, and this result parallels Suh (2000). In contrast, Brown et al. (2017) stated that the desire to benefit from consultancy services decreases as age increases.

In the third hypothesis discussed in the study, the effect of education level was evaluated, and this hypothesis was rejected. Studies in the literature generally state that there is a relationship between education level and the desire to benefit from pharmacy services. Brown et al. (2017) revealed that individuals with lower education levels benefitted more from ser-

vices. According to the results obtained in the study of Ünal and Bilgener (2019), as the education level of patients increases, they benefit from services, and satisfaction with the quality of care increases. Nielsen et al. (2008) found in their study that more meaningful results were achieved in patients with a lower education level than in patients with a higher education level.

As Cheng et al. (2013) mentioned, cultural differences, language barriers, low literacy, and low income can cause health disparities. Thus, in this study, factor scores were also evaluated to see the effect of income on the willingness to use pharmaceutical services. About the fourth hypothesis, which is that patients' income level affects their willingness to benefit from the services offered in pharmacies, it has been determined that income levels make a difference only in *APS*. In parallel with Brown et al. (2017), this study determined that low-income individuals were more willing to use these services.

Following Fernandez-Lazaro et al. (2019), managing their conditions is vital for patients with chronic diseases to improve their health outcomes. Therefore, to see the difference between patients with chronic disease and others, the fifth hypothesis of the study was conducted. This hypothesis was accepted, and individuals with chronic diseases were found to be more willing to use pharmacy services as expected.

## CONCLUSION

Within the scope of this study, the services offered by pharmacists in community pharmacies in Türkiye and the willingness of patients to use and pay for these services are discussed. In the study, services provided by community pharmacists were evaluated under three primary structures: *advanced pharmacy services, core pharmacy services*, and *providing information for usage*. As a result of the study, it was revealed that the participants were generally willing to benefit from the determined 24 services but not willing to pay for them. While the service the participants most wanted to benefit from was drug safety evaluation, the consultancy service for family planning was the least preferred one. When sorting as a service group, it is seen that the participants mostly want to benefit from *CPS*, followed by *PIU* and *APS,* respectively. In contrast, it is seen that the participants are more willing to pay for the services included in the *APS*.

From the research that has been carried out, it is possible to conclude that patients are willing to use pharmaceutical care services without any payment. This study is of great importance for developing pharmacy services in Türkiye. Additionally, demographic factors such as gender, age, income, and educational level were not found statistically effective on the factor scores. On the other hand, it is seen that patients with chronic diseases are more willing to use pharmaceutical services, so it can be said that focusing on some of the most common chronic diseases seems logical to improve these services. In this regard, the results of this study give essential clues for pharmacists and pharmacy policymakers to extend provided services in community pharmacies.

There are also several limitations in this study. The first limitation is about the data collection process. Since the survey conduction process coincided with the period when COVID-19 cases started to be seen in Türkiye and, therefore, there were many restrictions, the rate of conducting face-to-face surveys was lower than expected. However, since the study's sample size was above the required minimum, it did not negatively impact the analysis results. The second limitation of the study is that participants were only asked whether they would be willing to pay for services but not how much they would be willing to pay. So, future research will address how much they would be willing to pay for pharmaceutical care services.

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest

**AUTHOR CONTRIBUTION STATEMENT**

Concept: MA; Design: ZA, MA; Data Collection or Processing: ZA; Analysis or Interpretation: MA; Literature Search: ZA, MA; Writing: ZA, MA.

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# Linezolid-Loaded Nanoparticle for Topical Administration of Diabetic Foot Treatment: Formulation, *In Vitro* and *Ex Vivo* Characterization

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***Linezolid-Loaded Nanoparticle for Topical Administration of Diabetic Foot Treatment: Formulation, In Vitro and Ex Vivo Characterization***

***SUMMARY***

*The aim of this research is to formulate a topical gel containing nanoparticles for diabetic foot ulcers (DFU). In this respect, the nanoparticle formulations were prepared using the spontaneous emulsification technique. Linezolid (LZD)-loaded nanoparticle formulation exhibited low average particle size (PS) of 195.27±5.42 nm, low polydispersity index (PI) of 0.214±0.019, high zeta potential (ZP) of 20.57±0.35 mV and high drug entrapment efficiency (EE) of 99.746±0.021%. To enhance topical residence time, the LZD-loaded nanoparticles were dispersed in a gel formulation using Methocel TM K4M (HPMC) and Carbopol® 974 P NF. The formulated gels demonstrated favorable characteristics, including an appropriate pH value, suitable mechanical performance, and desirable viscosity and spreadability for topical application. All formulations displayed pseudoplastic flow and typical gel-type mechanical spectra at the specified frequency value. Moreover, the developed formulation achieved sustained drug release as intended for these systems. During ex vivo drug diffusion studies, 0.007±0.004% of LZD was found in receptor phase, indicating a local effect. The optimum formulation was stable for six months. The initial findings suggest that the formulated topical gel containing LZD-loaded nanoparticles holds promise as an effective drug delivery system for DFU management. However, further comprehensive investigations are required to substantiate this hypothesis.*

***Key Words:*** *Diabetic foot ulcer, linezolid, nanoparticle, gel*

***Diyabetik Ayak Tedavisinin Topikal Uygulaması için Linezolid Yüklü Nanopartikül: Formülasyonu, In Vitro ve Ex Vivo Karakterizasyonu***

***ÖZ***

*Bu çalışmanın amacı, diyabetik ayak ülseri (DFU) tedavisi için nanopartikül içeren bir topikal jel formülasyonu geliştirmektir. Bu bağlamda, nanopartikül formülasyonları, spontan emülsifikasyon tekniği kullanılarak hazırlandı. Linezolid (LZD) yüklü nanopartikül formülasyonu, düşük partikül boyutu (PS) 195.27±5.42 nm, düşük polidispersite indeksi (PI) 0.214±0.019, yüksek zeta potansiyel (ZP) 20.57±0.35 mV ve yüksek enkapsülasyon etkinliği (EE) %99.746$_{\pm 0.021}$ gösterdi. Topikal kalma süresini artırmak için, LZD yüklü nanopartiküller, MethocelTM K4M (HPMC) ve Carbopol® 974 P NF kullanılarak bir jel formülasyonunda disperse edildi. Formüle edilen jeller, uygun pH değeri, uygun mekanik özellik ve topikal uygulama için istenen viskozite ve yayılabilirlik gibi olumlu özellikler sergiledi. Tüm formülasyonlar psödoplastik akış sergiledi ve belirtilen frekans değerinde tipik jel tipi mekanik spektrumlar sergiledi. Ayrıca geliştirilen formülasyon, bu sistemler için amaçlandığı gibi sürekli ilaç salımı sağladı. Ex vivo ilaç difüzyon çalışmaları sonucunda LZD'nin %0.007$_{\pm 0.004}$'ünün reseptör fazında bulunması lokal etkinin bir göstergesidir. Optimum formülasyon 6 ay boyunca stabil bulundu. İlk bulgular, LZD yüklü nanopartikülleri içeren formülasyonun, DFU tedavisi için umut vadeden etkili bir dozaj şekli olduğunu göstermektedir. Ancak, bu hipotezi desteklemek için daha kapsamlı araştırmalar gerekmektedir.*

***Anahtar Kelimeler:*** *Diyabetik ayak ülseri, linezolid, nanopartikül, jel.*

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## INTRODUCTION

Diabetic foot ulcer (DFU) is a severe health problem with the increasing prevalence of diabetes. DFU is a multifaceted disease from various risk factors such as peripheral neuropathy, peripheral arterial disease, foot deformities, minor trauma, and compromised infection resistance (Rayate et al., 2023; Loera-Valencia et al., 2022). Topical administration of antimicrobial agents becomes crucial due to the heightened susceptibility to infections associated with this disease (Ezhilarasu et al., 2020).

Linezolid (LZD) is an antibiotic used to treat various bacterial infections. It belongs to the oxazolidinone class of antibiotics and inhibits protein synthesis in bacteria (Traunmüller et al., 2010). The molecular formula of LZD is $C_{16}H_{20}FN_3O_4$, and its molecular-weight, melting point, and LogP values are 337.351 g/mol, 181.5–182.5 C, and 0.9, respectively. LZD is sparingly soluble in water, its solubility at 25°C is approximately 3 mg/mL. It is more soluble in acidic solutions. LZD is a weak base, and its pKa values are reported to be approximately 1.8 (Fernandes et al., 2020). It is used in the treatment of gram-positive bacteria and mycobacteria. It is effective against gram-positive aerobic bacteria, gram-negative anaerobic bacteria and gram-positive anaerobes (Chen et al., 2020; Basetti et al., 2014). Due to the reported side effects of systemic LZD (such as nausea, vomiting, headache, dizziness, allergic reactions, diarrhea and abdominal discomfort), there is a need to develop a topical formulation that does not carry the previously mentioned risks in clinical practice.

The topical route of drug administration offers benefits such as reduced side effects, improved patient compliance and avoidance of the first-pass effect. Despite the limitations of poor skin permeability for some drugs, using nanoparticular drug delivery systems and other innovative formulation approaches can help overcome these barriers and improve the effectiveness of topical drug delivery (Wagas et al., 2022). Drug research aims to find new and effective ways of delivering medications. This situation includes prolonging their effectiveness, targeting specific areas, controlling release, preserving fragile drugs until absorption, and enhancing overall treatment efficiency (Wagas et al., 2022; İlhan et al., 2022).

Nanoparticles (NPs) offer several advantages in various applications, including DFU healing. One of the critical advantages of NPs is their versatility and ability to be used in different ways. They can be designed to have controlled sizes and unique physiochemical properties, making them suitable for particular purposes (Ezhilarasu et al., 2020; Jifar et al., 2021). A significant advantage of NPs in DFU healing is their larger surface area to volume ratio. This property allows for better cell adhesion, which is crucial for tissue regeneration. NPs can penetrate the skin more effectively, reaching deeper layers and ensuring better drug absorption. Furthermore, encapsulated drugs within NPs can be released in a sustained and controlled manner. This sustained release allows for a continuous and prolonged therapeutic effect, promoting the healing process over an extended period. The delivery rate of the encapsulated drugs can also be adjusted by altering the distribution of NPs, providing flexibility and customization in treatment. In summary, using NPs in wound healing treatments offers advantages such as targeted drug delivery, enhanced cell adhesion, sustained release of encapsulated drugs, and the ability to modify the delivery rate. These advantages make NPs an attractive approach to improve DFU healing outcomes (Ezhilarasu et al., 2020; Rajendran et al., 2018). Eudragit® (EUD) was chosen to prepare NPs. It is a non-biodegradable, cationic copolymer derived from acrylic and methacrylic acids or their esters. EUD RS contains a low percentage of quaternary ammonium groups, typically ranging from 4.5% to 6.8%. This polymer is insoluble in water under physiological pH conditions but exhibits swelling properties, making it suitable for dispersing active ingredients. EUD polymers have garnered

interest for their excellent stability, consistent release rates of active substances, and favorable mucosal tolerability, making them attractive candidates for various research applications (Rençber et al., 2016; Yenilmez, 2017).

Nevertheless, frequent administration is required for topical NP formulations due to their brief residence time on the skin. Semi-solid systems can be employed to extend the residence time of NPs. Topical gels provide numerous benefits, including easy administration to the skin, reduced frequency of application, enhanced patient compliance, and improved comfort compared to conventional dosage forms (Rençber & Karavana, 2020). Natural, synthetic or semi-synthetic polymers are used for gels, especially for controlled release. Hydroxypropyl methylcellulose (HPMC) is a nonionic, water-soluble synthetic polymer obtained from cellulose that is extensively utilized to formulate semi-solid preparations. Recognised for its lack of toxicity, non-irritating nature, exceptional mucoadhesive properties and swelling capacity, HPMC serves as a versatile ingredient in various formulations (Ghosal et al., 2011; Pan et al., 2023). Carbopol® polymer or carbomer, is a synthetic, high molecular-weight polymer derived from acrylic acid. Cross-linked acrylic acid polymers that swell in water to form transparent or slightly opaque gels. Carbopol® is known for its ability to increasing the viscosity of aqueous solutions at low concentrations, offering excellent rheological control. Additionally, it is pH-sensitive and can form gels over a wide pH range, making them versatile in various formulations (Shin et al., 2000; Kim et al., 2003).

The present work aims to develop a topical gel formulation containing NPs that can effectively alleviate the symptoms of DFU. This approach is designed to minimize the risk of undesirable side effects associated with existing oral formulations available on the market. The developed formulation was characterized for particle size (PS), polydispersity index (PI), zeta potential (ZP), morphology, drug entrapment efficiency (EE), pH, mechanical properties, viscosity, spreadability, flow, *in vitro* drug release, *ex vivo* drug diffusion, and stability.

## MATERIAL AND METHODS

LZD was donated by MS Pharma, Turkey. EUD RS 100 was donated by Karadeniz Chemical Company (Karadeniz, Turkey). Carbopol® 974 P NF was obtained from Noveon (Cleveland, OH). Methocel™ K4M was kindly gifted by Colorcon (Dartford, UK). All other chemicals used were of analytical grade.

### Preparation of NPs

The NPs were prepared using the spontaneous emulsification technique. A solution containing 2.5% EUD RS 100 and 0.6% LZD was dissolved in 25 mL of ethanol. The alcoholic solution was slowly dripped into 50 mL of distilled water with continuous stirring. The resulting NPs were stirred for 48 hours at room temperature (Rençber et al., 2016).

### Characterization of NPs

Determination of mean PS, PI, and ZP values for the formulations was carried out using the Dynamic Light Scattering method (Malvern NanoZS, Malvern Instruments, Malvern, UK) at ambient temperature.

The morphological analysis of both the blank and LZD-loaded NPs was carried out using a Scanning Electron Microscope (SEM) (Carl Zeiss 300VP). Before SEM analysis, the samples underwent a coating process with a thin layer of gold, facilitated by the QUORUM Q150 RES device.

The LZD content in the samples was measured spectrophotometrically (BMG Labtech- Clariostar) at 283 nm. The drug entrapment efficiency was determined through a validated spectrophotometric method involving ultracentrifugation of NP dispersion for 90 seconds at 15,000 rpm (Hettich Mikro 200R). The supernatant obtained after centrifugation was utilized for LZD analysis via spectrophotometry to determine the quantity of free drug. The quantity of encapsulated LZD was determined by subtracting the amount of free LZD from the total LZD content present in the dispersion (n=5, Equation 1) (Rencber & Tanrıverdi 2018).

$$\text{Encapsulation efficiency (EE) (\%)} \frac{\text{Total amount of LZD - The amount of free LZD}}{\text{Total amount of LZD}} \times 100 \quad \text{(Equation 1)}$$

**Preparation of gel formulations**

To prepare HPMC gels, varying proportions (2-3%) of Methocel™ were slowly added to distilled water with a magnetic stirrer. The gels were refrigerated until a transparent solution was achieved, typically within 24 hours.

To prepare Carbopol® gels, different ratios (1-2%) of Carbopol® 974 NF were kept in distilled water for 24 hours to ensure homogeneous swelling. After 24 hours, the pH was adjusted by adding triethanolamine, and transparent gels were obtained.

Gels prepared with different polymers in different ratios were evaluated for parameters such as pH, viscosity, mechanical properties, and rheological analysis. NPs with low viscosity were dispersed in gel formulations selected to give the appropriate viscosity for topical application.

The necessary amounts of Carbopol® 974 P NF, and Methocel™ were allowed to blend for 24 hours. Subsequently, the gels incorporating Carbopol® were neutralized with triethanolamine. The NP dispersion was introduced into the pre-prepared gels and stirred for 1 hour at ambient temperature. The concentration of LZD in the gels was set at 6 µg/mL as the conclusive level. Details regarding the composition of the gel formulations are outlined in Table 1.

**Table 1.** Composition of gel formulations

| Formulation Code | Content of Formulation | Polymer Ratio (%) | LZD NP |
|---|---|---|---|
| **F1** | Carbopol® 974 P NF | 1 | - |
| **F2** | Carbopol® 974 P NF | 1.5 | - |
| **F3** | Carbopol® 974 P NF | 2 | - |
| **F4** | Methocel™ K4M | 2 | - |
| **F5** | Methocel™ K4M | 2.5 | - |
| **F6** | Methocel™ K4M | 3 | - |
| **F1 + LZD NP** | Carbopol® 974 P NF | 1 | + |
| **F4 + LZD NP** | Methocel™ K4M | 2 | + |

**Characterization of gel formulations**

Macroscopic evaluation of gels: Macroscopic features of the gels, including color, uniformity, transition from clarity to opacity, and the identification of residues or phase separation, were evaluated through visual observation over a 48-hour duration.

Determination of pH: To evaluate the compatibility of the prepared gels with topical surfaces, pH measurements were carried out at room temperature using a pH meter (Hanna Edge, USA).

Mechanical properties of gels: The mechanical characteristics were evaluated, and assessed using a software-controlled penetrometer (TA-TX Plus, Stable Micro System, UK) operating in texture profile analysis (TPA) mode at temperatures of 25±0.5°C and 32±0.5°C. The formulations were transferred into a beaker, and the analytical probe (Perspex P/10) of the penetrometer was pressed into the formulations twice at a consistent speed to a certain depth. These compressions were performed at room temperature, with a 15-second interval between each compression. The mechanical parameters were derived from the force-time curves obtained during the analysis (n=6) (Rençber & Karavana 2020; Jones et al., 1997; Rencber et al., 2021).

Viscosity measurement of gels: The viscosity of the gel formulations was measured using a Brookfield viscometer (Brookfield Ametek DV2T Viscometer, Brookfield Engineering Laboratories, Inc., USA) at 25°C. The measurements were performed using a

rotational viscometer with a spindle 27. Viscosity parameters were measured at various rotations per minute (rpm) with a 1-minute equilibration time at each rpm. Samples were applied to the lower plate using a spatula to ensure proper shearing of the formulation (n=3).

Spreadability studies of gels: The spreadability of gel formulations was measured at 25°C and 32°C using a software-controlled penetrometer (TA-TX Plus, Stable Micro System, UK, Prob: TTC Spreadability Rig). The gel formulation was carefully added to a beaker, taking particular care to avoid the formation of bubbles. The force, quantified in Newtons, was monitored throughout the testing period, and spreadability was assessed by computing the area under the curve (AUC).

Flow behavior analysis of gels: The rheogram for the gels was generated using a Brookfield Viscometer (Brookfield Ametek DV2T Viscometer, Brookfield Engineering Laboratories, Inc., USA) at 25±1°C, employing spindle 27. Shear stress was measured by incrementally increasing the shear rate from 0.5 to 100 rpm.

*In vitro* drug release studies: The release of LZD from both NPs and gels containing NP was evaluated utilizing a dialysis bag submerged in PBS at 32±0.5°C. The system was continuously stirred with a magnetic stirrer for 24 hours. The drug content in the receiving solution was quantified through a validated spectrophotometric method. Sink conditions were upheld in the receptor compartment throughout the *in vitro* release studies (n=5).

Release kinetics: Five kinetic models were chosen to describe the release profiles of the prepared systems. Thus, the kinetic models were investigated using a computer program for empirical analysis.

*Ex vivo* drug diffusion study: The permeation study of LZD-NP-loaded gels was conducted using Franz diffusion cells. Sheep ear skin, sourced from a local slaughterhouse, was carefully mounted onto the diffusion cells following established protocols (Huong et al., 2009; Bayldon et al., 2014; Reddy et al., 2019). The donor and receptor chambers were filled with 1 mL of the formulation and 20 mL of PBS, respectively. The cells were kept at a temperature of 32±0.5°C with continuous magnetic stirring. Samples were collected at designated time intervals over 24 hours and analyzed using a validated spectrophotometric method. Sink conditions were maintained in the receptor compartment throughout the *ex vivo* drug diffusion studies (Senyiğit et al., 2010; Padula et al., 2019).

Stability studies: For stability evaluations, the optimum gel formulations containing NP were stored at 4±1°C in a refrigerator and at 25±2°C with a relative humidity of 60% for a period of 6 months in a stability cabinet (Nuve ID 300, Ankara, Turkey). The gels were scrutinized for alterations in macroscopic appearance, pH, and mechanical properties (n=3).

Statistical data analysis: All experiments were performed at least three times and data are expressed as mean values ± standard deviation. Statistical data analysis was performed using the Student's t-test with a minimum significance level of $p<0.05$.

## RESULTS AND DISCUSSION

### Preparation of NPs

NPs were prepared through the spontaneous emulsification technique, as outlined by Rençber et al. (Rençber et al., 2016). An inherent advantage of this method lies in its ability to circumvent the use of harmful organic solvents. The resulting NP formulation exhibited a clear and consistent appearance.

### Characterization of NPs

#### *Determination of PS, PI and ZP*

Particle size is a critical aspect of characterizing nanoparticle properties, given that particle size significantly influences various nanoparticle characteristics. The size of nanoparticles affects the penetration of drug molecules into the stratum corneum. It has been shown that the penetration and retention of nanoparticles into the skin depends on

the particle size. The optimal size range for topical application and improvement of topical delivery is around 100-700 nm (Xiang et al., 2023; Liu et al., 2018). PS is crucial for drug delivery and clearance. The PS of blank NP and LZD-loaded NP were found to be 184.93±4.87 nm and 195.27±5.42 nm, respectively (Table 2). The PS value slightly increased with the addition of LZD ($p < 0.05$). Similar results of 182.6±8.4 and 225.1±7.4 nm were obtained by Puglia et al. (Puglia et al., 2016).

The PI of blank NP and LZD-loaded NP were found 0.170±0.030 and 0.214±0.019, respectively (Table 2). The increase in PDI value of LZD-loaded NP was significant ($p < 0.05$) compared to blank NPs, but the PDI value was still around 0.3. PI of blank NP and LZD-loaded NP was low (PI<0.3), showing that this method of preparation resulted in appropriate size distribution, a narrow dispersity, high physical stability and uniform system (Table 2) (Aksu et al., 2014; Behbahani et al., 2017).

The stability of NPs is significantly indicated by the ZP, which is a crucial factor. The ZP of blank and LZD-loaded NP was found to be 26.40±1.04 and 20.57±0.35 mV, as represented in Table 2. ($p < 0.05$). The incorporation of LZD into nanoparticles appears to cause a significant decrease in zeta potential, which can be attributed to the preferential adsorption of counter ions or hydrogen ions on the nanoparticle shell with the addition of the active substance (Behbahani et al., 2017). The findings support a previous study by Salatin et al. (Salatin et al., 2017), who determined that NPs made with Eudragit have a positive charge surface.

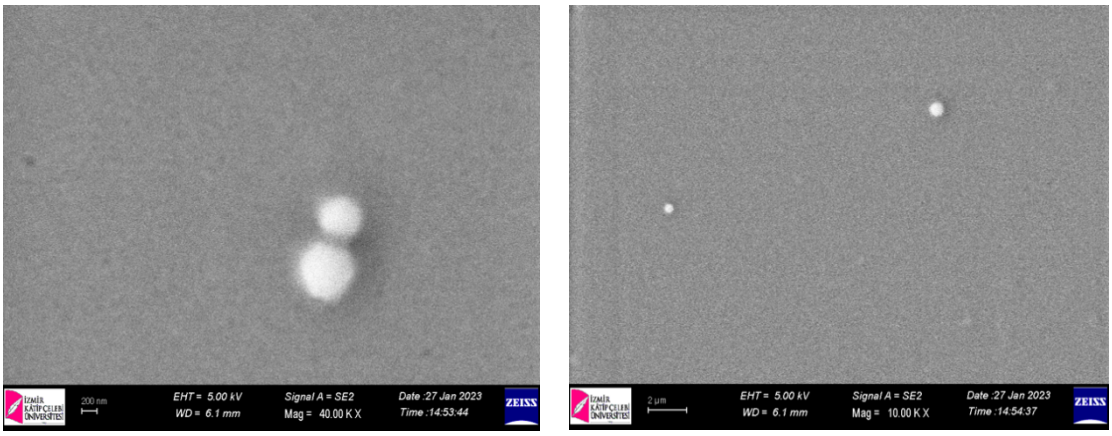
**Table 2.** PS, PI, and ZP of NP formulations

| | PS (nm) ± SD | PI ± SD | ZP (mV) ± SD |
|---|---|---|---|
| **Blank NP** | 184.93±4.87 | 0.170±0.030 | 26.40±1.04 |
| **LZD-loaded NP** | 195.27±5.42 | 0.214±0.019 | 20.57±0.35 |

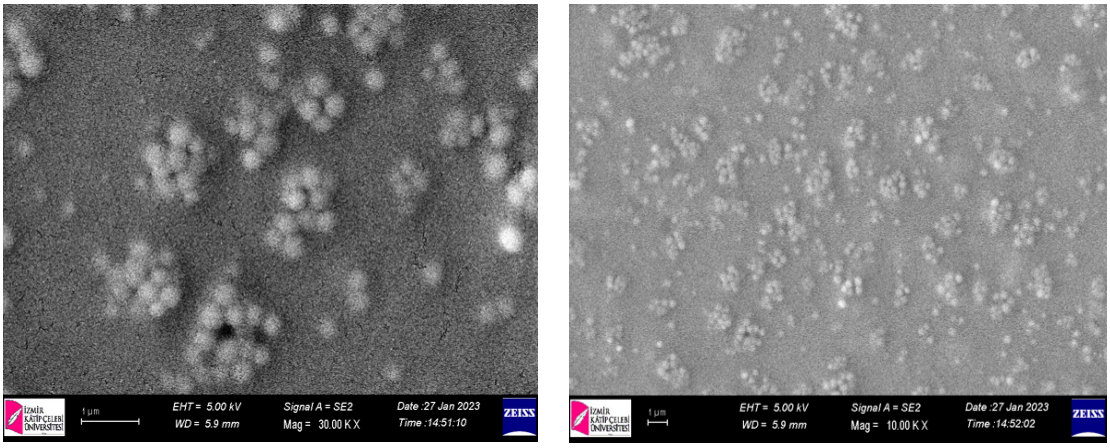
***Morphology analysis***

The SEM analysis of blank and LZD-loaded NP formulation showed spherical-shaped as shown in

Figure 1. The average particle size aligns with the results obtained using the Nano-ZS Zetasizer.




Blank NP formulation (at 40.00x and 10.00x magnifications)




LZD-loaded NP (at 30.00x and 10.00x magnifications)

**Figure 1.** SEM photographs of the NPs at 40.00x, 30.00x and 10.00x magnifications

***Drug entrapment efficiency***

The concentration of LZD in the aqueous phase was measured using a validated spectrophotometric method. The calibration curve for LZD exhibited linearity within the concentration range of 20-150 µg/mL, with a correlation coefficient ($r^2$) of 0.9999. The detection, and limit of quantification for LZD were determined to be 3.170, and 9.607 µg/mL, respectively.

As expected, the prepared LZD-loaded NPs exhibited a high drug EE of 99.746±0.021%. This elevated EE% is likely attributed to the high solubility of LZD in the selected solvent. The high EE% implies an outstanding level of protection against environmental stressors, including oxygen, moisture, pH fluctuations, and temperature variations (Bertrand et al., 2023). In the study of Enazy et al. (Enazy et al., 2023) in 2023, the EE of polymeric NP was 99.1%. The optimized NP dispersion was then introduced into the gel.

**Preparation of gel formulations**

In the present study, gels were successfully formulated using Methocel™ K4M (HPMC) and Carbopol® 974 P NF improve in terms of the patient compliance and comfort. Methocel™ is a biodegradable, polar, non-toxic, and non-irritant polymer with water solubility that undergoes swelling upon contact with a solution, leading to the formation of a gel mass (Yousaf et al., 2021; Rençber et al., 2019; Feroz & Dias 2021). It has received approval from the FDA for utilization in controlled-release formulations (Feroz & Dias 2021). Carbopol®, a poly(acrylic acid) (PAA) polymer, possesses good biocompatibility, biodegradability, and low toxicity. This polymer

is valuable as mucoadhesive agent, demonstrating controlled drug release profiles (Huei-Jen et al., 2006; Surassmo et al., 2015).

**Characterization of gel formulations**

***Macroscopic appearance of gels***

The gel formulations were observed to have a translucent appearance, providing a smooth feel upon application and exhibiting homogeneity.

***Determination of pH***

The pH of the formulation is significant for topical drug delivery systems. A small change in the pH of the topical formulation can cause skin irritation during application (Wagas et al., 2022; Rençber et al., 2019; Rençber & Tanrıverdi, 2018). The gel formulations exhibited pH values ranging from 6.15±0.107 to 7.23±0.092. The pH values of F1 + LZD NP and F4 + LZD NP were found to be 5.94±0.038 and 5.99±0.137, respectively (Table 3). The results suggest that the prepared gels are suitable, as their pH values are close to the normal pH of the skin.

**Table 3.** pH values of formulations

| Formulation Code | pH ± SD |
|---|---|
| **F1** | 6.15±0.107 |
| **F2** | 6.35±0.038 |
| **F3** | 6.28±0.065 |
| **F4** | 6.39±0.006 |
| **F5** | 6.44±0.072 |
| **F6** | 7.23±0.092 |
| **F1 + LZD NP** | 5.94±0.038 |
| **F4 + LZD NP** | 5.99±0.006 |

***Mechanical properties of gel formulations***

Mechanical properties (hardness, compressibility, adhesiveness, elasticity, and cohesiveness) were examined using software controlled penetrometer (Table 4).

The hardness is an essential parameter in TPA. This property refers to the force necessary to achieve a specific deformation in semi-solid systems. Hardness and compressibility characterize the stress and work needed to dispense a sample from the container and apply it to the desired site. For topical applications, low hardness and low compressibility for easy removal from the container are desirable. Furthermore, the literature demonstrates a correlation between viscosity and hardness (Jones et al., 1997; Toksoy et al., 2013; Rençber et al., 2019; Rençber et al., 2021). Gels prepared with Carbopol® and Methocel polymers were evaluated separately to decide the ideal polymer ratio. Both gel hardness and compressibility increased as a function of growing polymer concentrations. It was observed that the F1 formulation had the lowest hardness and lowest compressibility value among the gels prepared with Carbopol®. Among the gels prepared with Methocel, the F4 formulation had the lowest hardness and compressibility value (Table 4). Therefore, among the gel formulations, F1 and F4 formulations were selected as optimum formulation for both groups. After loading the nanoparticles into the gels, an increase in the hardness values of the gel formulations was observed. This situation can be explained by concentration-dependent effects on the formulations' viscosity. This is also supported by the literature (Karavana et al., 2012). F4 + LZD NP had lower hardness value, lower compressibility and the lower viscosity value than F1 + LZD NP (Table 4, Figure 2). Significant differences were observed in the values of hardness and compressibility ($p<0.05$).

Elasticity signifies the speed at which a deformed sample reverts to its original, undeformed state. Lower numerical values obtained through Texture Profile Analysis (TPA) indicate more excellent elasticity (Rençber et al., 2021). Among gel formulations containing NPs, F4 + LZD NP formulation has the most appropriate elasticity value. After loading the NPs into the F4 coded gel formulation, an decrease in the elasticity values of the gel formulation was observed. This situation is supported by the literature (Karavana et al., 2012).

**Table 4.** Mechanical properties of the gels

| Code | Temperature | H (g) ± SD | C (g·sec) ± SD | A (g·sec) ± SD | E ± SD | Ch ± SD |
|---|---|---|---|---|---|---|
| **F1** | 25°C | 0.133±0.002 | 0.511±0.008 | -0.380±0.026 | 1.026±0.046 | 1.045±0.020 |
| | 32°C | 0.104±0.007 | 0.309±0.084 | -0.256±0.072 | 0.854±0.038 | 1.060±0.047 |
| **F2** | 25°C | 0.157±0.006 | 0.634±0.071 | -0.478±0.063 | 1.055±0.015 | 1.052±0.022 |
| | 32°C | 0.121±0.016 | 0.202**±0.**032 | -0.190±0.088 | 1.009±0.083 | 1.053±0.066 |
| **F3** | 25°C | 0.153±0.003 | 0.564±0.083 | -0.398±0.077 | 1.006±0.018 | 1.089±0.042 |
| | 32°C | 0.201±0.011 | 0.840±0.207 | -0.665±0.200 | 1.014±0.029 | 1.047±0.047 |
| **F4** | 25°C | 0.013±0.000 | 0.048±0.004 | -0.042±0.003 | 0.877±0.008 | 1.127±0.012 |
| | 32°C | 0.009±0.000 | 0.026±0.000 | -0.022±0.000 | 0.891±0.000 | 1.238±0.000 |
| **F5** | 25°C | 0.017±0.001 | 0.064±0.002 | -0.068±0.001 | 0.909±0.007 | 1.149±0.009 |
| | 32°C | 0.012±0.000 | 0.038±0.004 | -0.047±0.011 | 0.870±0.021 | 1.195±0.014 |
| **F6** | 25°C | 0.020±0.001 | 0.076±0.005 | -0.087±0.008 | 0.939±0.018 | 1.119±0.014 |
| | 32°C | 0.013±0.000 | 0.033±0.002 | -0.053±0.007 | 0.913±0.016 | 1.187±0.004 |
| **F1 + LZD NP** | 25°C | 0.181±0.004 | 0.503±0.008 | -0.206±0.011 | 1.012±0.056 | 1.043±0.047 |
| | 32°C | 0.144±0.003 | 0.216±0.005 | -0.206±0.011 | 1.049±0.068 | 1.033±0.058 |
| **F4 + LZD NP** | 25°C | 0.038±0.001 | 0.068±0.009 | -0.163±0.001 | 0.973±0.013 | 1.063±0.001 |
| | 32°C | 0.031±0.002 | 0.060±0.014 | -0.136±0.013 | 0.990±0.049 | 1.140±0.006 |

*H: Hardness, C: Compressibility, A: Adhesiveness, E: Elasticity, Ch: Cohesiveness

***Viscosity Measurement***

The viscosity of the gel formulations was assessed using a Brookfield viscometer at a temperature of 25°C. The viscosity value increased significantly with increasing the hardness and compressibility of gels (Jones et al., 1997).

In gel formulations, Carbopol® and Methocel create a physically bonded network through the formation of junction sites, contributing to the mechanical strength of the gel. Incorporating of NPs in the F1 formulation resulted in a slight reduction in gel viscosity. This effect may be attributed to the low viscosity of the NPs and potential electrostatic interactions with the gel network (Saez et al., 2019). Contrastingly, the inclusion of NPs in the F4 formulation led to a slight increase in gel viscosity. This situation suggests no apparent interactions between the NPs and Carbopol® chains. The viscosity of F4+LZD NP increased slightly with the increase of particle size of NP. This situation is compatible with the literature (Liu et al., 2008). The viscosity values were ranked as F1>F1+LZD NP>F4+LZD NP>F4, respectively. These findings are consistent with TPA studies.

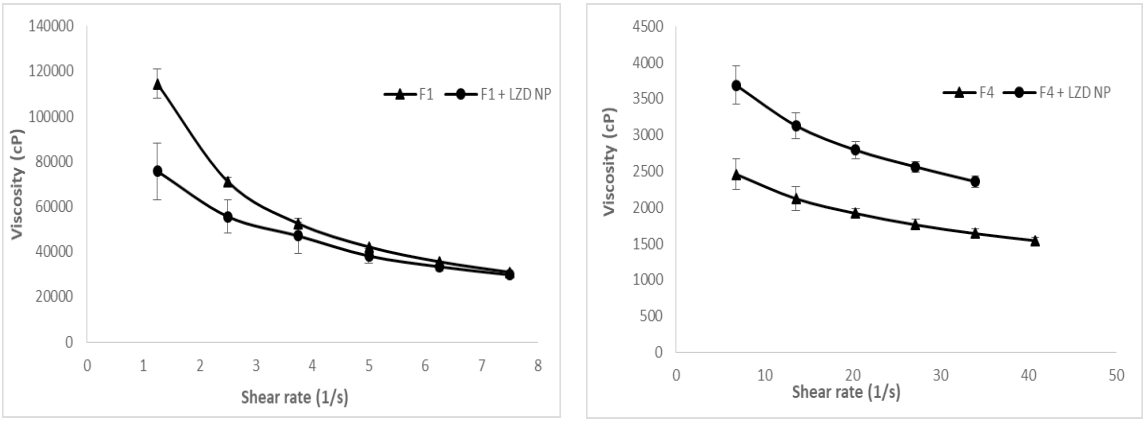



**Figure 2.** Viscosity values of the optimum formulations

***Spreadability studies***

Spreadability is a crucial property of gels as it reflects the behavior of the gel when applied to the skin. It is the term used to describe the ease with which the gel spreads over the skin surface (Nikumbh et al., 2015). Spreadability plays a crucial role in ensuring the stability and ease of application of topical gels (Hussain et al., 2016; Pathan et al., 2018). Moreover, spreadability has a direct impact on the therapeutic efficacy of the drug. It promotes the uniform application of the gel on the skin, contributing to increasing patient acceptance (Ergin et al., 2023; Lesieur et al., 1993). A gel's suitability for skin is determined by its effective spreadability, which is closely linked to its resilience against external forces. Endurance, measured by the force applied to surface deformation, is interconnected with spreadability, representing the overall structural response to external forces. Even if two products exhibit similar firmness, their skin application may differ. Hence, firmness and shear work are commonly assessed in tandem (Ergin et al., 2023).

The spreadability of the gel depends upon the polymer type, polymer rate, hardness, and viscosity of the gel. The spreadability of optimum gels selected from each group (Carbopol® and Methocel™ gels) is shown in Table 5. When the spreadability data are analysed, as the viscosity value increases in the formulations, the required work and firmness of the gels also increase. As this component increases, the spreadability of the prepared gel decreases. The spreadability of the prepared gels was significantly reduced with increasing the viscosity of formulations. The obtained result aligns with expectations and is consistent with findings reported in the literature ($p \geq 0.05$) (Ergin et al., 2023; Garg et al., 2022).

**Table 5.** Spreadability of gels

| Formulation Code | Temperature | Work of Shear ± SD (g.sec) | Firmness ± SD (g) | Stickiness ± SD (g) |
|---|---|---|---|---|
| **F1** | 25°C | 421.398±18.118 | 491.771±30.280 | -482.154±20.481 |
| | 32°C | 451.562±13.284 | 528.920±13.779 | -528.807±14.529 |
| **F4** | 25°C | 39.480±3.582 | 61.785±6.006 | -97.646±9.460 |
| | 32°C | 48.311±1.014 | 79.946±1.705 | -125.096±2.517 |
| **F1 + LZD NP** | 25°C | 339.866±2.104 | 359.980±9.031 | -314.640±0.437 |
| | 32°C | 367.459±21.572 | 394.207±49.815 | -334.006±37.946 |
| **F4 + LZD NP** | 25°C | 65.556±1.918 | 111.845±3.141 | -158.497±26.781 |
| | 32°C | 98.202±9.279 | 201.515±34.822 | -300.152±49.413 |

***Flow properties***

The flow property of a semi-solid formulation is crucial as it significantly influences the success of the formulation as a drug delivery system. The flow curves showed a decrease in the viscosity of all formulations with increased shear rate. All the prepared formulations were identified to exhibit a non-Newtonian pseudoplastic (shear-thinning) behavior, as evidenced by their observed flow curves (Figure 3). Our findings demonstrated sheer thinning, in line with the literatures (Rençber et al., 2019; Rençber & Karavana 2020; Mariane et al., 2023). Furthermore, as can be seen from the graphs, when the effect of LZD NP addition in gel formulations was examined, a decrease in viscosity values was observed in the F1 formulation and an increase in the F4 formulation. These findings are consistent with TPA studies.

The flow curves showed a decrease in the viscosity of all formulations with an increase in shear rate. This reflects the shear-thinning behavior of a pseudoplastic (non-Newtonian) system (Figure 2).

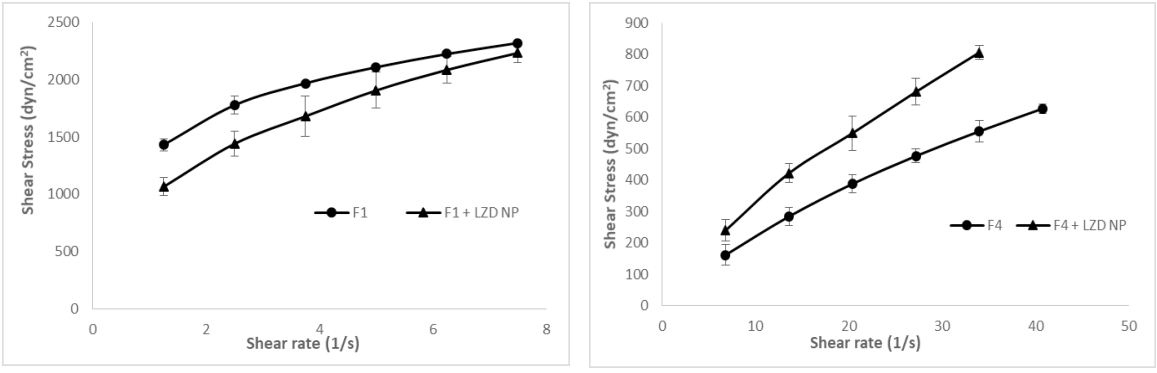



**Figure 3.** Flow curve of gel formulations

***In vitro drug release studies***

Figure 4 illustrates the *in vitro* release of LZD from NP and gel formulations containing NP over 24 hours, conducted using a dialysis bag. At the end of 8 hours, the LZD NP reached 84.86% release, the F1+LZD NP reached 100% release, and the F4+LZD NP reached 80% release. As a result, the release profile of gel formulations containing NP was found to be close NP dispersion. This is thought to be due to the rapid erosion of Carbopol® 974 P NF and HPMC K100M, which are polymers soluble in distilled water, in aqueous media, resulting in increased LZD release.

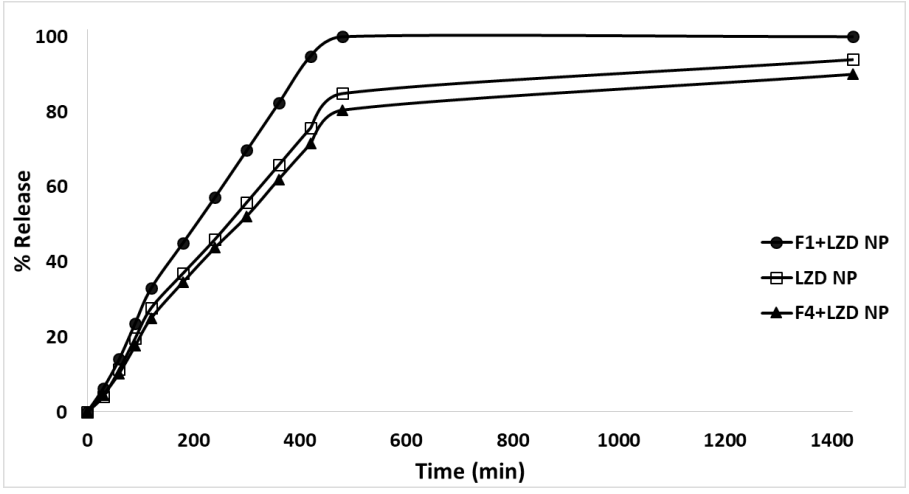



**Figure 4.** *In vitro* release profile of NPs

***Release kinetics***

Kinetic models are analyzed to determine their impact on the stability and release kinetics of drugs. Commonly used mathematical models are the zero-order kinetic model, first-order kinetic model, Higuchi model, Hixson-Crowell model and Korsmeyer-Peppas model. First order describes release from systems where the release rate is concentration dependent. Higuchi describes the release of drugs from the insoluble matrix as the square root of a time-dependent process based on Fickian diffusion. The Hixon–Crowell cube root law describes the release from systems where there is a change in the surface area and diameter of the particles or tablets. The zero-order describes the systems where the drug release rate is independent of its concentration.

As given in Table 6, according to the $r^2$ values obtained, LZD release kinetics from LZD NP, F1+LZD NP, F4+LZD NP formulations was determined as a zero-order kinetic model. Therefore, it can be said that the same amount of LZD is released from the formulation per unit time. The release is time-dependent and concentration-independent. This finding agrees with the study conducted by Dandagi et al. (Dandagi et al., 2020) in which zero-order release kinetics were obtained from LZD-loaded niosome gel. Although different release kinetics (Higuchi) have been reported for LZD in the literature, this result was obtained using chitosan NPs (Alkholief et al., 2023).

**Table 6.** The evaluation of the release kinetic of LZD from formulations

| | Zero order | | | First order | | | Higuchi | | | Hixon-Crowell | | | Korsmeyer-Peppas | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Code** | **$r^2$** | **n** | **M** | **$r^2$** | **n** | **m** | **$r^2$** | **n** | **m** | **$r^2$** | **N** | **M** | **$r^2$** | **N** | **m** |
| **LZD NP** | 0.9935 | 2.2773 | 0.1767 | 0.7165 | 0.7255 | 0.0031 | 0.9483 | 14.561 | 4.1629 | 0.7722 | 1.5432 | 0.007 | 0.9799 | 0.7988 | 1.033 |
| **F1+LZD NP** | 0.9966 | 2.3352 | 0.2226 | 0.7049 | 0.8084 | 0.0031 | 0.9439 | 5.2245 | 18.619 | 0.7792 | 1.6663 | 0.0075 | 0.9924 | 0.6156 | 0.9959 |
| **F4+LZD NP** | 0.9955 | 1.8188 | 0.1675 | 0.7301 | 0.7114 | 0.003 | 0.9453 | 14.013 | 3.936 | 0.7786 | 1.5114 | 0.0069 | 0.9907 | 0.7676 | 1.0081 |

***Ex vivo drug diffusion study***

Based on *in vitro* characterization studies, the F4+LZD NP formulation exhibited suitable hardness, compressibility, elasticity, and viscosity. To determine whether LZD would accumulate or penetrate the skin, diffusion cells were used in *ex vivo* drug penetration and permeation studies on tissue. In these studies, 0.007±0.004% of F1+LZD NP was detected in the receptor compartment. The low drug permeability results obtained are consistent with the fact that gels are semi-solid formulations and drug release is based on swelling and drug diffusion. Furthermore, topical drug delivery systems such as the developed gel do not cause systemic effects, making it a safe method of application for children and pregnant women. Therefore, the optimized gel formulation was determined to have the potential to deliver LZD by topical application through the skin (Singh et al., 2016; Okur et al., 2018; Ay Şenyiğit et al., 2021).

***Stability studies***

Stability studies for the optimal gel formulation were conducted at temperatures of 4±1°C and 25±2°C for 3 months. No significant changes in the macroscopic appearance, pH, and mechanical properties of the gels were observed during this period ($p \geq 0.05$) (Table 7-Table 8).

**Table 7.** pH results of the stability study (n=3)

| | Formulation Code | pH±SD | pH±SD | pH±SD | pH±SD |
|---|---|---|---|---|---|
| | | Beginning | 1. month | 2. month | 3. month |
| **5±2°C** | F4 | 6.39±0.006 | 6.37±0.006 | 6.37±0.006 | 6.37±0.006 |
| | F4+LZD NP | 5.99±0.006 | 5.98±0.010 | 5.97±0.006 | 5.98±0.01 |
| **25±2°C/ %60±5** | F4 | 6.39±0.006 | 6.38±0.006 | 6.37±0.006 | 6.37±0.006 |
| | F4+LZD NP | 5.99±0.006 | 5.98±0.006 | 5.98±**0.006** | 5.97±**0.01** |
| **40±2°C/%75±5** | F4 | 6.39±0.006 | 6.37±0.006 | 6.36±0.006 | 6.34±0.006 |
| | F4+LZD NP | 5.99±0.006 | 5.95±0.006 | 5.93±0.011 | 5.90±0.006 |

**Table 8.** Mechanical properties of gel formulations throughout stability

| Code | Months | Stability Conditions | H (g) ± SD | C (g·sec) ± SD | A (g·sec) ± SD | E ± SD | Ch ± SD |
|---|---|---|---|---|---|---|---|
| F4 | Beginning | - | 0.013±0.000 | 0.048±0.004 | -0.042±0.003 | 0.877±0.008 | 1.127±0.012 |
| F4 + LZD NP | | - | 0.038±0.001 | 0.068±0.009 | -0.163±0.001 | 0.973±0.013 | 1.063±0.001 |
| F4 | 1. month | 5±2°C | 0.013±0.000 | 0.047±0.002 | -0.040±0.004 | 0.865±0.001 | 1.127±0.030 |
| | | 25±2°C/ %60±5 | 0.015±0.000 | 0.045±0.002 | -0.039±0.003 | 0.874±0.009 | 1.176±0.023 |
| | | 40±2°C/%75±5 | 0.015±0.000 | 0.046±0.002 | -0.038±0.000 | 0.857±0.013 | 1.120±0.044 |
| | | 5±2°C | 0.039±0.000 | 0.062±0.002 | -0.157±0.002 | 0.969±0.010 | 1.055±0.004 |
| F4 + LZD NP | | 25±2°C/ %60±5 | 0.038±0.000 | 0.065±0.002 | -0.158±0.003 | 0.975±0.002 | 1.054±0.021 |
| | | 40±2°C/%75±5 | 0.039±0.001 | 0.059±0.001 | -0.149±0.005 | 0.948±0.32 | 1.030±0.040 |
| F4 | 2. month | 5±2°C | 0.014±0.000 | 0.042±0.005 | -0.041±0.003 | 0.904±0.097 | 1.127±0.005 |
| | | 25±2°C/ %60±5 | 0.013±0.000 | 0.046±0.009 | -0.039±0.012 | 0.890±0.083 | 1.121±0.076 |
| | | 40±2°C/%75±5 | 0.015±0.000 | 0.046±0.014 | -0.034±0.015 | 0.900±0.020 | 1.117±0.105 |
| F4 + LZD NP | | 5±2°C | 0.038±0.000 | 0.059±0.007 | -0.158±0.001 | 0.974±0.002 | 1.155±0.009 |
| | | 25±2°C/ %60±5 | 0.039±0.000 | 0.062±0.002 | -0.156±0.003 | 0.963±0.083 | 1.159±0.008 |
| | | 40±2°C/%75±5 | 0.040±0.001 | 0.056±0.002 | -0.149±0.003 | 0.962±0.025 | 1.144±0.013 |
| F4 | 3. month | 5±2°C | 0.015±0.000 | 0.045±0.003 | -0.042±0.002 | 0.902±0.081 | 1.116±0.068 |
| | | 25±2°C/ %60±5 | 0.014±0.000 | 0.046±0.004 | -0.040±0.004 | 0.917±0.111 | 1.129±0.024 |
| | | 40±2°C/%75±5 | 0.015±00.00 | 0.045±0.014 | -0.036±0.003 | 0.916±0.028 | 1.131±0.001 |
| | | 5±2°C | 0.039±0.001 | 0.064±0.011 | -0.158±0.002 | 0.968±0.012 | 1.058±0.017 |
| F4 + LZD NP | | 25±2°C/ %60±5 | 0.039±0.000 | 0.059±0.009 | -0.157±0.000 | 0.948±0.003 | 1.042±0.003 |
| | | 40±2°C/%75±5 | 0.040±0.001 | 0.039±0.006 | -0.148±0.003 | 0.933±0.026 | 1.050±0.006 |

*H: Hardness, C: Compressibility, A: Adhesiveness, E: Elasticity, Ch: Cohesiveness

## CONCLUSION

This study involved the preparation and evaluation of gel formulations containing NPs with LZD to treat DFU. The formulations were assessed for various properties, including PS, PI, ZP, morphology, drug entrapment efficiency, pH, mechanical properties, viscosity, spreadability, flow, *in vitro* drug release, *ex vivo* drug diffusion, and stability. The prepared NP showed a PS of 195.27±5.42 nm, PI of 0.214±0.019, a ZP of 20.57±0.35 mV and EE of 99.746±0.021%. The rheological analysis of the gels indicated a pseudoplastic flow and exhibited typical gel spectra. Furthermore, texture analysis demonstrated that the developed gel formulations had appropriate consistency. The optimized F4+ LZD NP showed sustained drug release over eight hour. In the *ex vivo* drug diffusion studies, it was determined that 0.007±0.004% of LZD permeated into the receptor phase, indicating a local effect.

The novelty and contribution of this research lie in the formulation of a topical gel incorporating nanoparticles for the treatment of DFU. The formulated gels demonstrated favorable characteristics, including an appropriate pH value, suitable mechanical

performance, and desirable viscosity and spreadability for topical application. The initial findings suggest that the formulated topical gel containing LZD-loaded NPs holds promise as an effective drug delivery system for DFU management. However, additional investigations, such as cytotoxicity assessments and *in vivo* animal studies, would be benefit for further evaluation.

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**AUTHOR CONTRIBUTION STATEMENT**

Concept – S.R., H.H.A.; Design – S.R., H.H.A.; Supervision – S.R.; Resources – S.R., H.H.A.; Materials – S.R., H.H.A.; Data Collection and/or Processing – Y.P., H.H.A., S.R.; Analysis and/or Interpretation – Y.P., H.H.A., S.R.; Literature Search – Y.P., S.R., H.H.A.; Writing – S.R., H.H.A.; Critical Reviews – Y.P., H.H.A., S.R.

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*RESEARCH ARTICLE*

# Linker and Tail Group Modifications on 2-((4-isopropyl-4H-1,2,4-triazol-3-yl)thio)-N-(4-phenoxyphenyl) Acetamide to Improve SIRT2 Inhibitory Potency

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***Linker and Tail Group Modifications on 2-((4-isopropyl-4H-1,2,4-triazol-3-yl)thio)-N-(4-phenoxyphenyl)Acetamide to Improve SIRT2 Inhibitory Potency***

***SUMMARY***

*Interest in SIRT2 has grown continuously over recent years, resulting in accumulated evidence that overexpression of SIRT2 is associated with many disorders, and its inhibition delays the progression of pathologies. Hence, targeting SIRT2 may be of therapeutic relevance, and inhibiting SIRT2 activity is a promising therapeutic strategy for severe diseases. The overarching aim of the work presented herein was to improve the SIRT2 inhibition potentials of our initial hits by modifying both linker and tail groups. Among the title compounds, ST49 (50.07%) and ST60 (54.03%) displayed the best inhibition rates against SIRT2 over SIRT1 and SIRT3. Predicted binding conformations of these compounds to SIRT2 highlighted the impact of the crucial interactions with SIRT2 active site residues on inhibitory activity. These results would provide structural guidance for future related design efforts.*

***Key Words:*** *Drug design, hit optimization, inhibitor, molecular modeling, SIRT2*

***SIRT2 İnhibitör Etkisini Geliştirmek Amacıyla 2-((4-izopropil-4H-1,2,4-triazol-3-il)tiyo)-N-(4-fenoksifenil)Asetamit Yapısında Köprü ve Kuyruk Grupları Üzerinde Gerçekleştirilen Modifikasyonlar***

***ÖZ***

*SIRT2 enziminin aşırı ekspresyonunun birçok hastalık ile ilişkili olduğuna ve SIRT2 inhibisyonunun patolojilerin ilerlemesini geciktirdiğine dair çalışmaların varlığı, SİRT2 enzimine olan ilgiyi artırmıştır. Bu nedenle, SIRT2 inhibisyonu ciddi hastalıkların tedavisi için umut verici bir terapötik hedef haline gelmiştir. Bu çalışmada, daha önce bildirilen öncü bileşiğin köprü ve kuyruk grupları üzerinde yapısal modifikasyonlar yapılarak SIRT2 inhibisyon potansiyellerinin geliştirilmesi amaçlanmıştır. Sentezlenen bileşikler arasında ST49 (%50.07) ve ST60 (%54.03), SIRT1 ve SIRT3'e kıyasla SIRT2'ye karşı en iyi inhibisyonu sergilemişlerdir. Bu bileşiklerin öngörülen bağlanma konformasyonları, inhibitor etki için SIRT2 aktif bölgesindeki önemli etkileşimlerin varlığını desteklemiştir. Bu sonuçlar, gelecekteki tasarım çalışmaları için yol gösterici veriler sağlamaktadır.*

***Anahtar Kelimeler:*** *İlaç tasarımı, öncü bileşik optimizasyonu, inhibitör, moleküler modelleme, SIRT2*

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## INTRODUCTION

Epigenetic-modifying enzymes, classified into writers, readers, and erasers, are gaining interest as a potential target for drug discovery (Biswas & Rao, 2018; Ganesan et al., 2019; Lu et al., 2020; Zhang et al., 2023). Sirtuins (SIRTs) are a host of nicotinamide adenine dinucleotide ($NAD^+$)-dependent histone deacetylases (HDACs), acting as epigenetic erasers (Biswas & Rao, 2018; Chen et al., 2015; Frye, 2000). SIRT2, the second member of the SIRT family, has predominantly cytoplasmic localization but shuttles into the nucleus to regulate nucleolar processes during mitosis (Min et al., 2018; Silva et al., 2023). SIRT2 plays a role in a wide range of physiological processes, such as metabolism and control of gene expression, through its deacylase activity on histones and non-histone proteins, including α-tubulin, p53, BubR1, BCL6, FOXO1, FOXO3a, and severe proteins involved in the regulation of metabolic enzymes (Avalos et al., 2002; Jing, Gesta, & Kahn, 2007; North et al., 2003; North et al., 2014; Penteado et al., 2023; Xu et al., 2014; Zhao et al., 2013). Accordingly, the importance of SIRT2 expression levels has been uncovered in a wide range of diseases, such as cancer, neurodegeneration, inflammation, and aging (Chen, Huang, & Hu, 2020; Hong et al., 2021; Kaya & Eren, 2023; Wang et al., 2019). Although research and development of small molecule SIRT2 inhibitors have gained significant momentum (Cai et al., 2023; Eren et al., 2019; Gozelle et al., 2022; Gozelle et al., 2023; Mellini et al., 2017; Quinti et al., 2016; Rumpf et al., 2015; Spiegelman et al., 2019; Sukuroglu et al., 2021; Tantawy et al., 2021; Trapp et al., 2006; Yagci et al., 2021; Yang et al., 2018; Yang et al., 2019), there is still a lack of clinically approved SIRT2 inhibitors and more effort should be focused on novel SIRT2 inhibitor scaffolds with improved efficacy and drug-like physicochemical properties.

Our initial attempt to identify novel scaffolds for SIRT2 inhibition let us obtain hit compounds with confirmed activities from virtual screening (Eren et al., 2019). Herein, motivated by a desire to drive further inhibitor optimization, among the obtained hits, **STH2** was selected and refined by linker and tail group modifications to improve SIRT2 inhibitory potency (Figure 1). The oxygen atom, as a linker between the phenyl ring which was accommodated in the substrate channel of SIRT2 and the central phenyl ring, was replaced by $-CH_2O-$, $-OCH_2-$, -NH-, $-CH_2NH-$, $-NHCH_2-$, $-CH_2-$, and -CO- groups to achieve the needed orientation of terminal phenyl ring allowing the crucial π-π interactions with the residues F119, F131, and F234. In the case of the tail group, which was directed toward the selectivity pocket, modification strategies, including fused ring cyclization, chain cyclization, and bioisosteric replacement, were adopted to access favorable moieties for selectivity pocket occupation, primarily by interacting with the residues Y139 and F190. As a result, fourteen novel analogs, seven of which were *N*-(4-phenoxyphenyl)aryl-carboxamides (**ST47**-**ST53**) and seven were *N*-(aryl)-2-(phenylthio)acetamides (**ST54**-**ST60**) were designed and synthesized, followed by evaluation of their inhibitory activities against SIRT2.

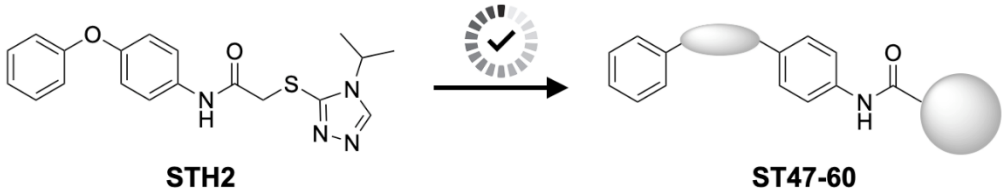



**Figure 1.** Structural modifications on **STH2** yielding novel analogues **ST47**-**60**.

## MATERIAL AND METHODS

### Chemistry

All chemicals used in the research were purchased commercially and employed without additional purification. Thin-layer chromatography (TLC) was applied to observe reactions on silica-coated aluminum plates (Silica gel 60 $F_{254}$, Merck) using UV light at 254 or 365 nm wavelengths. Using tetramethylsilane as the internal standard, $^{1}$H-NMR and $^{13}$C-NMR spectra were obtained using a Bruker Avance neo 500 MHz FT-NMR and an Agilent Varian Mercury 400 MHz High-Performance Digital FT-NMR spectrometers. The chemical shifts were identified as δ (ppm), whereas the coupling constants were expressed as Hertz. The Waters LCT Premier XE Mass Spectrometer was used to acquire high-resolution mass spectra data (HRMS). The equipment was utilized in electrospray ionization ($ESI^+$) mode and connected to an AQUITY Ultra Performance Liquid Chromatography system (Waters Corporation, Milford, MA, USA) with a UV detector set to monitor at 254 nm wavelength. The purity of all target compounds exceeded 95%. The melting points were determined using the Stuart SMP50 automated melting point instrument without correction.

4-Phenoxyaniline (1a): The synthesis of 1a was performed as previously reported (Lanning et al., 2016; Ma & Rao, 2003; Yagci et al.,2021). Yield: 37%, white solid. Mp 84.1-84.4 °C. CAS: 139-59-3. HRMS (ESI/TOF) *m/z*: $[M+ACN+H]^+$ Calcd for $C_{14}H_{15}N_2O$ 227.1184; Found 227.1182.

2-Bromo-*N*-(4-phenoxyphenyl)acetamide (1b): The synthesis of 1b was performed as previously reported (Han et al., 2012). Yield: 55%, white solid. Mp 109.5-109.9 °C. CAS: 36160-85-7. HRMS (ESI/TOF) *m/z*: $[M+H]^+$ Calcd for $C_{14}H_{13}BrNO_2$ 306.0130; Found 306.0121.

2-(Phenylthio)acetic acid (**2a**): The synthesis of **2a** was performed as previously reported (Xie et al., 2017). Yield: 85%, white solid. Mp 60.0-60.9 °C. CAS: 103-04-8. HRMS (ESI/TOF) *m/z*: $[M+ACN+H]^+$ Calcd for $C_8H_9O_2S$ 210.0589; Found 210.0587.

*N*-(4-Aminophenyl)-2-(phenylthio)acetamide (**2b**): Initially, *N*-(4-nitrophenyl)-2-(phenylthio)acetamide (CAS: 220518-16-1) was synthesized by amidation of **2a** (1 mmol) and 4-nitroaniline (1 mmol) in the presence of oxalyl chloride by using described method by Gozelle et al. (2022). Next, *N*-(4-nitrophenyl)-2-(phenylthio)acetamide (1 mmol), without performing additional purification, was refluxed in ethanol for 6 h in the presence of $SnCl_2.H_2O$ (5 mmol). After the reaction was finished, the reaction mixture was concentrated *in vacuo*, and the crude was dissolved in a 10% aqueous solution $NaHCO_3$ solution. The aqueous phase was extracted with ethyl acetate (3x10 ml), and the organic phase was subjected to washing with brine. After being dried over anhydrous $Na_2SO_4$, the organic phase was concentrated *in vacuo*. The pure 2b was obtained by purifying the crude product via silica-based column chromatography using an elution system of *n*-hexane:ethyl acetate (80:20). Yield: 61%, white solid. Mp >300 °C. CAS: 1019393-66-8. HRMS (ESI/TOF) *m/z*: $[M+H]^+$ Calcd for $C_{14}H_{15}N_2OS$ 259.0905; Found 259.0905.

*N*-(4-formylphenyl)-2-(phenylthio)acetamide (**2c**): Oxalyl chloride (2 mmol) was added to a solution of **2a** (1 mmol) and a catalytic amount of DMF in DCM at 0 °C, and the reaction mixture was stirred at room temperature for 2 h. The solvent was removed *in vacuo* after acyl chloride was formed. A solution of obtained 2-(phenylthio)acetyl chloride (theoretically 1 mmol) in DCM was added dropwise to a solution of 4-aminobenzaldehyde (1 mmol) and DIPEA (1.5 mmol) in DCM at 0 °C and stirred at room temperature for 4 h. Without additional purification. The resulting **2c** was used in the following step. Yield: 45%, yellowish oil. CAS: 1977315-84-6. HRMS (ESI/TOF) *m/z*: $[M+H]^+$ Calcd for $C_{15}H_{14}NO_2S$ 272.0745; Found 272.0737.

General synthesis method A through acyl chloride-mediated amide formation (ST47, ST48, ST52-ST56, ST59, ST60): Oxalyl chloride (2 mmol) was added to a solution of an appropriate carboxylic acid (1 mmol), and a catalytic amount of DMF in DCM at 0

°C. Then, the mixture was stirred for 2 h at room temperature. The solvent was removed *in vacuo* after acyl chloride was formed. A solution of obtained appropriate acyl chloride (theoretically 1 mmol) in DCM was added dropwise to a solution of an appropriate amine (1 mmol) and DIPEA (1.5 mmol) in DCM at 0 °C. The mixture was stirred at room temperature for 2-4 h. After the reaction was completed, the reaction mixture was diluted with DCM and subjected to sequential washing with 0.1 M HCl, 1% aqueous solution of $NaHCO_3$, and brine. After being dried over anhydrous $Na_2SO_4$, the combined organic phase was concentrated *in vacuo*. While ST47, ST48, and ST53 were purified by recrystallization from ethanol/water, ST52, ST54, ST55, ST56, ST59, and ST60 were purified by silica-based column chromatography using an elution system of *n*-hexane:ethyl acetate (85:15).

General synthesis method B through EDC/HOBt-mediated amide formation (ST50, ST51): A solution of an appropriate carboxylic acid (1 mmol), HOBt (1 mmol), EDC (1 mmol), and DIPEA (1.5 mmol) in DCM was stirred at room temperature for 30 min. After adding 1a (1 mmol), the reaction mixture was stirred overnight at room temperature until the reaction was completed. Then, the reaction mixture was diluted with DCM and subjected to sequential washing with 0.1 M HCl, 1% aqueous solution of $NaHCO_3$, and brine. After being dried over anhydrous $Na_2SO_4$, the combined organic phase was concentrated *in vacuo*. The crude product underwent purification via silica-based column chromatography using an elution system of *n*-hexane:ethyl acetate (85:15).

*N*-(4-Phenoxyphenyl)-3-phenylpropanamide (ST47): Obtained following the general synthesis method A from 3-phenylpropanoyl chloride (theoretically 1 mmol), 1a (1 mmol), and DIPEA (1.5 mmol). Yield: 45%, white solid. Mp 133.4-133.7 °C. $^1$H NMR ($CDCl_3$, 400 MHz): δ 7.37 (d, *J*=9.2 Hz, 2H), 7.27-7.33 (m, 4H), 7.19-7.25 (m, 3H), 7.14 (br s, 1H), 7.07 (t, *J*=7.4 Hz, 1H), 6.92-6.97 (m, 4H), 3.04 (t, *J*=7.6 Hz, 2H), 2.65 (t, *J*=7.6 Hz, 2H). $^{13}$C-NMR ($CDCl_3$, 100 MHz): δ 170.3, 157.5, 153.5, 140.6, 133.2, 129.7, 128.4, 128.4, 126.4, 123.1, 121.8, 119.5, 118.4, 39.3, 31.6. HRMS (ESI/TOF) *m/z*: $[M+H]^+$ Calcd for $C_{21}H_{20}NO_2$ 318.1494; Found 318.1478.

2-Phenoxy-*N*-(4-phenoxyphenyl)acetamide (ST48): Obtained following the general synthesis method A from 2-phenoxyacetyl chloride (theoretically 1 mmol), 1a (1 mmol), and DIPEA (1.5 mmol). Yield: 62%, white solid. Mp 137.3-137.5 °C. $^1$H NMR (DMSO-$d_6$, 500 MHz): δ 10.10 (s, 1H), 7.67 (d, *J*=8.9 Hz, 2H), 7.37 (t, *J*=8.0 Hz, 2H), 7.33 (t, *J*=8.0 Hz, 2H), 7.11 (t, *J*=7.4 Hz, 1H), 6.97-7.03 (m, 7H), 4.70 (s, 2H). $^{13}$C NMR (DMSO-$d_6$, 125 MHz): δ 166.9, 158.3, 157.7, 152.6, 134.7, 130.4, 130.0, 123.5, 122.0, 121.7, 119.8, 118.4, 115.2, 67.7. HRMS (ESI/TOF) *m/z*: $[M+H]^+$ Calcd for $C_{20}H_{18}NO_3$ 320.1287; Found 320.1295.

2-(Methyl(phenyl)amino)-*N*-(4-phenoxyphenyl) acetamide (ST49): A solution of *N*-methylaniline (1.1 mmol) and DIPEA (1.1 mmol) in ACN was stirred at room temperature for 15 min. After adding **1b** (1 mmol), the reaction was stirred overnight at 50 °C. Upon completion of the reaction, the reaction mixture was diluted with DCM and subjected to sequential washing with 1% aqueous solution of $NaHCO_3$ and brine. After being dried over anhydrous $Na_2SO_4$, the combined organic phase was concentrated *in vacuo*. The crude product underwent purification via silica-based column chromatography using an elution system of *n*-hexane:ethyl acetate (80:20) to afford ST49. Yield: 24%, white solid. Mp 134.8-135.5 °C. $^1$H NMR ($CDCl_3$, 400 MHz): δ 8.42 (br s, 1H), 7.47-7.51 (d, *J*=9.2 Hz, 2H), 7.26-7.34 (t, *J*=7.6 Hz, 4H), 7.09 (t, *J*=7.2 Hz, 1H), 6.96-7.01 (m, 4H), 6.91 (t, *J*=7.6 Hz, 1H), 6.84 (d, *J*=9.2 Hz, 2H), 3.97 (s, 2H), 3.09 (s, 3H). $^{13}$C NMR ($CDCl_3$, 100 MHz): δ 168.6, 157.5, 153.7, 149.4, 132.7, 129.7, 129.5, 123.1, 121.6, 119.6, 119.4, 118.4, 113.7, 60.0, 40.1. HRMS (ESI/TOF) *m/z*: $[M+H]^+$ Calcd for $C_{21}H_{21}N_2O_2$ 333.1603; Found 333.1610.

*N*-(4-Phenoxyphenyl)-5-phenylthiophene-2-carboxamide (ST50): Obtained following the general synthesis method B from 5-phenylthiophene-2-car-

boxylic acid (1 mmol), HOBt (1 mmol), EDC (1 mmol), DIPEA (1.5 mmol), and **1a** (1 mmol). Yield: 59%, white solid. Mp 197.4-197.8 °C. $^{1}$H NMR (DMSO-$d_6$, 500 MHz): δ 10.29 (s, 1H), 8.03 (d, *J*=3.9 Hz, 1H), 7.71 (d, *J*=8.6 Hz, 4H), 7.64 (d, *J*=3.9 Hz, 1H), 7.48 (t, *J*=7.4 Hz, 2H), 7.39 (t, *J*=7.9 Hz, 3H), 7.13 (t, *J*=7.4 Hz, 1H), 7.05 (d, *J*=8.6 Hz, 2H), 7.01 (d, *J*=7.9 Hz, 2H). $^{13}$C NMR (DMSO-$d_6$, 125 MHz): δ 160.0, 157.7, 152.9, 148.8, 139.3, 134.9, 133.5, 130.6, 130.5, 129.8, 129.2, 126.2, 124.9, 123.6, 122.6, 119.7, 118.6. HRMS (ESI/TOF) *m*/*z*: [M+H]$^+$ Calcd for $C_{23}H_{18}NO_2S$ 372.1058; Found 372.1059.

*N*-(4-Phenoxyphenyl)benzothiophene-2-carboxamide (ST51): Obtained following the general synthesis method B from benzothiophene-2-carboxylic acid (1 mmol), HOBt (1 mmol), EDC (1 mmol), DIPEA (1.5 mmol), and 1a (1 mmol). Yield: 54%, white solid. Mp 192.2-193.1 °C. $^{1}$H NMR (DMSO-$d_6$, 500 MHz): δ 10.56 (s, 1H), 8.36 (s, 1H), 8.06 (d, *J*=7.4 Hz, 1H), 8.02 (d, *J*=8.4 Hz, 1H), 7.80 (d, *J*=9.0 Hz, 2H), 7.48-7.51 (m, 2H), 7.40 (t, *J*=8.4 Hz, 2H), 7.13 (t, *J*=7.4 Hz, 1H), 7.07 (d, *J*=9.0 Hz, 2H), 7.02 (d, *J*=7.8 Hz, 2H). $^{13}$C NMR (DMSO-$d_6$, 125 MHz): δ 160.6, 157.6, 152.9, 140.9, 140.5, 139.6, 134.9, 130.5, 127.0, 126.2, 125.9, 125.5, 123.6, 123.3, 122.5, 119.7, 118.6. HRMS (ESI/TOF) *m*/*z*: [M+H]$^+$ Calcd for $C_{21}H_{16}NO_2S$ 346.0902; Found 346.0903.

*N*-(4-Phenoxyphenyl)-5-phenylfuran-2-carboxamide (ST52): Obtained following the general synthesis method A, from 5-phenylfuran-2-carbonyl chloride (theoretically 1 mmol), **1a** (1 mmol), and DIPEA (1.5 mmol). Yield: 42%, yellowish oil. $^{1}$H NMR (DMSO-$d_6$, 500 MHz): δ 10.21 (s, 1H), 7.98 (d, *J*=7.2 Hz, 2H), 7.79 (d, *J*=9.0 Hz, 2H), 7.51 (t, *J*=7.4 Hz, 2H), 7.36-7.44 (m, 4H), 7.18 (d, *J*=3.6 Hz, 1H), 7.13 (t, *J*=7.4 Hz, 1H), 7.06 (d, *J*=9.0 Hz, 2H), 7.02 (dd, *J*=8.7 and 1.0 Hz, 2H). $^{13}$C NMR (DMSO-$d_6$, 125 MHz): δ 157.7, 156.4, 155.7, 152.9, 147.1, 134.6, 130.5, 129.8, 129.4, 129.2, 125.0, 123.6, 122.9, 119.7, 118.6, 117.4, 108.3. HRMS (ESI/TOF) *m*/*z*: [M+H]$^+$ Calcd for $C_{23}H_{18}NO_3$ 356.1287; Found 356.1301.

*N*-(4-Phenoxyphenyl)benzofuran-2-carboxamide (ST53): Obtained following the general synthesis method A from benzofuran-2-carbonyl chloride (theoretically 1 mmol), **1a** (1 mmol), and DIPEA (1.5 mmol). Yield: 66%, white solid. Mp 159.0-159.4 °C. $^{1}$H NMR (DMSO-$d_6$, 500 MHz): δ 10.59 (s, 1H), 7.83-7.86 (m, 3H), 7.77 (d, *J*=0.8 Hz, 1H), 7.73 (dd, *J*=8.4 and 0.8 Hz, 1H), 7.51 (t, *J*=7.4 Hz, 1H), 7.35-7.43 (m, 3H), 7.13 (tt, *J*=7.4 and 1.1 Hz, 1H), 7.06 (d, *J*=9.0 Hz, 2H), 7.01 (d, *J*=7.7 Hz, 2H). $^{13}$C NMR (DMSO-$d_6$, 125 MHz): δ 157.6, 157.0, 154.9, 153.0, 149.3, 134.6, 130.5, 127.6, 124.3, 123.6 (2C), 123.4, 122.7, 119.7, 118.6, 112.4, 111.1. HRMS (ESI/TOF) *m*/*z*: [M+H]$^+$ Calcd for $C_{21}H_{16}NO_3$ 330.1130; Found 330.1126.

*N*-(4-(Benzyloxy)phenyl)-2-(phenylthio)acetamide (ST54): Obtained following the general synthesis method A, from 2-(phenylthio)acetyl chloride (theoretically 1 mmol), 4-(benzyloxy)aniline (1 mmol), and DIPEA (1.5 mmol). Yield: 28%, white solid. Mp 150.3-150.8 °C. $^{1}$H NMR (DMSO-$d_6$, 400 MHz): δ 10.07 (s, 1H), 7.30-7.47 (m, 11H), 7.20 (t, *J*=7.2 Hz, 1H), 6.95 (d, *J*=8.8 Hz, 2H), 5.06 (s, 2H), 3.82 (s, 2H). $^{13}$C NMR (DMSO-$d_6$, 100 MHz): δ 166.3, 154.4, 137.1, 135.9, 132.1, 129.0, 128.4, 128.0, 127.8, 127.7, 125.9, 120.7, 114.9, 69.3, 37.3. HRMS (ESI/TOF) *m*/*z*: [M+H]$^+$ Calcd for $C_{21}H_{20}NO_2S$ 350.1215; Found 350.1215.

*N*-(4-(Phenoxymethyl)phenyl)-2-(phenylthio)acetamide (ST55): Obtained following the general synthesis method A, from 2-(phenylthio)acetyl chloride (theoretically 1 mmol), 4-(phenoxymethyl)aniline (1 mmol), and DIPEA (1.5 mmol). Yield: 24%, white solid. Mp 139.5-139.8 °C. $^{1}$H NMR (DMSO-$d_6$, 400 MHz): δ 10.25 (s, 1H,), 7.56 (d, *J*=8.2 Hz, 2H), 7.38 (t, *J*=8.0 Hz, 4H), 7.27-7.33 (m, 4H), 7.19 (t, *J*=7.2 Hz, 1H), 6.98 (d, *J*=8.2 Hz, 2H), 6.92 (t, *J*=7.2 Hz, 1H), 5.01 (s, 2H), 3.85 (s, 2H). $^{13}$C NMR (DMSO-$d_6$, 100 MHz): δ 166.7, 158.2, 138.4, 135.8, 132.0, 129.4, 128.9, 128.4, 128.0, 125.9, 120.5, 119.0, 114.7, 68.7, 37.4. HRMS (ESI/TOF) *m*/*z*: [M+H]$^+$ Calcd for $C_{21}H_{20}NO_2S$ 350.1215; Found 350.1216.

*N*-(4-(Phenylamino)phenyl)-2-(phenylthio)acetamide (ST56): Obtained following the general synthesis method A, from 2-(phenylthio)acetyl chloride (theoretically 1 mmol), *N*-phenyl-1,4-phenylenediamine (1 mmol), and DIPEA (1.5 mmol). Yield: 42%, white solid. Mp 145.0-145.1 °C. $^{1}$H NMR (DMSO-$d_6$, 500 MHz): δ 10.01 (s, 1H), 8.03 (s, 1H), 7.41-7.45 (m, 4H), 7.33 (t, *J*=7.8 Hz, 2H), 7.19- 7.23 (m, 3H), 7.00-7.04 (m, 4H), 6.78 (t, *J*=7.3 Hz, 1H), 3.83 (s, 2H). $^{13}$C NMR (DMSO-$d_6$, 125 MHz): δ 166.6, 144.4, 139.6, 136.5, 132.1, 129.6, 129.5, 128.5, 126.4, 121.0, 119.6, 118.2, 116.4, 37.9. HRMS (ESI/TOF) *m/z*: $[M+H]^+$ Calcd for $C_{21}H_{19}N_{22}S$ 335.1218; Found 335.1228.

*N*-(4-(Benzylamino)phenyl)-2-(phenylthio)acetamide (ST57): A solution of benzaldehyde (1 mmol), 2b (1 mmol), and anhydrous $Na_2SO_4$ (6 mmol) in DCM was stirred at room temperature under an argon atmosphere until imine formation was completed. Following the filtration of the reaction mixture, the imine intermediate (filtrate) was concentrated *in vacuo* and dissolved in methanol. Then, $NaBH_4$ was slowly added and stirred for 30 min at room temperature. After the reaction was completed, the mixture was diluted with a 5% aqueous solution of $NaHCO_3$ until pH=~8 and subjected to extracting with DCM (3x10 ml). After being dried over anhydrous $Na_2SO_4$, the combined organic phase was concentrated *in vacuo*. The crude underwent purification via silica-based column chromatography using an elution system of *n*-hexane:ethyl acetate (85:15) to afford **ST57**. Yield: 24%, white solid. Mp 124.8-125.1 °C. $^{1}$H NMR (DMSO-$d_6$, 500 MHz): δ 9.78 (s, 1H), 7.38-7.41 (m, 2H), 7.30-7.36 (m, 6H), 7.18-7.23 (m, 4H), 6.52 (d, *J*=8.9 Hz, 2H), 6.10 (t, *J*=6.0 Hz, 1H), 4.24 (d, *J*=6.0 Hz, 2H), 3.78 (s, 2H). $^{13}$C NMR (DMSO-$d_6$, 125 MHz): δ 166.2, 145.7, 140.8, 136.6, 129.4, 128.7, 128.5, 128.4, 127.6, 127.0, 126.3, 121.4, 112.6, 47.2, 37.8. HRMS (ESI/TOF) *m/z*: $[M+H]^+$ Calcd for $C_{21}H_{21}N_2OS$ 349.1375; Found 349.1364.

*N*-(4-((Phenylamino)methyl)phenyl)-2-(phenylthio)acetamide (ST58): A solution of aniline (1 mmol), 2c (1 mmol), and anhydrous $Na_2SO_4$ (6 mmol) in DCM was stirred at room temperature under an argon atmosphere until imine formation was completed. Following the filtration of the reaction mixture, the imine intermediate (filtrate) was concentrated *in vacuo* and dissolved in methanol. Then, $NaBH_4$ was slowly added and stirred for 30 min at room temperature. After the reaction was completed, the mixture was diluted with a 5% aqueous solution of $NaHCO_3$ until pH=~8 and subjected to extracting with DCM (3x10 ml). After being dried over anhydrous $Na_2SO_4$, the combined organic phase was concentrated *in vacuo*. The crude product underwent purification via silica-based column chromatography using an elution system of DCM:methanol (99:1) to afford ST58. Yield: 43%, white solid. Mp >300 °C. $^{1}$H NMR (DMSO-$d_6$, 500 MHz): δ 10.26 (s, 1H), 7.50 (d, *J*=8.5 Hz, 2H), 7.40 (d, *J*=7.4 Hz, 2H), 7.32 (t, *J*=7.8 Hz, 2H), 7.29 (d, *J*=8.5 Hz, 2H), 7.20 (t, *J*=7.4 Hz, 1H), 7.03 (t, *J*=7.8 Hz, 2H), 6.55 (d, *J*=7.8 Hz, 2H), 6.50 (t, *J*=7.4 Hz, 1H), 6.15 (t, *J*=5.8 Hz, 1H), 4.19 (d, *J*=5.8 Hz, 2H), 3.85 (s, 2H). $^{13}$C NMR (DMSO-$d_6$, 125 MHz): δ 167.1, 149.1, 137.9, 136.4, 135.8, 129.5, 129.3, 128.5, 128.0, 126.5, 119.7, 116.2, 112.7, 46.5, 37.9. HRMS (ESI/TOF) *m/z*: $[M+H]^+$ Calcd for $C_{21}H_{21}N_2OS$ 349.1375; Found 349.1369.

*N*-(4-Benzylphenyl)-2-(phenylthio)acetamide (ST59): Obtained following the general synthesis method A, from 2-(phenylthio)acetyl chloride (theoretically 1 mmol), 4-benzylaniline (1 mmol), and DIPEA (1.5 mmol). Yield: 40%, white solid. Mp 136.0-136.3 °C. $^{1}$H NMR (DMSO-$d_6$, 400 MHz): δ 10.09 (s, 1H), 7.47 (d, *J*=8.4 Hz, 2H), 7.40 (d, *J*=7.6 Hz, 2H), 7.31 (t, *J*=8.4 Hz, 2H), 7.26 (d, *J*=7.6 Hz, 2H), 7.14-7.21 (m, 6H), 3.88 (s, 2H), 3.83 (s, 2H). $^{13}$C NMR (DMSO-$d_6$, 100 MHz): δ 166.5, 141.3, 136.8, 136.3, 135.8, 128.9 (2C), 128.5, 128.3, 128.0, 125.9, 125.8, 119.3, 40.4, 37.4. HRMS (ESI/TOF) *m/z*: $[M+H]^+$ Calcd for $C_{21}H_{20}NOS$ 334.1266; Found 334.1258.

*N*-(4-Benzoylphenyl)-2-(phenylthio)acetamide (**ST60**): Obtained following the general synthesis method A, from 2-(phenylthio)acetyl chloride (theoretically 1 mmol), 4-aminobenzophenone (1 mmol),

and DIPEA (1.5 mmol). Yield: 32%, white solid. Mp 130.7-131.1 °C. $^{1}$H NMR (DMSO-$d_6$, 400 MHz): δ 10.60 (s, 1H), 7.64-7.77 (m, 7H), 7.55 (t, *J*=7.6 Hz, 2H), 7.41 (d, *J*=8.0 Hz, 2H), 7.33 (t, *J*=7.6 Hz, 2H), 7.21 (t, *J*=7.6 Hz, 1H), 3.92 (s, 2H). $^{13}$C NMR (DMSO-$d_6$, 100 MHz): δ 194.5, 167.5, 142.9, 137.5, 135.6, 132.3, 131.6, 131.2, 129.4, 129.1, 128.5, 128.2, 126.1, 118.4, 37.6. HRMS (ESI/TOF) *m*/*z*: $[M+H]^+$ Calcd for $C_{21}H_{18}NO_2S$ 348.1058; Found 348.1064.

***In vitro* SIRT2 inhibition assay**

The inhibitory activities of the title compounds were examined using SIRT2 Direct Fluorescent Screening Assay Kits (Item No. 700280) following the manufacturer's protocol (Cayman Chemical, Ann Arbor, MI, USA) and a previously reported method (Gozelle et al., 2023). The percentage of inhibition in each well was calculated by comparing the fluorescence readings of compound-treated wells to those of control wells. The experiment was repeated three times.

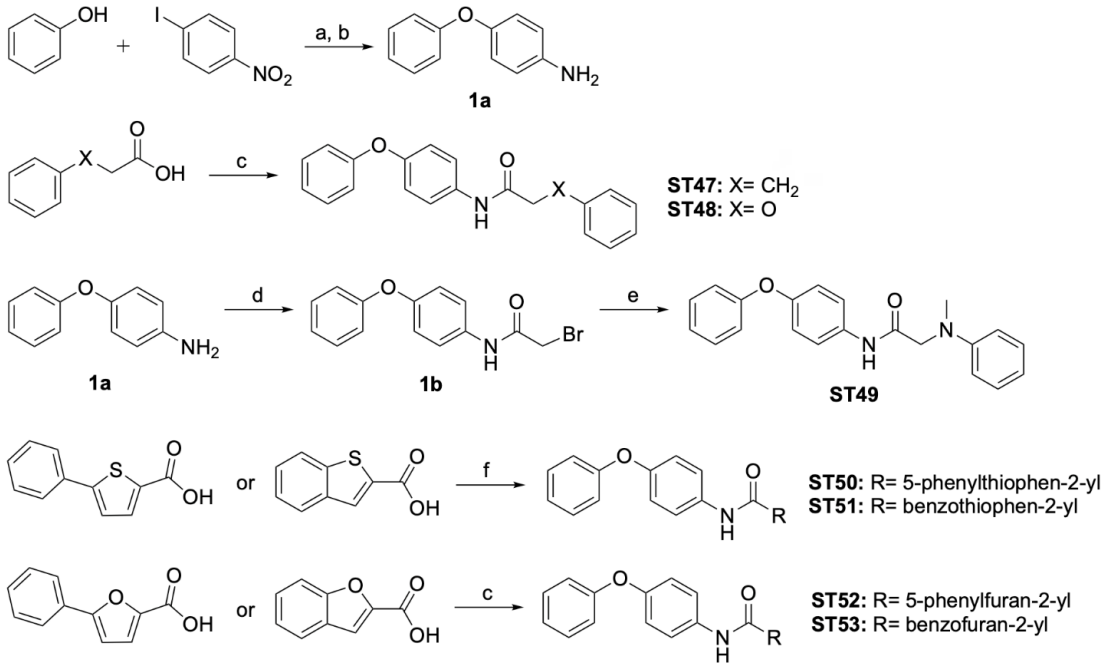
**Molecular docking**

The molecular docking simulations were performed using Glide within the Schrödinger Small-Molecule Drug Discovery Suite (Small-Molecule Drug Discovery Suite 2023-1, Schrödinger, LLC, New York, NY, 2023). The x-ray crystal structure of human SIRT2 (PDB: 5DY4) was retrieved from the RCSB Protein Data Bank and prepared by our previous protocol (Gozelle et al., 2022). The selected compounds were docked into the SIRT2 active site using the XP docking mode using a radius scaling factor of 0.80 vdW and a partial charge cutoff of 0.20 (Friesner et al., 2006).

## RESULTS AND DISCUSSION

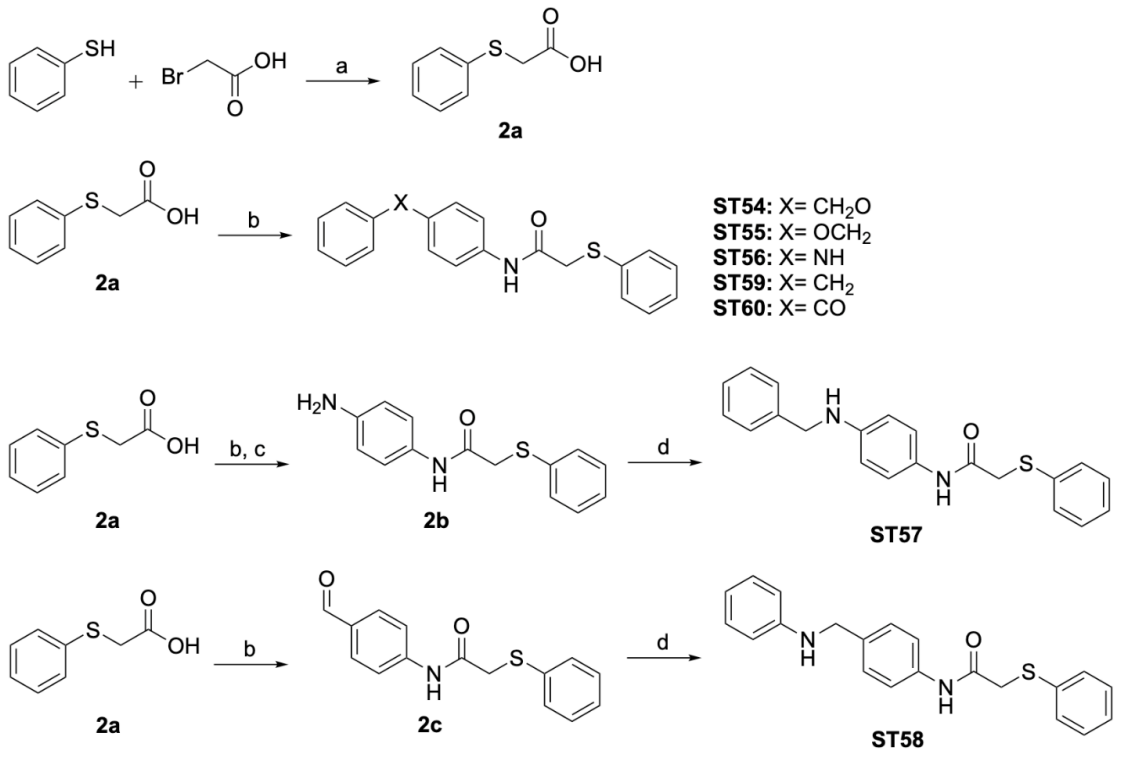
### Synthesis

The synthesis of *N*-(4-phenoxyphenyl)aryl-carboxamide derivatives (**ST47**-**ST53**) was carried out following the synthetic sequence depicted in Scheme 1. The Ullman coupling reaction of commercially available 1-iodo-4-nitrobenzene and phenol, catalyzed by CuI and *N*,*N*-dimethylglycine, afforded 1-nitro-4-phenoxybenzene, which was used without further purification in the next step. 4-Phenoxyaniline (1a) was obtained by reduction of the 1-nitro-4-phenoxybenzene in the presence of $SnCl_2.2H_2O$. The title compounds ST47, ST48, ST52, and ST53 were synthesized by the reaction of 1a with an appropriate acyl chloride, which was produced in the presence of oxalyl chloride from commercially available carboxylic acid derivatives with total yields ranging from 42% to 66%. **ST49** was obtained through the substitution reaction of *N*-methylaniline and the 2-bromo-*N*-(4-phenoxyphenyl)acetamide intermediate (1b), which was the product of the reaction between 1a and 2-bromoacetyl bromide in a yield of 24%. Moreover, amide coupling reactions of 5-phenylthiophene-2-carboxylic acid or benzothiophene-2-carboxylic acid with 1a in the presence of EDC, HOBt, and DIPEA afforded the desired compounds ST50 or ST51 with 54% and 59% yields, respectively.




**Scheme 1.** Synthetic route to compounds **ST47**-**ST53**. Reagents and conditions: (a) CuI, $Cs_2CO_3$, *N*,*N*-dimethylglycine, 1,4-dioxane, DMF, 100 °C, overnight; (b) $SnCl_2.2H_2O$, ethanol, reflux, 4 h; (c) *i.* appropriate carboxylic acid (3-phenylpropanoic acid for **ST47**, 2-phenoxyacetic acid for **ST48**, 5-phenylfuran-2-carboxylic acid for **ST52**, benzofuran-2-carboxylic acid for **ST53**), oxalyl chloride, cat. DMF, DCM, rt, 2 h, *ii.* **1a**, DIPEA, DCM, rt, 2-4 h; (d) 2-bromoacetyl bromide, TEA, rt, 3 h; (e) *N*-methylaniline, DIPEA, ACN, 100 °C, overnight; (f) appropriate carboxylic acid (5-phenylthiophene-2-carboxylic acid for **ST50** and benzothiophene-2-carboxylic acid for **ST51**), **1a**, EDC, HOBt, DIPEA, DCM, rt, overnight.

The synthesis of *N*-(aryl)-2-(phenylthio)acetamides was carried out under the synthetic sequence depicted in Scheme 2. Initially, 2-(phenylthio)acetic acid (**2a**), which is the starting material for *N*-(aryl)-2-(phenylthio)acetamide derivatives, was synthesized through the reaction of thiophenol and 2-bromoacetic acid in a basic medium. Subsequently, by reacting commercially available 4-substituted aniline derivatives with 2-(phenylthio)acetyl chloride, which was obtained from **2a** and oxalyl chloride, the desired compounds **ST54**, **ST55**, **ST56**, **ST59**, and **ST60** were produced with total yields ranged from 24% to 42%. The first step in the synthesis route to **ST57** was the reaction of 2-(phenylthio)acetyl chloride with 4-nitroaniline, yielding *N*-(4-nitrophenyl)-2-(phenylthio) acetamide. Next, by reducing nitro precursor in the presence of $SnCl_2.2H_2O$, *N*-(4-aminophenyl)-2-(phenylthio)acetamide (**2b**) was obtained in a yield of 55%. An indirect reductive amination procedure involving the condensation of **2b** with benzaldehyde and the subsequent reduction with $NaBH_4$ gave the desired product **ST57** with a 61% yield. In the case of compound **ST58**, 4-aminobenzaldehyde was reacted with 2-(phenylthio)acetyl chloride yielding *N*-(4-formylphenyl)-2-(phenylthio)acetamide (**2c**), which was used in the next step without further purification, followed by an indirect reductive amination reaction of **2c** and aniline producing the title compound **ST58** in a yield of 43%. Finally, the structures of the final compounds were confirmed by $^1$H-NMR, $^{13}$C-NMR, and HRMS spectra.




**Scheme 2.** Synthetic route to compounds **ST54**-**ST60**. Reagents and conditions: (a) NaOH, $K_2CO_3$, ethanol, water, rt, 2 h; (b) *i.* **2a**, oxalyl chloride, cat. DMF, DCM, rt, 2 h, *ii.* Appropriate amine (4-(benzyloxy)aniline for **ST54**, 4-(phenoxymethyl)aniline for **ST55**, *N*-phenyl-*p*-phenylenediamine for **ST56**, 4-benzylaniline for **ST59**, 4-aminobenzophenone for **ST60**, 4-nitroaniline for **2b**, 4-aminobenzaldehyde for **2c**), DIPEA, DCM, rt, 2-4 h; (c) $SnCl_2.2H_2O$, ethanol, reflux, 6 h; (d) *i.* benzaldehyde for **ST57**, aniline for **ST58**, $Na_2SO_4$, DCM, rt, overnight, *ii.* $NaBH_4$, methanol, rt, 30 min.

**Biological results**

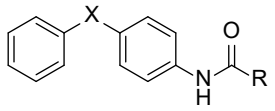
The inhibitory activities of the target compounds (ST47-ST60) against SIRT2 were tested in a fluorescence-based assay (Damonte et al., 2017; Yoon & Kim, 2016) at a screening dose of 100 μM. The results are listed in Table 1. According to the results, a significant increase in the inhibition rates of the tested compounds was observed compared to that of STH2.

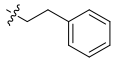
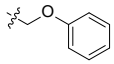
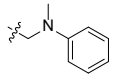
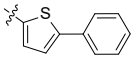
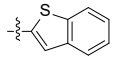
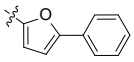
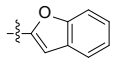
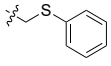
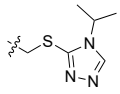
A brief overview of SAR related to the modifications performed revealed that among the compounds ST47-ST49 generated by bioisosteric replacement of sulfur atom close to the tail group, ST49 with *N*-methylamino group exhibited the best inhibition rate with a value of 50.07% at 100 μM. In comparison, STH2 had an inhibition value of 84.28% and 36.89% against SIRT2 at 300 μM and 100 μM screening concentrations, respectively. The title compounds ST50-ST53 bearing thiophene, benzothiophene, furan, and benzofuran rings were obtained due to fused ring cyclization and chain cyclization strategies implemented through the tail group. The fused ring compounds (ST51 and ST53) demonstrated a slight superiority in SIRT2 inhibitory effect compared to ST50 and ST52 with 5-phenylthiophene and 5-phenylfuran moieties, respectively. Besides, ST50 and ST51**,** with sulfur-containing rings, were more likely to show potent inhibition against SIRT2 than their counterparts with oxygen-containing rings (ST52 and ST53). Regarding the linker modification, the title compounds ST54-ST60 were obtained to contain various linkers instead of oxygen atom in the STH2 structure. Surprisingly, replacement of oxygen linker with -$CH_2O$- and

-$OCH_2$- groups in ST54 and ST55 led to significant loss of inhibitory effect on SIRT2 activity, while **ST56**, ST57, and ST58 with amine-containing linker (-NH-, -$CH_2$NH-, -$NHCH_2$-) displayed an enhanced SIRT2 inhibition ranging from 27.81% to 42.31% at 100 μM screening concentration. Moreover, SIRT2 inhibitions of 41.52% and 54.03% were obtained at 100 μM methylene linker-bearing ST59 and carbonyl linker-bearing ST60, respectively. Compared to the data gained in our previous study (Gozelle et al., 2023), the analogs with thiophene as the central ring exhibited a more potent inhibitory effect against SIRT2. In addition, the introduction of longer linker groups (n=2) resulted in a decrease in activity. All compounds tested displayed moderate inhibitory activity against SIRT2 compared to Suramin, the non-selective sirtuin inhibitor with an $IC_{50}$ value of 1.15 μM for SIRT2 (Trapp et al., 2007).

The compounds exhibiting 47-54% SIRT2 inhibition (ST49, ST51, ST60) were evaluated for their *in vitro* SIRT1 and SIRT3 inhibitory activities to predict the isoform selectivity. The results showed that all three compounds did not show significant inhibitory potency against SIRT1 compared to the selective SIRT1 inhibitor EX-527, which had an $IC_{50}$ value of 0.28 nM (Broussy, Laaroussi, & Vidal, 2020; Solomon et al., 2006). Furthermore, none of the compounds tested showed significant inhibitory potency against SIRT3, confirming the accuracy of our design approach to selectively inhibit SIRT2.

**Table 1.** *In vitro* inhibitory profiles of compounds tested at 100 μM against SIRT2



| ID | X | R | %inhibition±SD | | | Modification |
|---|---|---|---|---|---|---|
| | | | SIRT2 | SIRT1 | SIRT3 | |
| **ST47** | -O- |  | 29.73±4.88 | n.t. | n.t. | tail group |
| **ST48** | |  | 40.15±1.75 | n.t. | n.t. | |
| **ST49** | |  | 50.07±2.07 | 8.54±3.50 | 19.98±4.09 | |
| **ST50** | |  | 41.66±5.13 | n.t. | n.t. | |
| **ST51** | |  | 47.19±5.92 | 7.29±4.05 | 18.10±3.23 | |
| **ST52** | |  | 30.45±0.64 | n.t. | n.t. | |
| **ST53** | |  | 39.93±4.21 | n.t. | n.t. | |
| | -$CH_2O$- |  | n.i. | n.t. | n.t. | linker |
| **ST55** | -$OCH_2$- | | n.i. | n.t. | n.t. | |
| **ST56** | -NH- | | 33.57±4.60 | n.t. | n.t. | |
| **ST57** | -$CH_2NH$- | | 27.81±0.32 | n.t. | n.t. | |
| **ST58** | -$NHCH_2$- | | 42.31±7.24 | n.t. | n.t. | |
| **ST59** | -$CH_2$- | | 41.52±7.13 | n.t. | n.t. | |
| **ST60** | -CO- | | 54.03±1.52 | n.i. | 19.17±0.14 | |
| **STH2** | -O- |  | 84.28±5.19*<br>36.89±0.45 | n.i. | n.i. | - |
| **EX-527** | - | - | 76.39±2.49 | 99.28±1.07 | 44.61±3.81 | - |
| **Suramin** | | | 98.37±0.25 | 94.52±0.92 | 96.82±3.26 | |

*SD: standard deviation (n = 3); *Percent inhibition @300 μM; n.i.: no inhibition; n.t.: not tested.*

**Molecular modelling**

To predict the orientations of synthesized compounds bound to SIRT2, molecular docking studies were carried out compared to the binding pose of STH2. The information gathered from the x-ray crystal structure of SIRT2 (PDB: 5DY4) highlighted the importance of the critical interactions, including π-π stacking with F119, F131, and F234 at the entrance of the substrate binding channel, π-π stacking with Y139 and F190 at the selectivity pocket, and water-mediated H-bonding with P94 (Schiedel et al., 2016).

Although molecular docking is a valuable tool for understanding ligand binding predictions, it regularly fails to differentiate active from inactive compounds within each chemical family (Chen, 2015). In our case, the docking results, especially for ST60, could not pro-

vide supportive findings to establish a relationship between the binding conformation varying according to chemical structure and the inhibitory effect.

The SIRT2:docked STH2 complex showed that all critical interactions mentioned above were obtained. The replacement of 2-(4-isopropyl-4*H*-1,2,4-triazol-3-yl)thio moiety, the so-called tail group, by 2-methyl(phenyl)amino (ST49) led to the loss of interaction with F131 in the substrate channel, maintaining the water-mediated H-bond with P94. ST60**,** exhibiting the best inhibitory profile in the series, unexpectedly occupied the active site by interacting only with F190 in the selectivity pocket and F119 in the substrate binding channel. It was suggested that the reduced rotation of phenyl rings due to introducing the carbonyl group into the linker induced a conformational modification, yielding less interaction in the entrance of the substrate channel (Figure 2). The most notable conformational change is observed for the compounds with fused rings on the tail group (ST51 and ST53). Benzothiophene and benzofuran ring systems, which were used in fused ring cyclization, accessed the deeper inside the selectivity pocket due to the formation of more favorable π-π contacts than those of compounds with 5-phenylthiophene and 5-phenylfuran moieties (ST50 and ST52). They adopted less bent conformation that prevented interaction with P94 *via* structural water (Figure S4). The binding energies for STH2, ST49, and ST60 in the SIRT2 active site were calculated as -10.77, -11.43, and -10.82 kcal/mol, respectively.

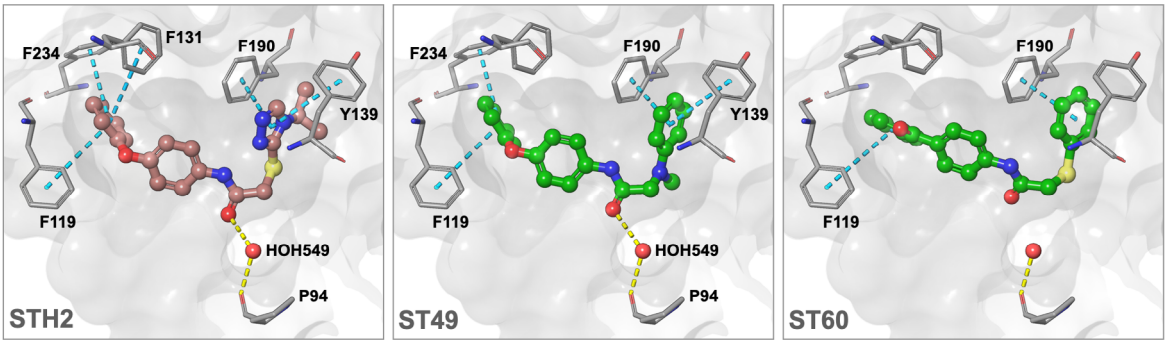



**Figure 2.** The proposed binding modes of docked compounds in the SIRT2 active site (PDB: 5DY4). H-bonds and π-π contacts are represented by yellow and cyan dashed-lines, respectively.

## CONCLUSION

In this work, we aimed to find analogs that exhibit more potent inhibition of SIRT2 by modifying the linker and tail groups on our initial virtual screening-derived hit. This eventually resulted in a significant increase in SIRT2 inhibitory activity. Our best SIRT2 inhibitors, **ST49** and **ST60,** exhibited 50.07% and 54.03% inhibition at 100 μM, respectively, while **STH2** inhibited SIRT2 by 84.28% and 36.89% at 300 and 100 μM screening concentrations, respectively. Moreover, ST49 and ST60 showed no significant inhibitory effect against SIRT1 and SIRT3 isoforms. Based on these findings, it may be suggested that the one-atom linker led to tighter binding to SIRT2 and more potent inhibition than the two-atom linker. Besides, the impact of the tail group involving π systems on inhibition ability was undeniable as maintaining the crucial π-π contacts with the aromatic residues of the selectivity pocket. This study, however, offers essential insights into structure-guided modifications for further hit expansion in the design of SIRT2 inhibitors.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

**AUTHOR CONTRIBUTION STATEMENT**

M.G.: Methodology, Investigation, Writing-Original Draft. Y.O: Methodology. G.E.: Conceptualization, Supervision, Methodology, Writing-Original Draft, Writing-Review & Editing. All authors reviewed the results and approved the final version of the manuscript.

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*RESEARCH ARTICLE*

# Psychiatrists' and Infectious Disease Physicians' Awareness and Knowledge Level of Drug-Drug Interactions Between Antiretrovirals and Psychotropics: A Comparative Questionnaire-Based Study

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***Psychiatrists' and Infectious Disease Physicians' Awareness and Knowledge Level of Drug-Drug Interactions Between Antiretrovirals and Psychotropics: A Comparative Questionnaire-Based Study***

***SUMMARY***

*The incidence of drug-drug interactions (DDIs) increases with psychotropics in people living with HIV (PLWH). This study aimed to compare the knowledge of infectious disease physicians (IDPs) and psychiatrists about DDIs as a primary outcome and to assess their knowledge, attitudes and awareness of DDIs as a secondary outcome. The quantitative and comparative questionnaire methods were administered to IDPs and psychiatrists in Türkiye via online survey. The questionnaire included open-ended and multiple-choice questions on physicians' sociodemographic and attitudes to DDIs and three hypothetical case scenarios. Information including patients' age, sex, social and medical history, laboratory findings and all drugs were provided in each scenario. After providing brief DDI information, the case scenario question was asked again with pretest-posttest design. Attitudes of 31 IDPs and 29 psychiatrists on ART-psychotropic DDIs were examined. Thirty-five (58.3%) of physicians emphasized that their perceived competence in DDIs knowledge was 'average'. Moreover, 53 physicians (88.3%) were affected by DDIs on prescription behavior. When we asked physicians how often they informed their patients about DDI, 45 (75%) responded 'often/always'. Major DDI was correctly defined by psychiatrists [12 (41.4%) vs. 23 (79.3%)] and IDPs [24 (77.4%) vs. 29 (93.5%)] before and after providing brief DDI information ($p<0.001$). To the best of our knowledge, this is the first study comparing knowledge, attitudes and awareness of psychiatrists and IDPs towards antiretroviral-psychotropic DDIs. It was determined that the presence of DDIs influenced decision-making of physicians. The majority of physicians reported that they had an above average perceived (subjective) level of knowledge about DDIs, but their objective level of knowledge about a DDI was insufficient.*

***Key Words:*** *Antiretrovirals, Human Immunodeficiency Virus, People Living with Human Immunodeficiency Virus, Psychotropic Drugs, Drug-Drug Interactions, Knowledge Level*

***Psikiyatristlerin ve Enfeksiyon Hastalıkları Hekimlerinin Antiretroviraller ve Psikotroplar Arasındaki İlaç-İlaç Etkileşimleri Konusundaki Farkındalık ve Bilgi Düzeyleri: Karşılaştırmalı Ankete Dayalı Bir Çalışma***

***ÖZ***

*HIV ile yaşayan kişilerde (PLWH) psikotroplarla ilaç-ilaç etkileşimlerinin (İİE) görülme sıklığı artmaktadır. Bu çalışmanın amacı, birincil sonuç olarak enfeksiyon hastalıkları uzmanları (EHU) ve psikiyatristlerin İİE'ler hakkındaki bilgilerini karşılaştırmak ve ikincil sonuç olarak İİE'ler hakkındaki bilgi, tutum ve farkındalıklarını değerlendirmektir. Kantitatif ve karşılaştırmalı anket yöntemleri ile EHU'lara ve psikiyatristlere online anket olarak Türkiye'de uygulanmıştır. Ankette hekimlerin sosyodemografik özellikleri ve İİE'lere yönelik tutumları ile ilgili açık uçlu ve çoktan seçmeli sorular ve üç varsayımsal vaka senaryosu yer almıştır. Her senaryoda hastaların yaşı, cinsiyeti, sosyal ve tıbbi geçmişi, laboratuvar bulguları ve tüm ilaçları içeren bilgiler verilmiştir. Kısa İİE bilgileri verildikten sonra, vaka senaryosu sorusu ön test-son test tasarımıyla tekrar sorulmuştur. 31 EHU ve 29 psikiyatristin antiretroviral-psikotropik İİE'lere karşı tutumları incelenmiştir. Hekimlerin 35'i (%58,3) İİE'leri konusunda 'ortalama' bir yeterlilik algısına sahip olduğunu vurgulamıştır. Ayrıca, 53 hekim (%88,3) reçete yazma davranışında İİE'lerden etkilenmiştir. Hekimlere hastalarını İİE konusunda ne sıklıkla bilgilendirdiklerini sorduğumuzda, 45 hekim (%75) 'sıklıkla/her zaman' yanıtını vermiştir. Majör İİE, bilgilendirme öncesi ve sonrasında psikiyatristler [12 (%41,4) vs. 23 (%79,3)] ve EHU'ları [24 (%77,4) vs. 29 (%93,5)] tarafından doğru tanımlanmıştır ($p<0,001$). Bu çalışma, psikiyatristlerin ve EHU'ların antiretroviral-psikotropik İİE'lere yönelik bilgi, tutum ve farkındalıklarının karşılaştırıldığı ilk çalışmadır. İİE'lerin varlığının hekimlerin karar verme süreçlerini etkilediği belirlenmiştir. Hekimlerin çoğunluğu algılanan bilgi düzeylerinin ortalamanın üzerinde olduğunu, ancak İİE'lerine ilişkin bilgi düzeylerinin yetersiz olduğunu belirtmiştir.*

***Anahtar Kelimeler:*** *Antiretroviraller, İnsan İmmün Yetmezlik Virüsü, İnsan İmmün Yetmezlik Virüsü ile Yaşayan Kişiler, Psikotrop İlaçlar, İlaç-İlaç Etkileşimleri, Bilgi Düzeyi*

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## INTRODUCTION

Mental disorders are 8 times more frequent among people living with HIV (Human Immunodeficiency Virus) (PLWH) than among the general population (Hill & Lee, 2013). Psychiatric disorders and psychotropic drug use could be as high as 50% and 27% among PLWH, respectively. Major depression can be found in up to 23% of PLWH, whereas its prevalence among the general population is 0.6% (Angelino & Treisman, 2001). Antidepressants are the leading co-medication used by 20.9% of PLWH, followed by anxiolytics (16.7%), antipsychotics (4.7%), and psychostimulants (3.0%) (Meade & Sikkema, 2005). Psychotropic drug usage is not related to the stage of HIV infection (Vitiello, Burnam, Bing, Beckman, & Shapiro, 2003). It is estimated that patient's adherent to psychotropic treatment is also adherent to antiretroviral treatment (ART) (Casaletto et al., 2016).

To minimize the risk of drug-drug interactions (DDIs) and to ensure safe and effective treatment, interventions such as dose adjustment, alteration of treatment or therapeutic drug monitoring should be performed or at least considered (Angelino & Treisman, 2001; Meade & Sikkema, 2005). The risk of potential DDIs is of concern as combination ART is recommended in the management of HIV infection which mandates physicians' awareness of the recognition and management of DDIs (Schlaeppi et al., 2020). The prevalence of red-flag DDIs among PLWH is 3.18% (Lopez-Centeno et al., 2019). While ART has gained notoriety for its association with a plethora of pharmacokinetic DDIs involving the CYP450 enzymes, the extent and clinical significance of these DDIs with psychotropics can vary, ranging from negligible effects on plasma concentrations to potentially life-threatening events like torsades de pointes, respiratory depression, or myelosuppression (Goodlet, Zmarlicka, & Peckham, 2019; Yalcin, Ak, & Demirkan, 2021). If psychiatric disorders and substance addiction cannot be managed accordingly, it may result in non-compliance.

It was recently shown that adherence to ART is high despite adverse effects which in turn, prone patients to drug-related adverse outcomes (Ceylan, Koc, Inkaya, & Unal, 2019). In line with the goals of the Joint United Nations Programme on HIV/AIDS (UNAIDS), no one should be left behind and any drug risks that impede outcomes should be eliminated. Commencement of a co-medication may lead to an increased risk of potential DDIs (Tseng & Foisy, 1999). It is estimated that 14.0% of PLWHs use more than 10 drugs simultaneously (Robertson, Penzak, & Pau, 2007), and polypharmacy is common among PLWHs (Hinkin, Castellon, Atkinson, & Goodkin, 2001; Kara, Inkaya, Aydin Hakli, Demirkan, & Unal, 2019; Mallet, Spinewine, & Huang, 2007). Polypharmacy enforces attending physicians' decision-making which results in overwhelmed physicians under the list of drugs and their potential DDIs. There are various online databases available for the determination of DDIs. Among them, *Liverpool HIV Interactions* (Liverpool Drug Interactions Group, Liverpool, UK) and *Medscape Drug Interaction Checker* (WebMD, New York, USA) databases were most commonly used. Different databases may reveal conflicting results which also incapacitate physicians' decision-making. In addition, understanding and implementation of DDI reports may also vary between clinicians, further affecting the management of PLWH.

It is essential that physicians are not only aware of DDIs, but also know the full details of DDIs and mitigation strategies and seek advice or prevention activities in these settings involving clinical pharmacists (Agu et al., 2014; Surmelioglu et al., 2021).

Limited data exist that address knowledge and attitudes of physicians about ART and/or psychotropic adverse effects and ART–psychotropic DDIs among PLWH. This study aimed to compare the knowledge of infectious disease physicians (IDPs) and psychiatrists about DDIs as a primary outcome and to assess their knowledge, attitudes and awareness of DDIs as a secondary outcome.

## MATERIALS AND METHODS

A total of 60 physicians with at least 6 months of clinical experience in infectious diseases (IDs) or psychiatry were enrolled over 6 months period in Türkiye. These physicians were selected through the contact network of the ID physicians and psychiatrists involved in the study. The questionnaire was administered at different times to doctors in both groups who were blinded to each other. The questionnaire was administered electronically (Google® Forms) to both groups via e-mail groups. To determine the participants' baseline knowledge of the case scenario questions, the questionnaire was designed so that brief information would not be opened without a required baseline response. The questionnaire included open-ended and multiple-choice questions on physicians' sociodemographic (age, sex, academic degree, duration of experience in a medical specialty, institution, examination of PLWH with a mental health problem in the last 6 months) and attitudes to DDIs with a 5-point Likert scale (perceived competence in DDI knowledge, the effect of potential DDIs on prescription behavior and the frequency of informing patients about DDIs) and three different hypothetical case scenarios (Supplementary Material). The questions and case scenarios in the questionnaire were prepared by the research group, including clinical pharmacists, by utilizing current literature and drug information databases.

In these case scenarios, patients' age, sex, social and medical history, laboratory findings and all drugs were provided. In this direction, these case scenarios included three different types of DDIs (e.g., Case 1: major interaction requiring replacement or discontinuation of the drug, Case 2: moderate interaction requiring additional follow-up, and Case 3: minor interaction requiring no additional follow-up or intervention). Following the case presentations, physicians were asked whether there was any DDIs related to the case. If the physician thought of a potential DDI, then their attitudes and management strategies were questioned by multiple choice questions with hypothetical case scenarios. As it was estimated that most physicians use online sources to check DDIs, information on DDIs adapted from online sources (Liverpool HIV Interactions Database, Medscape Drug Interaction Checker Database) were provided after survey questions (Supplementary Material).

The survey was designed so that volunteers could not see the brief DDI information before responding to the initial question. After providing brief information during the survey, the case scenario question was asked again. Brief information on severity, clinical implication and management of each DDI obtained from the current and scientific database Lexicomp® was given for each hypothetical case scenario after the pretest and before the posttest. The questionnaire took study participants 15-20 minutes to complete. There was no incentive to participate, and reminder e-mails were sent a week later only once to physicians who did not complete the survey. Additionally, the analysis of the study only included surveys that were fully completed (Supplementary Material).

### Statistical analysis

As there is no precedent study in the literature, the sample size could not be calculated. Instead, the power was calculated considering the data obtained by correct answer change after to be briefed of ID physicians and psychiatrists. With an effect size of 0.50, 95% power, 5% margin of error, the power of the 2-group study was found to be 95.07% (G*Power Version 3.0.10). Thirty-five physicians for each group were selected for comparison. While making the selection, physicians with at least 6 months of clinical experience were randomly selected by utilizing the working network and collaborations of psychiatrists and ID physicians who were the authors of the study.

In descriptive statistics, mean (SD) or median (minimum-maximum) values were given for numeric variables, and number (percentage) values were given for categorical variables. The normality of continuous variables was tested using the Shapiro–Wilk test. After

data extraction, continuous variables were defined as the mean (SD) and median (min-max), depending on the result of normality test. Mann-Whitney U tests were used to compare numerical data, and chi-squared and McNemar tests were used to compare categorical data. For all tests, $p<0.05$ was considered statistically significant. IBM SPSS Statistics 23.0 software was used to analyze and evaluate the data.

Those who volunteered to participate in the study responded to the questions and provided informed consent. The study was approved by the Hacettepe University Ethics Committee (decision no: GO 18/304-37).

## RESULTS and DISCUSSION

### Demographics

Despite the reminder, 4 (11.4%) ID physicians and 6 (17.1%) psychiatrists left the questionnaire unanswered. A total of 60 physicians were included; among them, 38 were female (63.3%), the mean (SD) age was 34.21 (5.10) years. Thirty-one (51.7%) were IDPs and 29 (48.3%) were psychiatrists (Table 1). The median duration of professional experience was 6 years for both IDPs (range: 1-30) and psychiatrists (range: 3-20) and the majority of physicians (78.3%) were employed in a tertiary hospital. There was no significant difference between the medical specialists and residents regarding demographic features. Also, 25 (41.7%) participants had treated a PLWH with mental health problems in the previous 6 months. IDPs (58.1%) encountered more PLWH with mental health problems than psychiatrists (24.1%) in the last 6 months. The duration of professional experience was similar across specialties.

**Table 1.** Sociodemographic characteristics of the study groups

| | Psychiatrists (n=29) | IDPs (n=31) | Total (n=60) | *p* |
|---|---|---|---|---|
| Age, mean (SD) | 34.21 (5.10) | 36.97 (8.88) | 35.63 (7.37) | 0.149 |
| Sex, n (%) | | | | |
| *Male* | 16 (55.2) | 6 (19.4) | 22 (36.7) | 0.009 |
| *Female* | 13 (44.8) | 25 (80.6) | 38 (63.3) | |
| Academic degree, n (%) | | | | |
| *Medical specialist* | 23 (79.3) | 12 (38.7) | 35 (58.3) | 0.003 |
| *Medical resident* | 6 (20.7) | 19 (61.3) | 25 (41.7) | |
| Duration of experience in a medical specialty, median (min-max) (years) | 6 (3-20) | 6 (1-30) | 6 (1-30) | 0.150 |
| Institution, n (%) | | | | |
| *Tertiary hospital* | 19 (65.5) | 28 (90.3) | 47 (78.3) | 0.042 |
| *Secondary hospital* | 10 (34.5) | 3 (9.7) | 13 (21.7) | |
| Examination of PLWH with a mental health problem in the last 6 months, n (%) | | | | |
| *Yes* | 7 (24.1) | 18 (58.1) | 25 (41.7) | 0.016 |
| *No* | 22 (25.9) | 13 (41.9) | 35 (58.3) | |

IDPs: infectious disease physicians, PLWH: people living with HIV

### General DDI information

Ninety percent of participants used online resources to learn about DDIs, and potential DDIs 'always or often' (88.3%) affected their prescribing decisions. Six physicians (10.0%, one psychiatrist and 5 IDPs) experienced an adverse drug reaction related to ART and psychotropic drugs.

Only 5 (8.3%) of the participants consulted a medical pharmacologist for information on drug-drug interactions (DDIs), while none consulted a pharmacist in their clinical practice. The reason why none of the participants consulted pharmacists for information on DDIs may be due to the recent introduction of clinical pharmacy practice within multidisciplinary teams in Turkey. Three-quarters of physicians stated that they had limited knowledge of DDIs and 35 (58.3%) reported moderate competence regarding DDI management. Three-quarters of physicians preferred to change psychotropic drugs upon encountering a DDI. After identification of a potential DDI, 45 (75%) often/always informed patients about potential DDIs and a potential DDI often/always affected prescribing decisions in 53 (88.3%). Psychiatrists used printed materials to check for DDIs more frequently than IDPs ($p$=0.019) (Table 2). Preferred information resources for physicians to determine the DDIs in given case scenarios are shown in Table 2. No significant relationship was found between the level of self-perceived competence and the frequency of informing patients about DDIs ($p$=0.343).

**Table 2.** Resource of information and attitude to DDIs among physicians

| | Psychiatrists (n=29) | IDPs (n=31) | Total (n=60) | *p* |
|---|---|---|---|---|
| Preferred information resources on DDIs | | | | |
| *Internet* | 28 (96.6) | 26 (83.9) | 54 (90.0) | 0.113 |
| *Smart device applications* | 12 (41.4) | 20 (64.5) | 32 (53.3) | 0.073 |
| *Printed materials* | 15 (51.7) | 7 (22.6) | 23 (38.3) | **0.019** |
| *Senior physician* | 9 (31.0) | 9 (29.0) | 18 (30.0) | 0.866 |
| *Drug leaflet* | 5 (17.2) | 5 (16.1) | 10 (16.7) | 0.590 |
| *Medical pharmacologist* | 1 (3.4) | 4 (12.9) | 5 (8.3) | 0.198 |
| *Pharmacist* | - | - | - | - |
| Perceived competence in DDI knowledge | | | | |
| *Above average/Very high* | 9 (31.1) | 6 (19.3) | 15 (25.0) | |
| *Average* | 17 (58.6) | 18 (58.1) | 35 (58.3) | 0.339 |
| *Very low/Below average* | 3 (10.3) | 7 (22.6) | 10 (16.7) | |
| The effect of potential DDIs on prescription behavior | | | | |
| *Often/Always* | 24 (82.8) | 29 (93.5) | 53 (88.3) | 0.185 |
| *Never/Seldom/Sometimes* | 5 (17.2) | 2 (6.5) | 7 (11.7) | |
| The frequency of informing patients about DDIs | | | | |
| *Often/Always* | 20 (69.0) | 25 (80.6) | 45 (75.0) | 0.296 |
| *Never/Seldom/Sometimes* | 9 (31.0) | 6 (19.4) | 15 (25.0) | |
| Evaluation of ART and psychiatric drug interactions by physicians | | | | |
| *I check DDI in every examination* | 7 (24.1) | 8 (25.8) | 15 (25.0) | 0.881 |
| *I just check DDI when a new psychiatric drug is initiated.* | 21 (72.4) | 22 (71.0) | 43 (71.7) | 0.901 |
| *I check DDI when an ART is added.* | 14 (48.3) | 15 (48.4) | 29 (48.3) | 0.993 |
| *I never check for a DDI.* | - | - | - | |

| Physicians' attitude to DDIs | | | | |
|---|---|---|---|---|
| *Alternating all drugs* | - | - | - | |
| *Switching all ART* | - | - | - | |
| *Switching interacting ART* | 1 (3.4) | 7 (22.6) | 8 (13.3) | |
| *Switching all psychiatric drugs* | 1 (3.4) | 1 (3.2) | 2 (3.3) | ** |
| *Switching interacting psychiatric drug* | 24 (82.8) | 21 (67.7) | 45 (75.0) | |
| *No change in any drug* | - | - | - | |
| *Other* | 3 (10.3) | 2 (6.5) | 5 (8.3) | |

* Percentages were taken over psychiatrists, infectious disease physicians and total physicians.

** *p-value* cannot be computed because the standard error of the difference is 0.

ART: antiretroviral treatment, DDI: drug-drug interaction, IDPs: infectious disease physicians

**Responses to clinical scenarios**

A total of 6 DDI questions in 3 case scenarios were directed to physicians before and after the brief information provided. The mean correct answer rate among psychiatrists and IDPs was 1.97 (1.11) and 2.19 (0.79), respectively ($p$=0.313). Before information, the number of correct answers given by psychiatrists and IDPs was 0.62 (0.67) and 0.97 (0.48), respectively, and after information was 1.34 (0.67) and 1.23 (0.56), respectively ($p$ <0.001).

Minor DDI was correctly recognized by 12.9% of IDPs but none of the psychiatrists. After brief information, the correct answer rate was 20.7% and 16.1% in psychiatrists and IDPs, respectively. Responses to the moderate DDI was not different between specialties and were not related to the information provided. Major DDI was initially defined correctly by 41.4% of psychiatrists and 77.4% of IDPs, respectively. After providing information, the correct response among psychiatrists and IDPs was 79.3% and 93.5%, respectively. After providing information, correct responses to only minor and major DDIs significantly increased among all physicians ($p$=0.001 and $p$<0.001, respectively).

There was no statistically significant correlation between the perceived level of knowledge (subjective assessment) and the number of correct answers to the case scenarios (objective assessment). Self-perceived competence level did not affect the correct decision rate ($p$=0.624). After being informed, the correct attitude among physicians who reported that potential DDI '*often/always affect their decision*' increased from 85.7% to 93.3%.

According to the specialty of physicians, their correct attitude rates before and after being informed are shown in Table 3.

**Table 3.** Distribution of physicians responding correctly to case scenarios

| Cases | Specialty | Before information | After information | *p*** |
|---|---|---|---|---|
| *Minor DDI* | Psychiatrists (n=29) | - | 6 (20.7%) | * |
| | IDs (n=31) | 4 (12.9%) | 5 (16.1%) | 1.000 |
| | Total (n=60) | 4 (6.7%) | 11 (18.4%) | 0.039 |
| | P*** | 0.113 | 0.903 | |
| *Moderate DDI* | Psychiatrists (n=29) | 6 (20.7%) | 10 (34.5%) | 0.289 |
| | IDs (n=31) | 2 (6.5%) | 4 (12.9%) | 0.687 |
| | Total (n=60) | 8 (13.4%) | 14 (23.4%) | 0.180 |
| | P*** | 0.140 | 0.095 | |
| *Major DDI* | Psychiatrists (n=29) | 12 (41.4%) | 23 (79.3%) | 0.001 |
| | IDs (n=31) | 24 (77.4%) | 29 (93.5%) | 0.125 |
| | Total (n=60) | 36 (60.0%) | 52 (86.7%) | <0.001 |
| | P*** | 0.010 | 0.140 | |

*The test could not be performed because the number of participants who answered correctly before the information was zero (not significant). ** within specialty, *** between specialties

DDI: drug-drug interaction, IDPs: infectious disease physicians

Here we have shown that physicians treating PLWH with psychiatric disturbances are not aware of potential DDIs which might be encountered during follow-up. Furthermore, the attitude to potential DDIs might change from physician to physician which may further hinder patient care. There are differences about DDIs between the US Food and Drug Administration and the European Medicines Agency or country-specific information. Therefore, healthcare professionals often rely on other sources (websites, apps) for their daily management of DDIs (Back, 2019). Our results indicate that despite physicians utilizing the same online sources to check and learn potential DDIs; their attitude to potential DDI-related risks is divergent.

According to the sociodemographic characteristics of both groups, differences between sexes, academic degrees and institutions were considered as chance associations due to the nature of the study design. The fact that ID physicians examined significantly more PLWH than psychiatrists was an expected result due to their specialty.

The complexity of ART regimens, adverse effects and inadequate reimbursement as well as DDIs are also identified as barriers to providing improved care (Defty, Smith, Kennedy, Perry, & Fisher, 2010). Potential and risky DDIs associated with initiating concurrent psychotropic drugs in PLWH are quite common (Vitiello et al., 2003). There is undoubtedly a risk of potential DDIs with ARTs as most psychotropic drugs are metabolized by cytochrome P450 (CYP) 3A isozymes whose activity may be inhibited or induced by antiretroviral agents (Cattaneo et al., 2018; Gallego, Barreiro, & Lopez-Ibor, 2012). Furthermore, some psychotropic drugs may inhibit the activity of CYP enzymes and act as instigators of DDIs when simultaneously administered with ARTs, thus creating a complex scenario that may lead to inadequate psychotropic or ART doses and, consequently, sub-optimal clinical responses (Gallego et al., 2012).

In this study, we evaluated the attitudes of clinicians to potential DDIs subjectively and as well as objectively. Despite more than 80% of physicians reporting an average/high level of perceived competence in potential DDIs, the first correct response to clinical scenarios was 0.80 (0.60) (whereas the full score should be 3). Furthermore, the correct response to clinical scenarios were 1.28 (0.61) after brief information. Physicians' level of perceived competence and objective DDI knowledge was not

related. Therefore, there is a fundamental gap in physicians' knowledge and attitudes toward DDIs. According to a study, 46% of general practitioners cited ART-related DDIs as a barrier to prescribing for PLWH, highlighting physicians' concerns (Defty et al., 2010).

(Nabovati et al., 2017) reported that physicians utilized books (42.7%) and smart device applications (33.5%) to check for potential DDIs. However, the ART field is an ever-changing field and new drugs emerge in the market faster than the book publishing rate. In this study, the internet (90.0%) and smart device applications (53.3%) are preferred over hard-copy-published resources. They (Nabovati et al., 2017) also observed that physicians used smart device applications (24.9%) and consulted pharmacists (11.7%) when their patients were exposed to potential DDIs. Clinical pharmacy is in its infancy in our country, so many physicians do not have access to clinical pharmacy consultation. That is why almost all physicians use online resources to check for potential DDIs.

Online resources pose several challenges to their users. Firstly, some of the results are not evidence-based in the light of clinical practice. Secondly, they report on pairs of medicines rather than all treatment regimens, which makes it difficult to manage patients from a broad perspective. Finally, they report potential DDIs using different nomenclatures. For example, any DDI may be described as 'contraindicated' in one database, while the same DDI may be described as a 'serious' interaction in another database.

According to van Stiphout et al., when task analysis, including electronic prescribing training, was performed for physicians (intervention group), the frequency of missed DDIs was less than the control group. This result may improve this situation by making available real-life examples of missed DDIs (van Stiphout et al., 2018). It is estimated that the frequency of potential and clinically significant DDIs will decrease with the increasing use of technologies such as machine learning, electronic health records, and alert systems in healthcare (Obermeyer & Emanuel, 2016).

In addition, the interpretation of potential DDIs and their relevance to the clinical experience of people in this field is of concern. Benni et al. recently showed that professors are more vigilant about drug-food interactions than their junior colleagues, and almost a third of clinicians' face drug-food interactions in clinical practice (Benni, Jayanthi, Basavaraj, & Renuka, 2012; Zawiah et al., 2020). Our findings demonstrate that the level of DDI knowledge and correct DDI management was not related to the seniority of the physicians.

In another study the attitudes of patients about drug-drug interactions were evaluated (Arumugam, Murali, Biju, & Thankachan, 2015). Three-quarters of patients did not believe there was a risk of interaction with other drugs and 58.0% started allopathic and other drugs without consulting their physicians, and 64.0% of the patients stated that they did not communicate regularly with their physicians.

In a study (Immadisetti, Poka, Rajesh, & Varma, 2016), there were 267 DDIs in 107 (44.6%) of 240 PLWH receiving ART. Of these interactions, 14 (5.2%) were contraindicated and 253 (94.8%) were found to require additional follow-up. In a prospective questionnaire-based study, 129 (38.6%) patients had at least one clinically significant DDI, which was not recognized and/or mismanaged in 56 (43%) patients (Kuemmerle et al., 2021). In another study, the search identified 82 PLWH concomitantly receiving antiretroviral and psychotropic drug treatment, 55% of whom had plasma psychotropic drug concentrations that were below minimum adequate plasma levels. The same result was found in only 26% of the samples taken from people without HIV infection (Cattaneo et al., 2018). These results suggest that physicians should be cautious about potential DDIs in PLWH with comorbid diseases, and may change doses or drugs if necessary.

In a study assessing how concerns about adverse effects and DDIs affects medication adherence in

transgender women living with HIV on both hormone therapy (HT) and ART, only 49% of transgender women living with HIV discussed ART–HT DDI with their provider. This highlights the need to improve ART integration due to imperfect ART combinations and limited provider communication (Braun et al., 2017).

Physicians are aware of the adverse effects related to DDIs and the incidence of adverse effects decreases with the drug education given to physicians (Agu, Oparah, & Ochei, 2012; Tetteh et al., 2017). The majority of psychiatrists (72.4%) and IDPs (71.0%) stated that they would check for a DDI when prescribing a psychotropic drug. At the same time, when they found ART-psychotropic DDIs, the majority of psychiatrists (82.7%) and IDPs (67.7%) stated that they would change the interacting psychiatric drug. This result shows that the physicians do not consider changing ARV regime and the majority of infectious physicians are considering interfering with the psychotropic drug even though this decision falls beyond the scope of specialty. Our findings reveal that many physicians overreact to potential minor and moderate DDIs whereas follow-up will suffice. Switching to a second-line regimen because of a minor/moderate risk of DDI may in turn hinder HIV suppression and immune recovery.

Familiarity with the potential interactions and adverse effects associated with certain antiretrovirals and psychotropic medications will empower clinicians to make well-informed prescribing choices, thereby enhancing the health and well-being of this vulnerable patient group (Goodlet et al., 2019). Despite strong guidelines, many patients continue to use ARTs with a high risk of toxicity and clinically significant DDI due to patient reluctance to change therapeutics. Therefore, it is imperative that clinicians encountering PLWH have knowledge of all commercially available ART agents and relevant potential DDIs. To minimize the DDI rate, it is recommended to use resources such as DDI databases (Lexicomp, Medscape, Micromedex, Liverpool, etc.), case reports, clinical trials, DDI alert programs integrated into electronic health record systems, and the development of clinical pharmacist-led antiretroviral stewardship programs (Billedo, Berkowitz, & Cha, 2016; Johnston, Heavner, Liu, Casal, & Akgun, 2023).

In a survey including non-psychiatrist HIV specialist physicians, they failed to routinely evaluate neuropsychiatric comorbidities, follow guideline recommendations, and use questionnaires, highlighting opportunities for improved detection and management in PLWH (Perez-Valero, Blanch, & Martinez, 2022). On the other hand, it is estimated that the integration of education-oriented clinical pharmacy practices in countries with PLWH using psychotropics will enable the identification, prevention and management of clinically significant DDIs.

## CONCLUSION

In our study, we have shown that after providing brief information regarding DDI risk, physicians' attitude to DDI management has been improved. However, physicians tended to overreact to minor and moderate DDI risks which necessitated a clinical pharmacist/pharmacologist consultation. Also, we have evaluated the knowledge and attitudes of attending physicians towards DDIs between antiretrovirals and psychoactive drugs via three didactic case scenarios. We have performed this survey on 2-different specialist groups experienced in the care of PLWH. Our results stress the importance of external consultation in managing DDIs between antiretrovirals and psychoactive drugs.

### *Study Limitations*

However, our results have some limitations, including their cross-sectional design and the limited number of participants. Nevertheless, this study will contribute to the literature and increase the awareness and perspective of psychiatry and infectious physicians, especially against DDIs. On the other hand, as there is no precedent in the literature, the sample size could not be calculated.

In this study, it was found that all of the physicians controlled potential DDIs, the presence of a DDI influenced their decision-making, had partial information about DDIs, and informed their patients about DDIs. Although the vast majority of physicians reported an above-average perceived level of knowledge, their attitudes towards a potential DDI were inadequate, necessitating consultation with a clinical pharmacist. This result indicates that information on the internet is highly questionable and that consulting with current evidence-based databases or clinical pharmacists rather than the internet will allow for more health responses for potential DDIs. It is estimated that repeated DDI training to be applied to psychiatrists and IDPs in clinical practice will improve the awareness and knowledge level of physicians against DDIs in PLWH.

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**AUTHOR CONTRIBUTIONS STATEMENT**

ACI, NY, EK, KD contributed to design; ACI, OK, KB, SU contributed to conception; ACI, NY, EK, OK contributed to acquisition, analysis, or interpretation; KD, KB, SU contributed to interpretation; ACI, NY, EK drafted manuscript; KB, OK, KD, SU critically revised manuscript.

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*RESEARCH ARTICLE*

# A Qualitative Study Exploring Self-Management Behavior of Patients with Chronic Kidney Disease During COVID-19

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***A Qualitative Study Exploring Self-Management Behavior Of Patients with Chronic Kidney Disease During COVID-19***

***SUMMARY***

*Chronic kidney disease (CKD) is a significant public health issue. COVID-19 immensely affects people with chronic diseases, including patients with CKD. We aimed to explore the factors that might impact the self-management of patients receiving haemodialysis (HD) and patients with kidney transplants (KTx) during COVID-19 using the Health Belief Model (HBM). A qualitative phenomenological research design was used to build evidence on the effects of the COVID-19 pandemic on the self-management behavior of patients with CKD. Data were analyzed using framework analysis to identify critical factors influencing patients' perceptions regarding the self-management of their conditions during COVID-19. Eighteen patients with KTx and seven with HD were interviewed during the lockdown. Having CKD was discussed as a major factor contributing to the perception of susceptibility. The fear of death expressed by the participants had a profound impact on self-management. Delays in check-ups and monitoring, staying at home, the financial burden of treatment, lack of trusted information, safety of test sites, and fatalistic attitudes of patients were identified as barriers to the self-management behaviors of patients. External and internal triggers were the motivators of adopting self-management behavior. This study provides an in-depth view of factors impacting the adoption of self-management behavior of patients with CKD during COVID-19 and may offer healthcare providers and policymakers knowledge about factors contributing towards compromised patient care.*

***Key Words:*** *COVID-19, chronic kidney disease, health belief model, qualitative research, self-management.*

***COVİD-19 Sürecinde Kronik Böbrek Hastalarının Öz Yönetim Davranışlarını Araştıran Nitel Bir Çalışma***

***ÖZ***

*Kronik böbrek hastalığı (KBH), önemli bir halk sağlığı sorunudur. COVID-19, KBH hastaları da dahil olmak üzere kronik hastalığı olan kişilerin yaşamını büyük ölçüde etkilemektedir. Bu çalışmada, Sağlık İnanç Modeli kullanılarak, COVID-19 sırasında hemodiyaliz (HD) ve böbrek nakli (KTx) olan hastalarının öz yönetimini etkileyebilecek faktörleri keşfetmek amaçlanmıştır. KBH hastalarının öz yönetimini etkileyen COVID-19 pandemisinin etkilerini belirlemek için nitel bir fenomenolojik araştırma tasarımı tercih edilmiştir. Veriler, nitel yöntemler kullanılarak COVID-19 sürecinde hastaların öz yönetimlerine ilişkin algılarını etkileyen temel faktörleri belirlemek için analiz edilmiştir. COVID-19 pandemisi karantinası döneminde on sekiz böbrek nakli hastası ve yedi hemodiyaliz hastası ile görüşülmüştür. KBH'na sahip olmanın COVID-19'a karşı duyarlılık algısına etki eden temel bir faktör olduğu anlaşılmıştır. Katılımcılar tarafından ifade edilen ölüm korkusu, öz yönetim üzerinde derin bir etkiye sahiptir. Hekim kontrollerinin ve hastanedeki tetkiklerin gecikmesi, evde kapalı kalmak, tedavinin finansal yükü, güvenilir bilgi eksikliği, test merkezlerinin güvenliği ve hastaların kaderci tutumları, çalışmada hastaların kendi kendilerini yönetim davranışlarını engelleyen faktörler olarak belirlenmiştir. Dış ve iç tetikleyiciler, öz yönetim davranışlarını benimsemenin motivasyon kaynaklarını oluşturmaktadır. Bu çalışma, COVID-19 sürecinde KBH hastalarının öz yönetim davranışlarını etkileyen faktörlerin derinlemesine bir şekilde anlaşılmasına katkı sunmaktadır ve sağlık hizmeti sunucuları ile sağlık politika yapıcılarına, hastaların öz bakımlarının tehlikeye girmesine neden olan faktörler hakkında bilgi sağlamaktadır.*

***Anahtar Kelimeler:*** *COVID-19, kronik böbrek hastalığı, sağlık inanç modeli, nitel araştırma, öz-yönetim.*

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## INTRODUCTION

Chronic kidney disease (CKD) is defined as abnormalities in kidney structure or function that last longer than three months, with significant health consequences ('Summary of Recommendation Statements', 2013). CKD is a major public health issue whose global prevalence is predicted to be 13.4% (Murton et al., 2021). A study by Süleymanlar et al. shows that the prevalence of CKD in Turkey is around 15.7% (Süleymanlar et al., 2011). According to the 2020 Turkish Society of Nephrology (TSN), the total number of patients receiving chronic hemodialysis (HD) in Turkey was 60,558, and patients with kidney transplantation (KTx) are 19,405 (Seyahi, Koçyiğit, Ateş, & Süleymanlar, 2022).

Based on the 2020 TSN report, the COVID-19 pandemic resulted in remarkable changes in treatment offered to patients with CKD in Turkey. Many kidney transplant centers were forced to halt their operations, resulting in a considerable drop in the number of kidney transplants and a disruption in the follow-up of transplant patients compared to prior years (Seyahi et al., 2022). It is well established that individuals with chronic conditions are more vulnerable to the COVID-19 virus (Yang & Wang, 2020). In accordance with the data of the same report, the incidence of COVID-19 was 19.6% in patients receiving hemodialysis (HD) and 8.7% in patients with KTx, while the mortality rate due to COVID-19 was 24.4% in patients receiving HD and 11.3% in patients with kindney transplant (KTx) (Seyahi et al., 2022). Other studies demonstrated that individuals with CKD were more susceptible to COVID-19 and had a higher risk of death from the virus than healthy individuals (Al Raiisi et al., 2019).

A high level of patient engagement and interest is a requirement for long-term CKD treatment management, both in decision-making and in the implementation of care (Peng et al., 2019a). Treatment adherence is vital in patients with CKD, due to the use of excessive medications and the need for frequent hospital visits during the treatment process (Cukor, 2017). Many comorbidities, including hypertension and diabetes, are common in patients with CKD, and on average, these individuals reportedly use around 8-10 tablets of medication per day (Nielsen, Juhl, Feldt-Rasmussen, & Thomsen, 2018). During COVID-19, social distancing, health service disruptions, and lockdowns were reported to impact treatment and management, potentially leading to adverse outcomes due to lack of follow-up (Al Raiisi et al., 2019; Iwashita et al., 2018; TC Sağlık Bakanlığı, 2018). Thus, self-management of patients with CKD gained importance during COVID-19.

Self-management behavior in patients with chronic diseases is defined as the dedicated commitment of patients to healthcare activities aimed at maintaining a satisfying quality of life. This includes solving problems, controlling the chronic disease, and adjusting daily life to monitor their own condition and emotions (Ma et al., 2022). Self-management in patients with CKD may include spotting initial warnings, self-adjustments of home-care regimes, and adhering to medication, exercise, and diet recommendations (Peng et al., 2019a). Since CKD is irreversible and patients with HD and KTx need lifelong therapy and, their self-management behaviors play a critical role in determining their life quality and quantity (Ma et al., 2022). However, how people value and make changes to adopt these self-management practices is crucial for the success of such management plans.

The Health Belief Model (HBM) was the mainly used model to understand how patients with CKD view health threats, adherence, and self-management behavior (Chironda, Bhengu, & Manwere, 2019; Kung, Yeh, Lai, & Liu, 2017; Nooriani et al., 2019; Peikani, Shahgholian, & Kazemi, 2018; Sutherland et al., 2021). The HBM states that an individual's adoption of appropriate behavior to prevent a health problem may be influenced by certain individual factors such as perceived susceptibility (beliefs about the possibility

of getting a disease or condition), perceived severity (subjective assessment of the severity of a health problem and its possible consequences), perceived benefits (perceived benefits of an action to decrease the risk of disease), perceived barriers (belief about the obstacles of taking action), cues to action (perceived motivators towards taking action), and self-efficacy (an individual's confidence of his or her competence to perform a behavior successfully) (Champion and Skinner, 2008).

The aim of this study was to explore factors that might have an impact on the adoption of self-management behavior of patients with CKD during COVID-19 by using the HBM in Turkey.

## MATERIALS AND METHODS

### Study Design

As this study aimed to explore the in-depth views and perceptions of patients with CKD, it utilized a qualitative phenomenological research design to investigate the effects of COVID-19 on the self-management behavior of these patients.

### Study Population, Setting, and Recruitment Criteria

The study population consisted of patients with CKD who were receiving treatment or follow-up at the Nephrology Clinic of Ibn-i Sina Hospital, Ankara University, at the beginning of the study. The patients who met the inclusion criteria of receiving HD or a KTx were included in the study. The participants were informed about the purpose of the study and the expertise of the researchers. Patients under 18 years old and/or who cannot decide for themselves were excluded from the study. The interviews were conducted within the Nephrology Clinic of the Ibn-i Sina Hospital in Ankara, Turkey, during the COVID-19 pandemic lockdown period.

This study received ethical approval from the Ankara University School of Medicine Human Research Ethics Committee (approval date: 17/06/2021; protocol number: İ6-397-21). Written informed consent was obtained from each participant. Data confidentiality was ensured, and all data were anonymized.

### Data Collection

Data collection was carried out with a qualitative method using semi-structured face-to-face interviews between July and December 2021. The semi-structured interview allows for conveying the participants' perceptions or experiences about a certain phenomenon of interest to the researcher(s) (Willig, 2013). The interview guide was designed by three authors (MBU, GG, AP) after reviewing relevant literature, and investigating the impacts of previous pandemics on chronic patients' behavior related to disease management. The framework of the HBM guided the development of the interview guide and was also used as a guiding framework for presenting data in this study (Table 1.). The interview guide was piloted by interviewing two patients to assess the face and content validity. No changes were made. These interviews were not included in the study sample for analysis. The interviews were conducted by three authors (MBU, AP, GG) and lasted for 15 minutes to 45 minutes. Participants provided written consent before the interviews. Audio-recording of the interviews was taken after obtaining participants' consent and participants who did not accept to take part in the study were excluded. Data was collected till data saturation was agreed upon by the authors conducting interviews.

**Table 1.** General outline of the interview guide mapped onto the Health Belief Model

| Theme/HBM Components | Questions |
|---|---|
| Opening Questions | What do you think about the relationship between COVID-19 and chronic patients? |
| Perceived Threat | How do you consider the severity of COVID-19 for people who do not have any chronic conditions? |
| Perceived Severity | How do you consider the severity of COVID-19 for people who have a chronic condition? |
| Perceived Susceptibility | Have you tested positive before?<br>If yes, please describe your experiences and their effect on your health.<br>If no, what would be the impact on your health if you were to get COVID-19? |
| Self-Efficacy | How did you manage your treatment during the pandemic?<br>Can you explain your confidence in managing your treatment?<br>Were you confident in managing your treatment when you tested positive?<br>Do you think you would be confident in managing your treatment if you were to test positive? |
| Perceived barriers to self-management | Have you experienced any barriers to managing your treatment during this pandemic?<br>If yes, how did you feel and how did you solve the problems you faced?<br>If no, what did you need to solve the problems you faced? |
| Perceived benefits of self-management | What were the benefits of self-managing your treatment during the pandemic? |
| Cues to Action | What were the driving factors that activated you to engage with your treatment management during the pandemic?<br>Did you get any support from somewhere or somebody? |
| Closing Questions | Do you think there are missing points you want to bring up? |

**Data Analysis**

The framework analysis was performed for data analysis by three authors manually (MBU, GG, AP). The framework analysis was applied in a six-step approach adopted from (Gale, Heath, Cameron, Rashid, & Redwood, 2013): transcription, familiarization, coding, applying a framework, charting the data and, interpreting the data. First, all interview recordings were anonymized using a protocol number (coded as 'DP' for haemodialysis patients and 'TP' for transplant patients) and a sequence number for each interview [e.g. TP4]. All the interviews conducted in Turkish were transcribed verbatim. Second, a naive reading of the verbatim transcripts was done to become familiar with the data. Third, the authors (MBU, GG, AP) independently assigned codes to transcripts capturing underlying concepts of the HBM and charted them into a framework matrix. Charting is where data is deductively coded and summarized, each column being the construct of HBM and each row representing participants. The similar data codes were grouped into sub-themes and discussed every two weeks. With the consensus of the entire research team, the reviewed sub-themes were mapped to the constructs of the HBM. Finally, a detailed report was drafted by MBU, AP, and GG to give an overview of the exploratory findings of the study. The research principles of COREQ for reporting qualitative research were closely followed in this study (Tong, Sainsbury, & Craig, 2007).

**Data Trustworthiness and Validity**

Trustworthiness was achieved through credibility, dependability, and transferability. Credibility was attained by using data triangulation and recruiting both HD and KTx patients. Additionally, to ensure credibility, a peer debriefing was used to get feedback not just on data analysis but also on the various details of the research. Dependability was established by reviewing and comparing the research procedure with other studies (Amin et al., 2020). Additionally, a transparent description of study procedures, data collection, and analysis by three independent authors provides the dependability of this study. In addition, the four authors who analyzed the data provide different expertise due to their diverse backgrounds in social pharmacy, clinical pharmacy, and internal medicine (nephrology), which contributes to the reliability and confirmability of the findings. Thick descriptions of the phenomena, setting, and participants' quotes have been provided, which assist researchers in making judgments about transferability (Schwandt, Lincoln, & Guba, 2007) of the study findings to other contexts.

## RESULTS AND DISCUSSION

### Results

Eighteen patients with KTx and seven patients receiving HD were interviewed. The interviewed patients consisted of nine women and sixteen men. The patient's mean age was 44.7, ranging from 19-74. Considering the employment status of the patients, eleven patients were retired, seven patients were actively working, and seven patients were not working. Of the patients who participated in the study, six were elementary school graduates, twelve were high school graduates, and six were university graduates. Twenty-three of the patients interviewed had received the COVID-19 vaccine, while two of them had preferred not to get vaccinated. No participants withdrew from the study.

The results of the study show the perceived susceptibility due to COVID-19, the perceived severity of COVID-19, the perceived barriers to self-management behavior, the perceived benefits of self-management, self-efficacy, and cues to action underpinned by the essential components of the HBM which can be seen in (Figure 1.). Quotes related to constructs and sub-constructs were provided in Table 2.

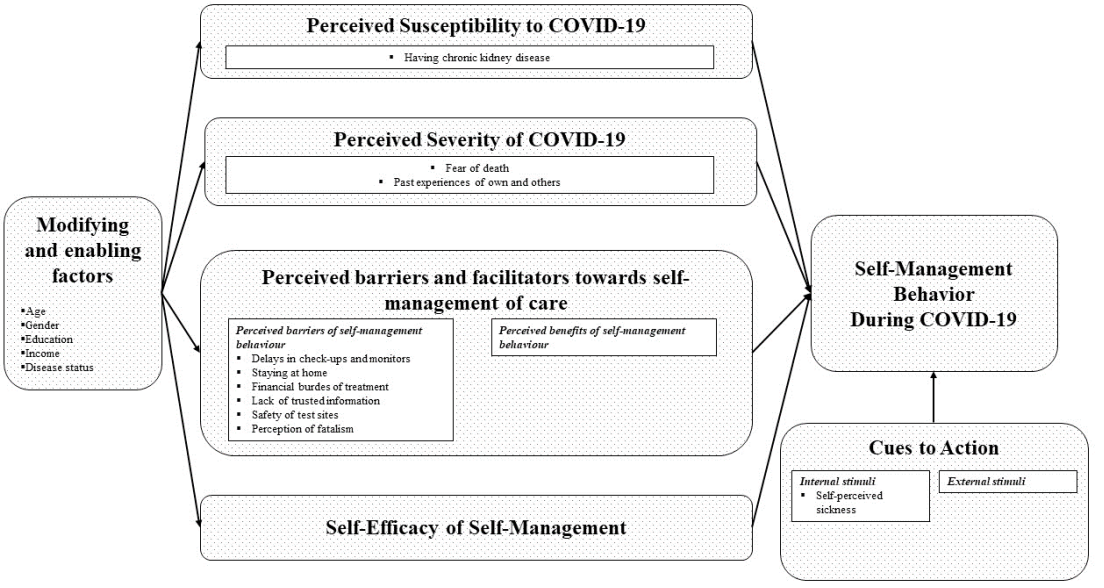


**Figure 1.** Constructs of self-management behavior of patients with CKD during COVID-19

### Perceived Susceptibility to COVID-19

#### Having chronic kidney disease

Most of the participants (16) were aware of the symptoms of COVID-19, and they had a perception that having a KTx or receiving HD would increase the risk of experiencing the symptoms of COVID-19 worse. Patients shared that they felt susceptible to the worsened effects of COVID-19.

### Perceived Severity of COVID-19

Most of the participants (14) perceived COVID-19 as more severe when they adopted self-management behavior. Two sub-themes were identified that had an impact on the participants' perception of the severity of having caught COVID-19.

#### Fear of death

Participants highlighted that they had to cope with the constant feeling of losing their lives due to COVID-19.

#### Past experiences of own and others

Patients who had terrible memories, such as kidney transplant rejection in their past, were more likely to perceive COVID-19 as severe. Moreover, losing friends or loved ones due to COVID-19 had an impact on the perception of severity.

**Perceived Barriers to Self-Management Behavior During COVID-19**

The following factors were identified by the participants that could have an impact on the patients' self-management behavior during COVID-19.

**Delays in check-ups and monitors**

Some patients (5) shared that they had to either delay or cancel their regular hospital visits due to the fear of catching COVID-19. This led to a disruption in their treatment, and they felt that they were unable to self-manage the process on their own.

**Staying at home**

Patients shared the impact of staying at home voluntarily or being in quarantine due to COVID-19 on self-management behavior. They shared that changes in dietary habits, sedentary lifestyles, and lack of exercise caused them to gain weight. Patients felt that being unhealthy was a factor they perceived was going to become an obstacle toward CKD self-management. Some of the patients expressed that staying at home impacted their mental health.

**Financial burdens of treatment**

Participants shared that increased health-related expenditures due to COVID-19 were a barrier against self-management. Some patients (4) mentioned the financial burden of COVID-19, which they experienced while traveling and trying to find a suitable place to stay near the hospital where they received treatment.

**Lack of trusted information**

Patients shared that too much information on COVID-19 left them uncertain about what information to believe. People shared that having access to information from various sources seemed to have a negative impact on the self-management including whether to be vaccinated or to use alternative medicines to boost their immune systems.

**Safety of test sites**

Participants expressed that they were self-managing their testing to continue their dialysis during COVID-19. They mentioned that they were aware that they had to be tested to continue their dialysis during COVID-19. Patients shared that while getting dialysis, because of the high risk of COVID-19, they were advised against using testing sites that were conveniently located inside the hospital. This was an additional challenge, and patients believed it impacted their usual care.

**Perception of fatalism**

Patients shared that the perception of fatalism negatively affected their self-management behavior towards CKD. If patients believed that there is nowhere to run from destiny, this behavior has been called the fatalism effect. This attitude also impacted their behaviors to get vaccinated, paying attention to social distancing, and following home-care regimens. Some patients shared a resignation and shared there might be no escape from COVID-19.

**Perceived Benefits of Self-Management Behavior During COVID-19**

Some of the participants (4) shared that they believed in the benefits of practicing self-management behaviors such as exercising and following a proper diet during the pandemic.

**Self-Efficacy of Self-Management Behavior During COVID-19**

Participants shared that as they have been coping with CKD for many years, they feel more in control and self-efficient in handling unfortunate circumstances and difficulties in assessing care pathways and facilities.

**Cues To Action**

The following subthemes show participants' views on external and internal factors triggering their self-management behaviors:

**External Stimuli**

Participants reported that getting moral support from families, relatives, and their workplaces helped them to cope with the burden of the pandemic and its effect on their health. Some of the participants (3) who were employed shared they needed a supportive

approach regarding flexibility of working hours when their work performance decreased due to COVID-19.

CKD patients expressed that due to COVID-19 related impact on their ability to work and earn a livelihood, having government funds for chronic patients might help support them financially and avoid any health-related impacts.

**Internal stimuli**

**Self-perceived sickness**

Self-perception of illness had an impact on one's motivation to adopt self-management behavior during the pandemic. Patients who considered themselves in a more high-risk situation felt obliged to adhere to routine check-ups, COVID-19 protective behaviors, and develop a healthy lifestyle.

**Table 2.** Quotes representing constructs and sub-constructs

| Construct or sub-construct | Quotes |
|---|---|
| Having chronic kidney disease | "This is a fearful process for us! Lots of drugs like immunosuppressants make us delicate. For instance, if I'm caught… I think I have only a ten percent chance to survive." (TP23) |
| Fear of death | "I have been on dialysis for seventeen years and in the end had a chance to be transplanted... There were times that I found myself in fear… If I get this virus, would I be hospitalized? Maybe I will die before losing my kidney…" (TP11). |
| Past experiences of own and others | "A friend next to my bed got COVID-19. He passed away... it wasn't easy to handle. We, all in the dialysis unit, were very negatively affected... This was a turning point for me!" (DP24) |
| Delays in check-ups and monitors | "The last time I came for a check-up was in October 2019. I haven't been here [means hospital] since the pandemic started. I tried to control my urine at home by not using the toilet and collecting it in a bottle to check if its colour was abnormal, but these tasks were difficult to manage at home." (TP23) |
| Staying at home | "I am always eating across the TV and do not want to go outside even for shopping." (TP11)<br>"…very boring, depressing atmosphere at home. You cannot see your friends and go out for a dinner. When you become stressed, it hits your whole body, especially us." (TP13) |
| Financial burdens of treatment | "I took out a loan and bought a car, which put me in a tight spot… and when we come to this city for hospital bits, we must stay at a hotel… There's no other place to stay in a pandemic. Some of our friends who cannot afford to stay at a hotel had to spend the night in the hospital garden. What a shame!" (TP22) |
| Lack of trusted information | "I have congenital kidney disease. Someone says the vaccine causes infertility, the other says it damages the kidney, and I'm a little bit nervous about what to believe." (TP14) |
| Safety of test sites | "Hospital staff warned me that the test place was extremely contagious as there were a lot of COVID-19 patients inside. He said, "We can't get your test here, because you have kidney disease. Let's direct you to the dialysis unit, they can take the test and send your sample to us". When I got the unit, they turned me down. They referred me to give my test in the emergency room at midnight. I was tired of rushing around and became angry. It shouldn't take too long to find a suitable place!" (DP25) |
| Perception of fatalism | "There was fear in the first three months of the pandemic, but now I am relaxed… Allah Almighty has given me this life and will take it back. No matter how cautious you are, COVID-19 will find you anyway." (TP13) |
| Perceived Benefits of Self-Management Behavior During COVID-19 | "I know monitoring myself has benefits for my chronic conditions… I am also trying to maintain my diet to live healthier." (TP6) |
| Self-Efficacy of Self-Management Behavior During COVID-19 | "The pandemic did not affect my treatment process much. I could manage… have trust … because I have been diagnosed for a long time." (TP14) |
| External stimuli | "I feel so lucky that my colleagues at work, especially our managers, are not complaining while putting up with me as I couldn't come to the office several times. We need to see tolerance (patience) for our decreased work performance." (TP10)<br>"If the government helped us to cover some of our expenses, that would have been nice!" (TP10) |
| Self-perceived sickness | "I am a newly transplanted patient and I know I am at risk. My values are constantly fluctuating. I had to come to the hospital every weekend, I couldn't put off my visits… I am coming with a double mask, with these things (means face coverings and shields)" (TP10) |

**Discussion**

Deeply exploring the factors affecting the self-management of patients with CKD has allowed us to understand the needs of patients during the pandemic as well as the barriers and triggers that have an impact on the adoption of self-management behavior.

In this study, as observed in some other studies, patients with CKD had a strong sense of perceived susceptibility toward getting infected with COVID-19 and were found to be seemed anxious during this period due to their susceptibility to COVID-19 (Jiang et al., 2021; Rabb, 2020). This is congruent to other study findings that also report being a patient with CKD increased patients' perception of susceptibility to COVID-19 (Duculan et al., 2021; Kirchberger, Berghaus, von Scheidt, Linseisen, & Meisinger, 2021). This has been reported in a study that shows that patients with CKD in COVID-19 experienced an increased risk of hospitalization, severity in COVID-19 symptoms, admission to intensive care units, and high mortality rates (Jdiaa et al., 2022).

In our study, the perceived severity of COVID-19 was a major concern among the study population and was reported to have an impact on the adoption of self-management behavior, which was mostly related to the fear of death. This is also reported in a study exploring the self-management of people with diabetes, which found that COVID-19 was related to an increase in fear of death and its impact on the self-management of individuals (Kaplan Serin & Bülbüloğlu, 2021). Even though there are studies (Frontini, Sousa, Ribeiro, & Figueiredo, 2021; Luo, Ghanei Gheshlagh, Dalvand, Saedmoucheshi, & Li, 2021) showing fear as a motivator of adopting a self-management behavior in the face of COVID-19, further research is needed to explore the difference in the perception of the severity of COVID-19. In this study, patients who had witnessed people suffering due to COVID-19 were more likely to perceive and fear death from COVID-19. This is shown in another study where the perceived severity of leptospirosis was reported to be reinforced by seeing other patients die or having experienced severe consequences due to leptospirosis infection (Sukeri et al., 2020).

This study reports that people might delay follow-up or monitoring for CKD during COVID-19. People with CKD also shared that they might be able to self-manage their CKD despite the inability to visit hospitals regularly (Jiang et al., 2021; Okoro, 2021; Rabb, 2020). Our study reports that people with CKD might be experiencing mental health issues due to COVID-19, which remains consistent with other studies reporting the negative effect of COVID-19 on mental health during quarantine (Mattioli, Ballerini Puviani, Nasi, & Farinetti, 2020; Vindegaard & Benros, 2020; Xiong et al., 2020).

Apart from delayed follow-ups and the mental consequences of quarantine, patients shared that the reduction in physical activity and lack of a healthy diet were inhibiting their self-management. This has also been reported in other studies, which discuss how staying in quarantine may be inhibiting people from adopting and practicing better health behaviors (Louvardi, Pelekasis, Chrousos, & Darviri, 2020; Mattioli et al., 2020).

COVID-19-related financial burden was also a barrier to self-management in this study. This is also reported in another study from Turkey, which reported that patients receiving HD with high incomes were better at managing their treatment (Bağ & Mollaoğlu, 2010). Similar challenges have been reported in people with cancer, where financial incompetence was related to reduced healthcare visits during the pandemic and recommended government support vital for chronic patients (Chan, Ho, Li, Tam, & Tang, 2021; Ou et al., 2022).

People in this study also shared their confusion and mistrust in available information, which compromised their ability to self-manage their disease. This issue has also been reported in other studies (Jimenez et al., 2020). As a unique finding of our study, the perception of fatalism caused patients

with CKD to be unresponsive to events and feeling a state of belief, of not having control over their lives. This was reported to be a sign of a lack of interest in self-management and losing the opportunity to self-manage their condition and could potentially lead to patient harm.

Previous studies show the perceived benefits of self-management might have a direct impact on self-management behaviors (Sharifirad, Azadbakht, Feizi, Kargar, & Mohebi, 2013). Self-management can enhance the well-being of patients with CKD by helping with weight and blood pressure control, encouraging regular exercise, and improving mental health (Lin, Liu, Hsu, & Tsai, 2017; Peng et al., 2019b). Since perceived benefits are one of the predictors of engaging in positive health behavior (Sulat, Prabandari, Sanusi, Hapsari, & Santoso, 2018), informing patients with CKD about the benefits of self-management is crucial.

Previous studies examining the self-efficacy of patients with CKD to self-manage reveal that several factors, including age, marital status, family support, stage of disease, and education level, have an impact on self-efficacy (Curtin et al., 2008; Qalawa, Eltahry, & Aly, 2022). This study adds that years of experience with CKD might be another factor affecting the self-efficacy of patients with CKD in self-management during COVID-19. Furthermore, studies exploring how fatalism might lead to low self-efficacy, which in turn affects perceived severity (Jimenez et al., 2020) might be a future direction for researchers to understand the causality between self-efficacy, perceived fatalism, and severity among CKD patients.

Supportive behavior from family and relatives was determined to encourage patients to adopt self-management behaviors positively (Qalawa et al., 2022). Moreover, having flexibility in the employment sector is more likely to prompt patients with CKD to be able to self-manage. Although some are in favour of flexibility in the working environment (Nerobkova, Park, Park, & Jang, 2022), others mentioned that shifts in work hours and flexibility can lead to increased work stress, fatigue, burnout, and time conflict (Golden, Henly, & Lambert, 2013). New studies should look at understanding the relationship between flexibility in work schedules and employee well-being in CKD and other chronic patients. Internally, the self-perception of illness might act as a motivator for adopting new behaviors such as self-management. In a study exploring diabetes patients' decision-making process regarding contraceptive usage by using HBM, patients who identified themselves as having poor health are avoiding pregnancy and have a likelihood to use contraceptives as a cue to action (Johnson et al., 2021).

**Strengths and limitations**

This study extends previous work by exploring the self-management behavior of patients receiving a KTx and HD during COVID-19. The thick rich descriptions give exemplars that may be valuable to clinicians, researchers, and health policymakers who might wish to reflect on their daily practices.

This study provides valuable insights into the self-management of patients with CKD during COVID-19. The findings in this study have shed some light on the factors affecting the adoption of self-management behaviors in patients with CKD. This study was conducted during the COVID-19 pandemic; therefore, factors impacting self-management behavior may be examined differently now that COVID-19 has reduced its impact on individuals, societies, and cultures. Future studies should also carefully consider the factors identified in the study to help inform policymakers to develop scientific emergency measures. New interventions, self-management care pathways and virtually supporting and monitoring of people with chronic conditions could lead to reduced patient-related mortality and morbidity and help reach better patient outcomes.

The demographic limitations of this qualitative study on CKD patients' self-management behavior

include potential biases stemming from the sample's limited diversity. The participants may predominantly represent certain age groups, ethnicities, socio-economic statuses, or geographic regions, which can affect the generalizability of the findings.

The study was conducted across a single hospital in Turkey and hence the findings may not be transferable to the other parts of the country. Due to the sampling methodology used to recruit patients, the data collection phase of this study was conducted at the dialysis units. The researchers could not spend extended time with the participants due to COVID-19 restrictions in the hospital at that time. The participants' expressions might have been limited due to restrictions on both interviewer and interviewees.

The COVID-19 pandemic is a period of rapid change. This study was carried out during a period when quarantine measures were strict due to the pandemic. Therefore, the resulting structure is suitable for explaining the quarantine period of the pandemic and may be insufficient to explain the entire pandemic period.

## CONCLUSION

Patients with CKD perceive themselves as susceptible to excessive harm from COVID-19 and expressed a fear of death. Many factors in a patient's care pathway, both external and internal, may impact the self-management behavior of people with CKD. The knowledge of this study highlights the need for patient inclusivity and their respective needs to be considered by healthcare teams while designing therapeutic regimes. Policymakers should develop protocols and guidelines for people with chronic conditions in case of emergencies like COVID-19 and ensure patients are offered full access and support to treatment and lifesaving facilities.

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## AUTHOR CONTRIBUTION STATEMENT

MBU: Conceptualization, Methodology, Investigation, Interviewing, Data Curation, Data Analysis, Writing—Original Draft Preparation

GG: Conceptualization, Methodology, Investigation, Interviewing, Data Curation, Data Analysis, Writing— Review & Editing

AP: Conceptualization, Methodology, Investigation, Interviewing, Data Curation, Writing— Review & Editing

ŞŞ: Conceptualization, Writing— Review & Editing Supervision

## CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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*RESEARCH ARTICLE*

# A Network Toxicology Analysis of the Molecular Pathways and Novel Targets in TCDD-Induced Cardiovascular Toxicity

Fuat KARAKUŞ*°

***A Network Toxicology Analysis of the Molecular Pathways and Novel Targets in TCDD-Induced Cardiovascular Toxicity***

***SUMMARY***

*2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), an environmental contaminant, disrupts multiple systems including endocrine, immun, nervous, reproductive, developmental, and cardiovascular. This study aimed to identify the molecular pathways and potential therapeutic targets for TCDD-induced cardiovascular toxicity using CTD, ShinyGO, STRING, GeneMANIA, ChEA3, MIENTURNET, and Cytoscape computational tools. The analysis identified the AGE-RAGE signaling pathway, blood circulation, and cytokine receptor binding as the top 3 among ten key molecular pathways, biological processes, and molecular functions associated with TCDD-induced cardiovascular toxicity. Additionally, ten hub proteins/genes were found to play a critical role, with NFKB1 being the most essential regulating transcription factor and hsa-miR-19a-3p and hsa-miR-125b-5p as the most crucial microRNAs. This study sheds light on the molecular mechanisms underlying TCDD-induced cardiovascular toxicity, revealing novel potential targets for therapeutic intervention.*

***Key Words:*** *Cardiovascular toxicity, hsa-miR-19a-3p, hsa-miR-125b-5p, NFKB1, TCDD.*

***TCDD ile İndüklenen Kardiyovasküler Toksisitede Moleküler Yolakların ve Yeni Hedeflerin Ağ Toksikolojisi Analizi***

***ÖZ***

*2,3,7,8-Tetraklorodibenzo-p-dioksin (TCDD), çevresel bir kirleticidir ve endokrin, immün, sinir, üreme, gelişimsel ve kalp-damar sistemleri dahil olmak üzere birçok sistemi bozmaktadır. Bu çalışma, CTD, ShinyGO, STRING, GeneMANIA, ChEA3, MIENTURNET ve Cytoscape gibi hesaplamalı araçlar kullanarak TCDD kaynaklı kalp-damar toksisitesi için moleküler yolları ve potansiyel terapötik hedefleri belirlemeyi amaçlamaktadır. Analiz, TCDD kaynaklı kalp-damar toksisitesi ile ilişkili 10 önemli moleküler yol, biyolojik süreç ve moleküler fonksiyon arasında AGE-RAGE sinyal yolu, kan dolaşımı ve sitokin reseptör bağlanmasının en önemli 3'ü olduğunu belirlemiştir. Ek olarak, 10 hub protein/genin kritik bir rol oynadığı, NFKB1'in en önemli regüle edici transkripsiyon faktörü ve hsa-miR-19a-3p ile hsa-miR-125b-5p'nin en önemli mikroRNA'lar olduğu bulunmuştur. Bu çalışma, TCDD kaynaklı kalp-damar toksisitesinin altında yatan moleküler mekanizmaları aydınlatarak terapötik müdahale için yeni potansiyel hedefler ortaya koymaktadır.*

***Anahtar Kelimeler:*** *Kardiyovasküler toksisite, hsa-miR-19a-3p, hsa-miR-125b-5p, NFKB1, TCDD.*

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## INTRODUCTION

Dioxins are a class of persistent organic pollutants, wherein 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) stands out as a well-known representative (Figure 1A). TCDD is unintentionally produced during various processes, including the combustion of organic materials and as a side product in organic synthesis (Ogino et al., 2016). This highly stable compound exhibits a long half-life in humans (5–10 years) and persists in the environment at varying concentrations (0.01-1.31 ng/g) (Sorg et al., 2009; Colombo et al., 2011; Sforzini et al., 2014). While human exposure to TCDD primarily occurs through contaminated food or occupational environments, the industrial accident that occurred in Seveso, Italy, in 1976 also caused years of exposure. With the international legal measures taken in accordance with the Stockholm Convention, the level of TCDD in the environment, and therefore human exposure, has gradually decreased, especially in developed countries. TCDD is one of the most toxic chemicals. While short-term high-levels exposure can cause liver damage and chloracne, long-term exposure is linked to a wide range of health problems affecting the endocrine, immune, nervous, reproductive, and cardiovascular systems (Gogal and Holladay, 2008; Humblet et al., 2008; Sorg et al., 2009; Marinković et al., 2010; Pelcl et al., 2018; Gaspari et al., 2021).

TCDD-induced cardiovascular toxicity has been observed in various animals, such as fish, birds, and rodents. While animal studies have identified the aryl hydrocarbon receptor pathway as a critical contributor, this pathway alone does not fully explain the observed effects in humans (Kopf and Walker, 2009; Mohsenzadeh et al., 2018; Mi et al., 2023). This knowledge gap hinders the development of effective preventive and therapeutic strategies for TCDD-related cardiovascular diseases in humans. Therefore, this study aims to explore the molecular pathways, biomarkers, and potential therapeutic targets associated with TCDD-induced cardiovascular toxicity in humans using computational tools.

## MATERIAL AND METHOD

### Identification of common genes between TCDD and cardiovascular diseases

The identification of genes linked to TCDD and their associations with cardiovascular diseases (CVDs) was accomplished using the Comparative Toxicogenomics Database (CTD; https://ctdbase.org) and its tools (Davis et al., 2023). CTD curates and connects a wide range of data on chemical exposures and their biological effects across various species. This process involves manually curating and connecting data on chemicals, genes, phenotypes, anatomies, diseases, taxa, and exposures from published literature. CTD currently provides 45 million toxicogenomic relationships for over 16,300 chemicals, 51,300 genes, 5,500 phenotypes, 7,200 diseases, and 163,000 exposure events from 600 comparative species (Davis et al., 2023). However, the limitations of the CTD rely on the manual curation of scientific literature, which can lead to incomplete coverage. Not all relevant studies might be included, and newly published data may not be immediately available.

To analyze genes related to TCDD, the term "TCDD" was entered in the "Chemicals" section of CTD, and all resulting genes were downloaded. The genes associated with CVDs were acquired from the "Direct Evidence" section of CTD, where "M" indicates "marker/mechanism" and "T" represents "therapeutic." To identify common genes related to both TCDD and CVDs, the MyVenn CTD tool (Davis et al., 2023) was utilized. It can process up to three input lists and display the results as a Venn diagram. All findings in this study are based on data collected in March 2024.

### GO bioprocess analysis and KEGG enrichment analysis

Gene Ontology (GO) terms were analyzed for annotated genes associated with TCDD and CVDs using ShinyGO 0.80 (Ge et al., 2020). ShinyGO is a gene list enrichment analysis tool accessible at (Ge et al., 2020). It is based on a vast annotation database

that includes 1,678 bacterial species, 59 plant species, 256 animal species, and 115 archaeal species from Ensembl and STRING-db. Among its attributes are the graphical representation of enrichment outcomes and gene attributes. Additionally, it offers program interface access to KEGG (Kanehisa et al., 2023) and STRING (Szklarczyk et al., 2015) for retrieving pathway diagrams and protein-protein interaction networks, respectively. However, ShinyGO has some limitations; for instance, it does not offer dynamic or real-time updates to reflect changes in GO annotations or underlying databases, and the enrichment analysis may be influenced by bias in GO annotations, such as overrepresentation of well-studied genes or biological processes.

For the analysis, the common gene list was input into ShinyGO, with *Homo sapiens* selected as the target species. For gene ontology analysis, the top 10 molecular pathways (MP), biological processes (BP), and molecular functions (MF) were determined. The significance of the results was established by applying a false discovery rate (FDR) correction and adhering to a recommended p-value cut-off of 0.05.

**Protein-protein interaction and centrality analysis**

For protein-protein interactions (PPIs) of the common genes between TCDD and CVDs, STRING v.12.0 (https://string-db.org/cgi) was used. The STRING database systematically gathers and consolidates information on protein-protein interactions, including direct physical associations and functional relationships. This data is sourced from various channels, including automated text mining of scientific literature, computational predictions based on coexpression and conserved genomic context, databases housing interaction experiments, and established complexes/pathways curated from reliable sources (Szklarczyk et al., 2015). However, it's important to note some limitations of STRING. Firstly, the accuracy of predicted interactions can vary, and false positives may be present due to the inherent complexity of protein interactions and limitations in computational algorithms. Secondly, STRING's reliance on existing annotations and curated data may not capture newly discovered or less well-studied protein interactions, leading to potential gaps in coverage.

For the analysis, the protein set was entered into the "Multiple Proteins by Names/Identifiers" section, with *Homo sapiens* designated as the chosen species. A minimum interaction score of 0.4 was established. The final PPI network was constructed using Cytoscape version 3.10.1 (http://www.cytoscape.org/). Cytoscape is an open-source software for interactive analysis, integration, and visualization of network data. It provides a versatile platform for researchers to explore biological networks, including protein-protein interactions, gene regulatory networks, and metabolic pathways. Additionally, Cytoscape supports the integration of additional plugins, expanding its functionality for specific analysis tasks such as functional enrichment, network clustering, and pathway analysis. However, Cytoscape also has some limitations. Firstly, while it offers extensive flexibility, the complexity of the software may present a steep learning curve for users unfamiliar with network analysis tools. Secondly, Cytoscape's performance may degrade when handling large-scale networks with thousands of nodes and edges, requiring substantial computational resources.

Additionally, centrality analysis was evaluated using the Network Analyzer Cytoscape plugin and the cytoHubba plugin (https://apps.cytoscape.org/apps/cytohubba) to identify core proteins TCDD-induced CVDs. This evaluation was based on six topological algorithms: degree, closeness, radiality, edge percolated component (EPC), maximum neighborhood component (MNC), and stress.

**Gene network analysis**

The common genes between TCDD and CVDs

were inputted into GeneMANIA (http://genemania.org) to study the network of gene-gene interactions (Warde-Farley et al., 2010). GeneMANIA identifies other genes associated with a group of input genes by utilizing an extensive array of functional connection data, encompassing protein and genetic interactions, pathways, coexpression, co-localization, and protein domain similarity. Currently, it maps 166,691 genes from nine organisms (Warde-Farley et al., 2010). However, GeneMANIA also has some limitations. Firstly, while it provides valuable insights into functional relationships among genes, the accuracy of predictions may vary depending on the quality and completeness of input data. Secondly, the reliance on pre-existing databases and computational algorithms means that GeneMANIA's results may not capture emerging or context-specific gene associations, potentially leading to incomplete or biased interpretations.

In this study, *Homo sapiens* was chosen as the target organism for analysis, and an automatically selected weighting method was employed.

**Analysis of transcription factors and microRNAs**

The common genes between TCDD and CVDs were input into ChIP-X Enrichment Analysis Version 3 (ChEA3) (https://maayanlab.cloud/chea3) to identify the transcription factors (TFs) responsible for their regulation. ChEA3 is an online tool for TF enrichment analysis, which evaluates and prioritizes TFs linked to gene sets submitted by users (Keenan et al., 2019). The ChEA3 background repository encompasses various gene set libraries compiled from diverse origins, such as TF-gene coexpression data derived from RNA-seq investigations, TF-target associations identified through ChIP-seq experiments, and TF-gene cooccurrence patterns computed from gene lists submitted by users (Keenan et al., 2019). However, ChEA3 also has a limitation. The accuracy of TF enrichment analysis heavily depends on the quality and coverage of underlying datasets, which may vary across different experimental sources and conditions.

Next, the common genes were also subjected to the MIcroRNA Enrichment TURned NETwork (MIENTURNET) tool (http://userver.bio.uniroma1.it/apps/mienturnet/), and *Homo sapiens* was selected as the target to determine potential miRNA networks from miRTarBase that were experimentally confirmed. MIENTURNET uses computationally predicted or experimentally validated miRNA-target interactions from several organisms, including *Homo sapiens, Mus musculus, Rattus norvegicus, Caenorhabditis elegans, Drosophila melanogaster,* and *Danio rerio* (Licursi et al., 2019). MIENTURNET integrates multiple sources of miRNA-target interaction data, including databases curated from literature mining, computational predictions based on sequence complementarity, and experimental validation assays. Additionally, it offers advanced analysis features such as network visualization, pathway enrichment analysis, and prioritization of crucial regulatory hubs. The limitation of MIENTURNET is that the accuracy of predicted miRNA-target interactions may vary depending on the underlying computational algorithms and experimental validation methods. False positives and negatives in the predicted interactions could lead to misinterpretation of regulatory networks.

In the analysis, the threshold for the minimum number of miRNA-target interactions was set at 2, and the adjusted p-value (FDR) was 0.5.

**Chemical-gene binary interaction analysis**

To establish correlations between protein/genes linked to CVDs and genes associated with TCDD, a manual analysis was conducted using CTD (https://ctdbase.org). This involved scrutinizing the "gene interaction" card in the CTD chemical profile, specifically identifying interactions between proteins/genes and TCDD regarding protein activity, mRNA expression, and protein expression. The resulting table enumerates the interactions between TCDD and selected genes, excluding interactions involving a combination of two or more chemicals and their collective impact on the genes.

## RESULTS AND DISCUSSION

TCDD, a persistent organic pollutant, causes various adverse effects, including cardiovascular toxicity. While animal studies suggest mechanisms involving AhR activation, the underlying causes of TCDD-induced CVD in humans remain unclear. This study aimed to elucidate potential molecular mechanisms and targets associated with this toxicity using toxicogenomic analysis and other bioinformatics tools.

### Common genes associated with TCDD and CVDs

A search of the CTD database showed that TCDD targets 17,055 genes. Additionally, the database identified 3,782,920 genes associated with CVDs, of which 1,674 were marked as "markers/mechanisms" and/or "therapeutics" in the "Direct Evidence" section. Furthermore, 1,438 genes were common between TCDD and CVDs (Figure 1). All common genes are presented in Table S1.

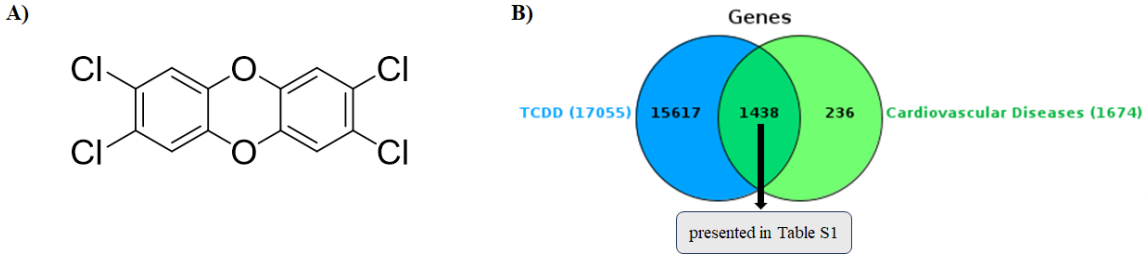


**Figure 1. A)** Molecular structure of TCDD.

**B)** Venn diagram illustrating the 1,438 genes common between TCDD and CVDs.

### GO enrichment analyses of common genes

GO analysis identified the top 10 critical molecular pathways, biological processes, and molecular functions involved in the development of CVDs induced by TCDD (Figure 2). The results highlight the AGE-RAGE signaling pathway as a primary molecular pathway related to the 1,438 common genes (Figure 2A). Advanced Glycation End Products (AGEs) are harmful molecules formed naturally during metabolism and aging. These arise when sugars bond with proteins or fats in a non-enzymatic process. Significantly, AGEs bind to receptors for AGEs (RAGE), triggering a cascade of cellular events contributing to CVD development. Chronic inflammation, oxidative stress, and endothelial dysfunction are all hallmarks of CVD, and research shows the AGE-RAGE pathway plays a crucial role in their activation (Lee et al., 2019). Clinical studies support this connection. Sabbatinelli et al. (2022) found that higher levels of circulating AGEs and RAGEs were linked to increased mortality and major cardiovascular complications in patients with type 2 diabetes. Similarly, Singh et al. (2022) suggested that AGEs are not only associated with arterial stiffness and atherosclerosis but also with impaired cellular signaling in the endothelium, vascular smooth muscle cells, and platelets. These detrimental effects on blood vessel function and blood cell activity further solidify AGEs as crucial risk factors for CVD. Identifying of the AGE-RAGE signaling pathway as the most crucial molecular pathway associated with common genes suggests that TCDD may exert its cardiovascular effects, at least in part, through modulation of this pathway.

Among biological processes, blood circulation (Figure 2B) was identified as the most significant contributor to TCDD-induced CVDs, while cytokine receptor binding was found to be the most critical molecular function (Figure 2C). Efficient blood circulation is crucial for maintaining cardiovascular health. Disruptions in blood flow, often due to atherosclerotic plaque formation, lead to ischemia and infarction. The

association of blood circulation as the critical biological process with the common genes implies that these genes may play a crucial role in mediating the effects of TCDD on vascular health. TCDD exposure has been shown to disrupt normal vascular function and impair blood circulation, contributing to the development of CVDs such as atherosclerosis and hypertension (Kopf et al., 2010; Walsh-Wilcox et al., 2019; Bock, 2019).

The identification of cytokine receptor binding as the primary molecular function associated with the common genes suggests that TCDD may influence cardiovascular health by modulating cytokine signaling pathways and cytokines play a central role in mediating inflammatory responses. Dysregulated cytokine receptor binding could lead to aberrant immune responses and chronic inflammation, contributing to the pathogenesis of TCDD-induced CVDs (Bock, 2019).

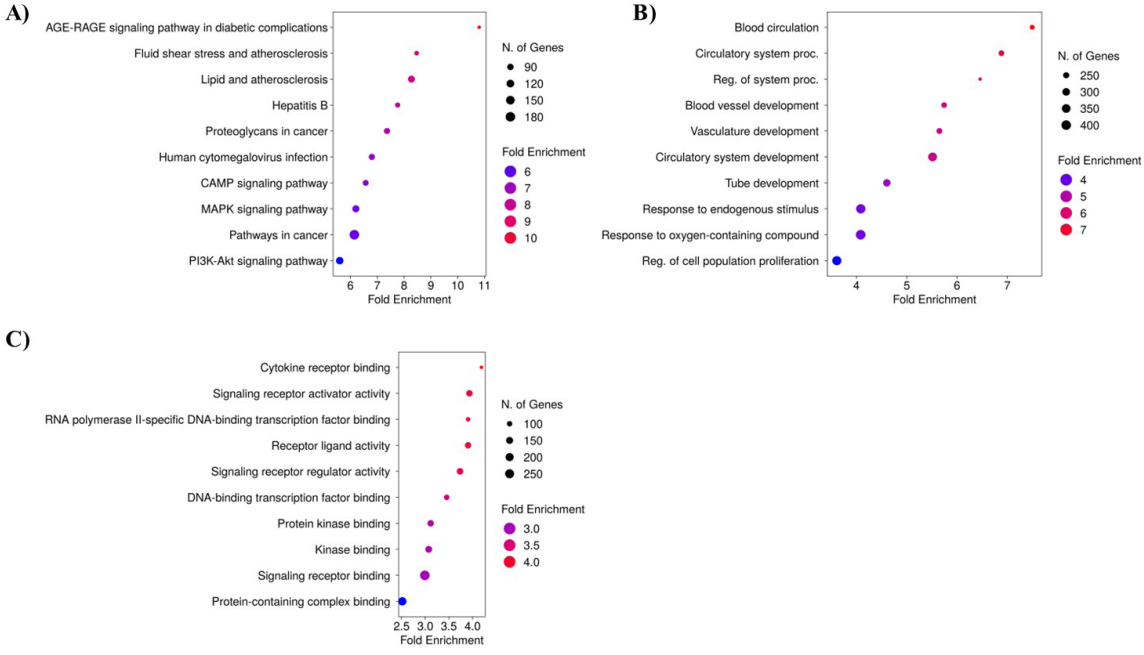


**Figure 2.** GO enrichment analysis of 1,438 common genes: **A)** Top 10 KEGG pathways of the common genes. **B)** Top 10 biological processes associated with the common genes. **C)** Top 10 molecular functions associated with the common genes.

**PPI interactions, hub proteins/genes involved in TCDD-induced CVDs, and gene-gene interactions**

The PPI network revealed 1,371 nodes and 43,318 edges, as illustrated in Figure 3A, with a PPI enrichment p-value of $<1.0x10^{-16}$. Centrality analysis was also performed to determine the core proteins. As a result of the analysis, top 10 hub proteins/genes of 1,438 common proteins/genes in the list was selected based on their six topological algorithms (Figure 3B, Table 1). This hub proteins/genes alphabetically: Actin, cytoplasmic 1 (ACTB), RAC-alpha serine/threonine-protein kinase (AKT1), Albumin (ALB), Epidermal growth factor receptor (EGFR), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Interleukin-1 beta (IL1B), Interleukin-6 (IL6), Insulin (INS), Tumor necrosis factor (TNF), and Cellular tumor antigen p53 (TP53).

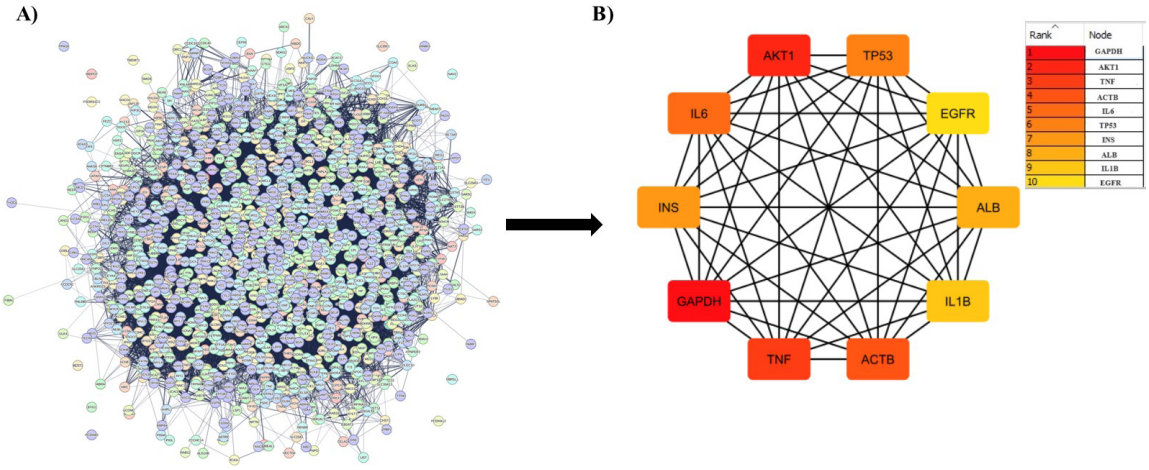


**Figure 3. A)** PPI network from STRING analysis of 1,438 proteins/genes common between TCDD exposure and CVDs. **B)** Centrality analysis of the common proteins/genes by Cytoscape. The darker-colored nodes score higher in most of the six topology algorithms (Table 1).

**Table 1.** Centrality analysis of 1438 common proteins revealed the ten most essential proteins.

| Hub proteins | Degree score | Closeness Score | Radiality Score | EPC Score | MNC Score | Stress Score |
|---|---|---|---|---|---|---|
| GAPDH | 1 | 1 | 1 | 2 | 1 | 1 |
| AKT1 | 2 | 2 | 2 | 3 | 2 | 2 |
| TNF | 3 | 3 | 3 | 1 | 3 | 5 |
| ACTB | 4 | 4 | 4 | 8 | 4 | 4 |
| IL6 | 5 | 5 | 5 | 4 | 5 | 9 |
| TP53 | 6 | 6 | 6 | 9 | 6 | 3 |
| INS | 7 | 7 | 7 | 7 | 7 | 6 |
| ALB | 8 | 8 | 8 | 6 | 8 | 7 |
| IL1B | 9 | 9 | 10 | 5 | 9 | 10 |
| EGFR | 10 | 10 | 9 | 10 | 10 | 8 |

The GeneMANIA online plug-in created a linked network among hub genes. The results showed that most genes associated with CVDs were involved in coexpression (54.37%) and physical interactions (25.82%). Other types of interactions were less pronounced: genetic interactions (9.94%), predicted interactions (7.06%), colocalization (2.48%), and pathways (0.33%) (Figure 4). Coexpression, the dominant interaction among hub genes, suggests similar expression levels across conditions in a gene expression study. Namely, when one gene is upregulated or downregulated, the others coexpressed with it also show a similar expression pattern. Co-expressed genes may be involved in the same biological processes, pathways, or cellular functions.

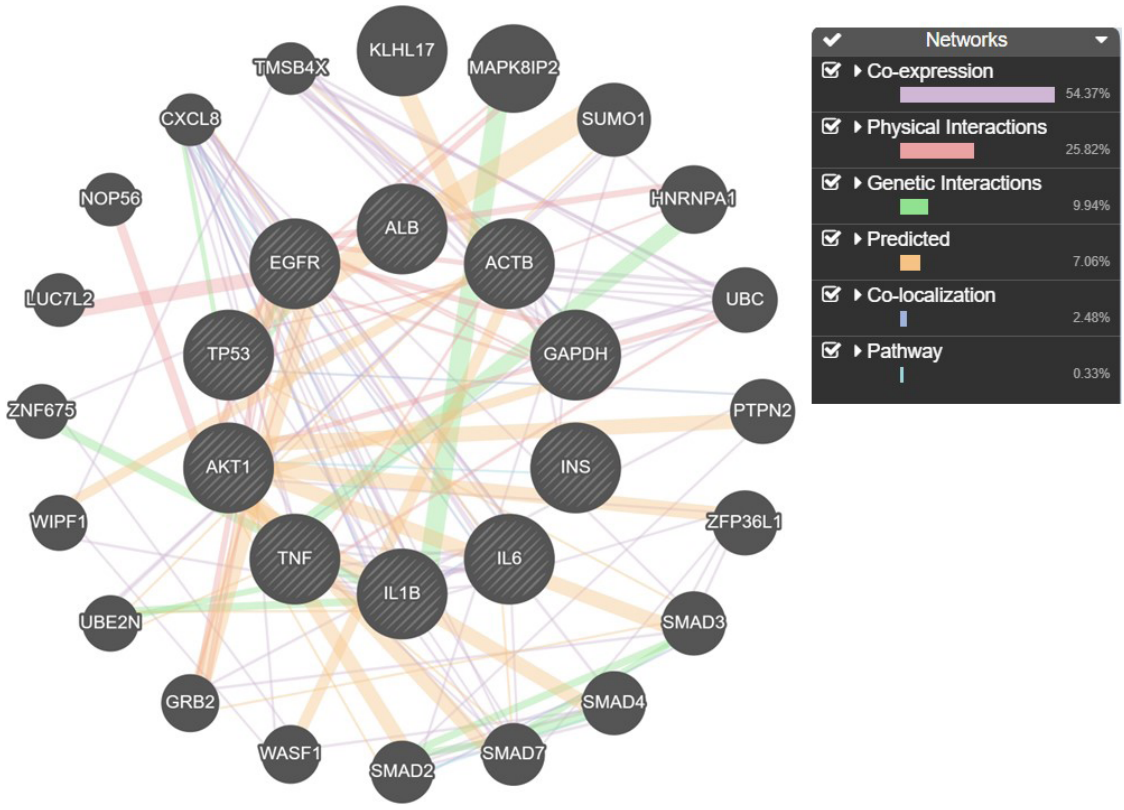


**Figure 4.** Gene network from GeneMANIA analysis of 10 hub genes involved in TCDD-induced CVDs.

**Core transcription factors and miRNAs involved in TCDD-induced CVDs**

After analyzing the hub genes for TFs in ChEA3, the top 10 TFs were identified. Subsequently, nodes and edges representing the relationships between TFs and hub genes were manually prepared in Excel and schematized in Cytoscape 3.10.1, as shown in Figure 5A. The results are sorted by mean rank (Table S2). MeanRank refers to the mean rank of each TF across all libraries containing that TF, serving as the score by which a composite list of TFs is reranked.

In Figure 4A, green nodes represent TFs, whereas light brown nodes represent hub genes. ChEA3 revealed that the most significant TF regulating these hub genes, with eight connections and a mean rank of 13.0, was Nuclear Factor Kappa B Subunit 1 (NFKB1) (Table S2). These results are supported by previous studies. For instance, Luo et al. (2022) identified the NFKB1 polymorphism as associated with susceptibility to coronary heart disease in populations of different genetic backgrounds. In a meta-analysis, authors suggested that a polymorphism in the NFKB1 promoter region was associated with susceptibility to coronary artery disease in both Asian and Caucasian populations (Chen et al., 2016).

As a result of miRNA-target analysis, the top ten miRNAs are shown in Figure 4B, while the corresponding p-values, FDR, odds ratios, and target genes are presented in Table S3. Among these 10 miRNAs, hsa-miR-19a-3p and hsa-miR-125b-5p emerged as the most critical miRNAs regulating these hub genes in TCDD-induced CVDs, which is consistent with the findings of prior studies. Bulent Vatan et al. (2016) reported that hsa-miR-19a-3p is downregulated in patients with mitral valve rupture. Jia et al. (2016) suggested that hsa-miR-125b-5p has diagnostic value for early diagnosis of acute myocardial infarction. Overall, these TFs and miRNAs may be used as predictors of TCDD-induced CVDs.

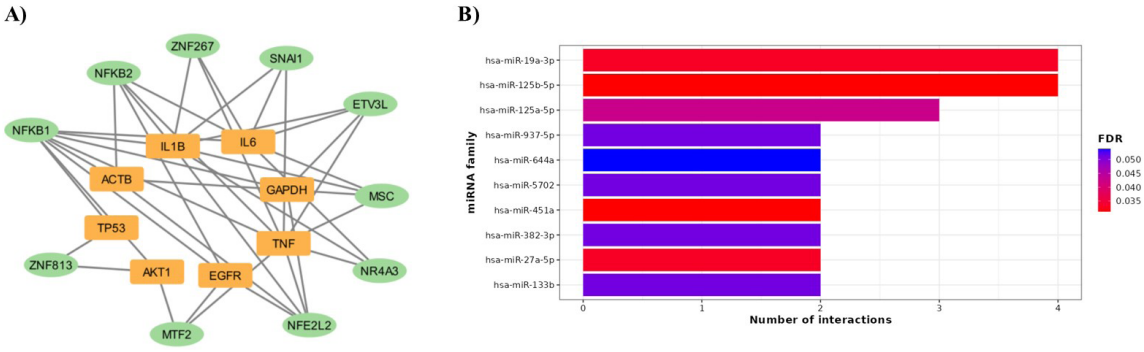


**Figure 5. A)** The top 10 transcription factors related to hub genes are highlighted, with green nodes indicating TFs and light brown color indicating the hub genes. **B)** miRNA-target analysis showed that the top 10 miRNAs related to the hub genes were predicted by MIENTURNET.

### TCDD-hub protein/gene binary analysis results

After identifying ten hub proteins/genes involved in TCDD-induced CVDs (Figure 3B, Table 1), a manual search of the CTD database revealed the impact of TCDD on the mRNA and protein expression as well as the protein activity of these proteins/genes, except for INS. Additionally, only studies where TCDD was applied alone were considered and focused on human plasma/serum or human cells.

TCDD was found to increase the protein activities of AKT1 and EGFR; mRNA expression of TNF, IL1B, and EGFR; and protein expression of ACTB, ALB, and EGFR. Interestingly, TCDD had a bidirectional effect on the mRNA expression of IL6 (Table 2). However, the studies summarized in Table 2 are not direct cardiovascular system studies, so they offer a prediction that TCDD could similarly affect these hub proteins/genes in the cardiovascular system.

**Table 2.** TCDD – hub proteins/genes interactions.

| **Hub genes** | **TCDD** (2,3,7,8-Tetrachlorodibenzo-*p*-dioxin) | | |
|---|---|---|---|
| | Protein activity | mRNA expression | Protein expression |
| GAPDH | | ↓ | |
| AKT1 | ↑ | | |
| TNF | | ↑ | |
| ACTB | | | ↑ |
| IL6 | | ↑↓ | |
| TP53 | | ↓ | ↓ |
| INS | | | |
| ALB | | ↓ | ↑ |
| IL1B | | ↑ | ↑ |
| EGFR | ↑ | ↑ | ↑ |

↑—increase; ↓—decrease; ↑↓—both increase and decrease

## CONCLUSION

This study elucidates how TCDD may contribute to the development of CVDs by modulating ten hub proteins/genes, as presented in Table 2. Additionally, TFs (especially NFKB1) and miRNAs (especially hsa-miR-19a-3p and hsa-miR-125b-5p) were predicted as potential mediators of TCDD-induced CVDs. Furthermore, these proteins/genes, TFs, and miRNAs may be potential biomarkers or therapeutic targets for TCDD-induced CVDs. Importantly, these molecules hold promise as not only biomarkers for disease diagnosis and prognosis but also as therapeutic targets for mitigating TCDD-induced cardiovascular toxicity. By targeting these key molecules, it may be possible to modulate the pathological processes underlying TCDD-induced CVDs and alleviate their adverse effects. Future research should focus on validating the therapeutic potential of these targets and developing innovative intervention strategies to combat TCDD-induced cardiovascular toxicity effectively.

## AUTHOR CONTRIBUTION STATEMENT

Concept, Design, Data Collection and/or Processing, Analysis and/or Interpretation, Writing manuscript (FK)

## CONFLICT OF INTEREST

The author declares no conflicts of interest.

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*REVIEW ARTICLE*

# Nanoparticles, Aptamers, and Aptamer-Conjugated Nanoparticles in Cancer Therapy

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***Nanoparticles, Aptamers, and Aptamer-Conjugated Nanoparticles in Cancer Therapy***

***SUMMARY***

*Cancer represents one of the main causes of death around the world, causing about 10 million deaths in the year 2020. Large progress has been made in the discovery of targeted therapy for cancer in recent years. Targeted therapy is a branch of cancer treatment that targets a specific site, without affecting the surroundings. The current review focused on recent achievements in the field of targeted anti-cancer drug delivery based on aptamers, nanoparticles, and aptamer-conjugated nanoparticles. It included a comprehensive survey of almost all recent studies published on this topic and comprehensively discussed the properties, advantages, and disadvantages of using these formulations as delivery vehicles in cancer therapy. We also provided examples of nanoparticles, aptamers targeting cancer biomarkers, aptamer-drug conjugates, and aptamer-conjugated nanoparticles, employed in targeted cancer therapies, and discussed the cytotoxicity of these formulations on cancer and non-cancer cell lines. The factors that can trigger nanoparticles for optimal drug release were also discussed. We hope that this review will provide additional information that will facilitate advanced applications of nanoparticle/ aptamer-based drug delivery systems for cancer therapy.*

***Key Words:*** *Cancer, biomarkers, nanoparticles, aptamer, drug delivery, aptamer-conjugated nanoparticles.*

***Kanser Tedavisinde Nanopartiküller, Aptamerler ve Aptamer-Konjuge Nanopartiküller***

***ÖZ***

*Kanser, 2020 yılında yaklaşık 10 milyon ölüme neden olan tüm dünyada ölümlerin başlıca nedenlerinden biridir. Son yıllarda kanser için hedeflendirilmiş tedavinin keşfinde büyük ilerleme kaydedilmiştir. Hedeflendirilmiş tedavi, kanser tedavisinin bir parçasıdır ve çevre dokuları etkilemeden spesifik bir alanı hedefler. Mevcut derleme, aptamer, nanopartiküller ve aptamer-konjuge edilmiş nanopartiküller ile ilgili hedeflendirilmiş anti-kanser ilaç tedavisi alanındaki son gelişmelere odaklanmıştır. Bu makalede konuyla ilgili son yıllarda yayınlanan neredeyse tüm çalışmaların kapsamlı bir incelenmesi ve kanser tedavisinde bu formülasyonların tedavi aracı olarak kullanımının özellikleri, avantajları ve dezavantajları kapsamlı olarak tartışılmıştır. Hedeflendirilmiş kanser tedavilerinde kullanılan nanopartiküller, kanser biyobelirteçlerini hedef alan aptamerler, aptamer ilaç konjugatları ve aptamer-konjugat nanoparküllerin örnekleri de sunulmuş ve bu formülasyonların kanser ve kanser olmayan hücre hatları üzerindeki sitotoksisitesi tartışılmıştır. İlaçların optimum şekilde salım yapması için nanopartikülleri tetikleyebilecek faktörler de tartışılmıştır. Bu derlemenin, kanser tedavisi için nanopartikül/aptamer temelli ilaç taşıyıcı sistemlerinin gelişmiş uygulamalarını kolaylaştıracak ilave bilgiler sağlayacağını umuyoruz.*

***Anahtar Kelimeler:*** *Kanser, biyobelirteçler, nanopartiküller, aptamer, ilaç taşıyıcı sistem, aptamer-konjuge nanopartiküller.*

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## INTRODUCTION

Cancer represents one of the main causes of death around the world, causing about 10 million deaths in the year 2020. One of the hallmarks of this disease is the rapid creation of abnormal cells that grow outside their usual boundaries, which may later go on to invade neighboring parts of the body and spread to other organs. The latter process is referred to as "metastasis" (WHO, 2022). Anti-cancer drugs can be synthesized chemically, or derived from natural sources (Tewari et al., 2019). However, more than 60% of contemporary anti-cancer drugs, in all their forms, come from natural sources (Joujeh and Joujeh, 2023).

Large progress has been made in the discovery of targeted therapy for cancer in recent years. But, even for the most impactful drugs that have been accepted, acquired and innate resistance mechanisms are common (Ward et al., 2021).

Current cancer therapies, including radiotherapy and chemotherapy, often lack specificity for tumor cells, leading to severe toxic effects in cancer patients undergoing the treatments (Liu et al., 2014). Among these include peripheral neuropathy, hair loss, diarrhea, and loss of appetite (Awasthi et al., 2018).

Targeted therapy is a branch of cancer treatment that targets a specific site, without affecting the surroundings. This significantly increases the specificity and reduces toxic effects (Pucci et al., 2019). Available drug delivery systems (DDS) include liposomes, micelles, vesicles, and nanospheres that act to transport anticancer agents into the body (Ghasemi et al., 2022). The combination of chemotherapy and nanotechnology can offer several advantages such as improved drug bioavailability and prolonged release of the chemotherapeutic agent. To achieve active targeting, biomolecules such as peptides, antibodies, and aptamers can be conjugated to nanoparticles (Kadkhoda et al., 2022). This combination could provide a promising candidate with the potential for an effective and safe delivery option in oncotherapy (Sheikh et al., 2022).

The current review focused on recent achievements in the field of targeted DDS based on aptamers, nanoparticles, and aptamer-conjugated nanoparticles, and comprehensively discussed the properties, advantages, and disadvantages of using these formulations as delivery vehicles in cancer therapy.

## METHODOLOGY

Data was obtained from extensive literature searches using internet databases, mainly PubMed and google scholar, using the keywords 'aptamers', 'nanoparticles', 'drug delivery', 'aptamer- conjugated nanoparticles', 'cancer diagnosis', and 'cancer therapy'. Online search was mainly devoted to publications written in English.

### Nanoparticles And Cancer

Nanoparticles (NPs) are materials ranging in size from 1 to 100 nm. They assume various forms, including nanoparticles, nanotubes, nanofilms, and bulk nanomaterials like dendritic structures. These entities possess unique properties, such as novel reactivity, and mechanical, electrical, and magnetic properties (Yetisgin, et al., 2020; Shrestha et al., 2020). They have unique physico-chemical properties, such as biocompatibility, biodegradability, and environmental sustainability (Hazra et al., 2023).

Being smaller than cells, nanoparticles can cross biological barriers to deliver the drug to the targeted site, increasing drug durability in the bloodstream and enabling targeted drug delivery (Aghebati-Maleki et al., 2019).

Nanotechnology offers new frontiers for cancer therapy, specifically through Nano Drug Delivery Systems (Ahmed et al.,2022). Loading oncology drugs inside the carrier or adsorbing them on the carrier surface helps to protect the drugs from premature elimination and enhance the solubility of insoluble drugs (Ahmed et al., 2022). There are various types of targeted drug delivery systems using nanoparticles (Figure 1). NPs can be categorized into mesoporous silica, liposomal, polymer, metal, carbon, and protein-based NPs (Herdiana et al., 2021). Some of the nanoparticles employed in targeted cancer therapies are listed in Table 1.

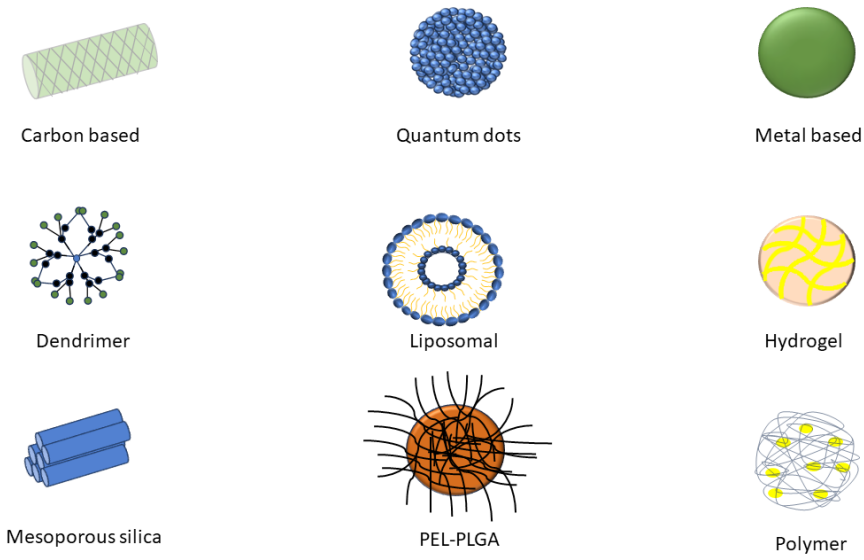


**Figure 1.** Types of nanoparticles for targeted drug delivery systems

**Table 1.** Some of the nanoparticles employed in targeted cancer therapies

| Nanoparticle | Target Cells | Targeted Cancer | Loaded drug / Therapy | Ref |
|---|---|---|---|---|
| GNP | HeLa | Cervical Cancer | DOX | (Bansal et al., 2023) |
| PCL-Chitosan | HCT 116 | Not specified | Berberine, Curcumin | (Ghaffarzadegan et al., 2023) |
| NC-NP | 4T1 | Not specified | DACHPt | (Xiang et al., 2023) |
| Chitosan-based microgels | 4T1 | Not specified | piperine | (Wang et al., 2023) |
| MSLN | MDA-MB-231 | Breast cancer | ATS, VIN | (Shinde et al., 2023) |
| PMNP-D | CAFs | ACC | DOX | (Liu et al., 2023) |
| Nanomicelles (PLGA,PCL,PSt) | 4T1, SPC-A1 MHCC-97H, | Not specified | PTX | (Miao et al., 2023) |
| D-g-PAA-GNP | MDA-MB-231, MCF10A | TNBC | Photodynamic therapy | (Warren et al., 2023) |
| CS- NZIF-8 | MCF-7 | breast cancer | curcumin, 5-FU | (Radhakrishnan et al., 2023) |
| BPCA1-BPCA4 | MCF-7 | breast cancer | DOX | (Du et al., 2023) |
| QD@Ca | 4T1, MDA-MB-231 | breast cancer | Sonodynamic therapy with DOX | (Cai et al., 2023) |
| Tc -HAS | MCF-7, 4T1 | breast cancer | Methotrexate (MTX) Trastuzumab (TRZ) | (Ekinci et al., 2023) |
| (TB) (PCL-PEG-PCL) | HCT-119, HT-29 | Colorectal cancer | PGZ, CAP | (Pouya et al., 2023) |
| CDs-PEG4-β-Cdx | tumors overexpressing PDE-5 | Not specified | Sildenafil | (Mauro et al., 2023) |

(GNPs) gold nanoparticles, (DOX) doxorubicin, (PCL) Polycaprolactone, (HCT116) human colorectal carcinoma cells, (NC-NP) Norcantharidin-Platinum Codelivery Nanoparticles, (4T1) Mouse breast cancer cell line, (DACHPt) 1,2-diamino cyclohexane-platinum (II) a parent drug of oxaliplatin, (PLGA) poly (lactic-co-glycolic acid), (MSLNs) Mannose-conjugated Solid Lipid Nanoparticles, (MDA-MB-231) human breast cancer cell line, (ATS) Atorvastatin Calcium, (VIN) Vinpocetine, (PMNPs-D) CAF-associated ITGB1-inactivating peptide-enriched membrane nano delivery system, (CAFs) Cancer-associated fibroblasts, (ACC) adenoid cystic carcinoma, (PSt) polystyrene, (SPC-A1) human lung carcinoma cell line, (MHCC-97H) Human hepatocellular carcinoma cell line, (PTX) Paclitaxel, D-g-PAA-GNP) Dextran-grafted-polyacrylamide encapsulated with gold nanoparticles, (TNBC) Triple Negative Breast Cancer, (MCF-7) human breast cancer cell line, (5-FU) 5-Fluorouracil, (BPCA1-BPCA4) Biotin-linked Amphiphilic Calix arene-based Supramolecular Micelles, (QD@Ca) Quantum Dots @ Calcium, (Tc) Technetium-99m, (MTX) Methotrexate, (TRZ) Trastuzumab, (HSA), Human Serum Albumin, (CAP) Capecitabine, (PGZ) pioglitazone hydrochloride, (TB) triblock (CDs-PEG4-β-Cdx) β-Cyclodextrin-decorated sulfur-doped carbon nanodots.

### Aptamers

Aptamers are short, single-stranded oligonucleotides (Zhang et al., 2019). They are classified as DNA or RNA aptamers or peptide aptamers (Odeh et al., 2019). The most important merits of using aptamers are that there are no limitations on their targets, their ease of generation, low production cost, reversible folding properties, and low immunogenicity (Kim et al., 2016). Aptamers can target a wide range of entities, from small molecules to biomacromolecules, infected cells, stem cells, and cancer cells (Zhu and Chen 2018). Aptamers mimic antibodies by binding to a specific target molecule (Byun et al., 2021) with high affinity and specificity by folding into tertiary structures (Zhang, et al., 2019). However, unlike antibodies, aptamers are more stable, especially after chemical modifications (Subjakova et al., 2021). Therefore, they can be synthesized in large quantities and stored without or with minimal loss in activity (Odeh al., 2019). They can withstand temperatures of up to 95°C without losing their structure upon cooling. They also can be stored in a freezer (-18°C) under dry conditions for up to a year (Subjakova et al., 2021). They are chemically synthesized under in vitro conditions, avoiding the use of experimental animals, and offer advantages such as relative resistance to changes in pH, temperature, and ionic concentrations, as well as cost-effective production (Reid et al., 2020).

### Selex Technique

Even though some aptamers exist naturally, most of them are generated in vitro by selecting them from a large random sequence pool (Qian et al., 2021). The procedure for *in vitro* aptamer synthesis is known as the "Systematic Evolution of Ligands by Exponential Enrichment (SELEX)" (Srivastava et al., 2021). SELEX is a comprehensive, multidisciplinary project involving various fields, including molecular biology, analytical chemistry, bioinformatics, materials chemistry, and nucleic acid chemistry.

SELEX is used to isolate aptamers with high affinity and specificity for a variety of target molecules from a carefully designed oligonucleotide library (Qi et al., 2022). To achieve this, target-related sequences were iteratively selected and amplified to preferentially enrich those sequences with the highest affinity for the target (**Figure 2**). Traditionally, after 10 to 15 iterations, one or more aptamers can be identified. This process can take several months to complete (Szeto et al., 2013). In recent decades, the SELEX protocol has been further innovated and developed, leading to the emergence of new SELEX technologies to simplify the procedure and expand the variety of aptamers, such as Graphene oxide (GO)-SELEX, Capillary electrophoresis (CE)-SELEX, Cell-SELEX, and Fluorescence-activated cell sorting (FACS)-SELEX (Lyu et al., 2021).

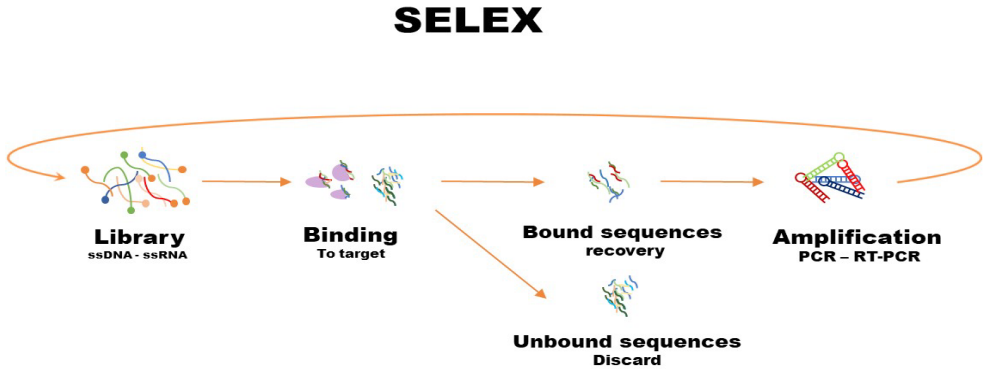


**Figure 2.** Schematic representation of in-vitro SELEX procedure

**Aptamers and Cancer**

Aptamers' specificity has been harnessed in targeting cancer cells. As mentioned earlier, aptamers are novel oligonucleotides that recognize and bind to their cognate targets, including tumor surface receptors (Zhu et al., 2014). Aptamers can act as antagonists, inhibiting the interaction of tumor-related targets (proteins or receptor-ligands), or as agonists, activating the function of anti-cancer target receptors, contributing to potential anti-tumor therapeutic strategies (Li et al., 2021). Some *in vivo* experiments indicate that aptamers can inhibit the growth of tumors overexpressing receptor-related targets, positioning them as promising anti-tumor therapeutic agents (Wang and Li, 2011). Cancer biomarkers are molecules that indicate abnormalities in cancer and play an important role in many biological processes, including cell migration, cell-cell interactions, signal transduction, and cell proliferation (Sawyers., 2008).

Numerous biomarkers have been identified as key players in different types of cancer. For example, mucin 1 (MUC1) and nucleolin are frequently overexpressed in breast cancer cells (Yang et al., 2023). Similarly, prostate-specific membrane antigen (PSMA), a carboxypeptidase, is known to be upregulated at various stages of prostate cancer development (Cruz-Hernández et al., 2022). Another important biomarker is the human epidermal growth factor receptor 2 (ErbB-2/HER2), a receptor tyrosine kinase, which is commonly overexpressed in various human cancers, such as gastric and breast tumors (Kara et al., 2023). EpCAM, a tumor-associated antigen, is highly expressed in common epithelial cancers and their tumor-initiating cells, making it a crucial focus of research (Holz et al., 2023). Additionally, PTK7-receptor is another biomarker known to be overexpressed in various types of tumors (Sicco et al., 2021). Aptamer has proven to be sensitive in detecting the overexpressed biomarker on the surface of cancer cells and has emerged as a new targeting material due to its high affinity for target molecules. It recognizes and binds to its corresponding target by spontaneously forming a three-dimensional (3D) structure (Figure 3), aiming to improve therapeutic effects and minimize toxicity to non-cancerous cells (Kim et al., 2018). Table 2 displays some of the aptamers targeting biomarkers that have been studied for the diagnosis and treatment of cancer.

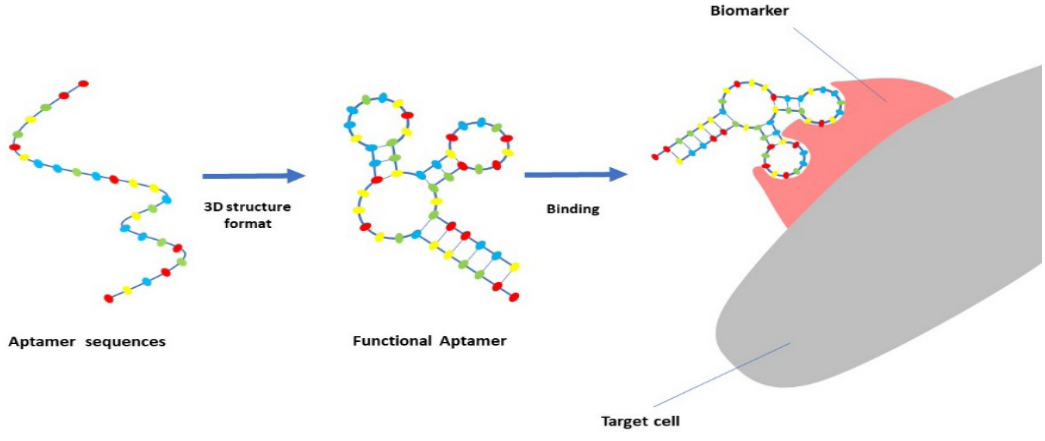


**Figure 3.** Schematic diagram of aptamer targeting cancer biomarker

**Table 2.** Aptamers targeting cancer biomarkers

| Aptamer | Target | Target cells | Cancer type | Ref |
|---|---|---|---|---|
| AB3<br>5'-TGCGTGTGTAGTGTGTCT-GTTGTTTGTATTGTTGTCTATCCTCT-TAGGGATTTGGGCGG-3' | OFA/RP | hematologic tumor cells | Hematologic malignancies cancer | (Rus et al.,2023) |
| S1–4 | (ER+) | MCF-7 | breast cancers | (Cong et al.,2023) |
| AS1411<br>5'-GGTGGTGGTGGTTGTGGTGGTG-GTGG-3' | nucleolin | MCF-7 | breast, cervical, and colon cancer | (Gupta et al.,2023) |
| W3<br>AGCAGCGTGGAGGATAGGGGTC-GGAGTGGGTGGTTATGATTG-GCTCTTCTGCGCTGC | CTCs and exosomes derived from CRC cells | | colorectal cancer | (Lu et al.,2023) |
| A2<br>CACCACGCGAATGCTATCGGGGCTA-AGTATCAAAATGAGC | β1-integrin | KYSE410 | ESCC | (Zhang et al.,2022) |
| yly12<br>AGGATAGGGGGTAGCTCGGTC-GTGTTTTTGGGTTGTTTGGTGG-GTCTTCTG | L1CAM | LoVo, PC3 | colon/prostate cancer | (Long et al.,2023) |
| GreenB1<br>5'-FAM-ATCCAGAGTGACGCAGCAG-GTGGAAGGGGTAACTACGTGGG-GAGGTGGTAGGGGTGGGTGGACAC-GGTGGCTTAGT-3' | β1-integrin, | MCF-7, MDA-MB-231, and MDA-MB-436 | triple-negative breast cancer | (Pleiko et al.,2023) |

(OFA) Oncofetal antigen, (RP)immature laminin receptor protein, (HR+) Hormone Receptor-Positive, (CTCs) Circulating tumor cells, (ESCC) Esophageal squamous cell carcinoma, (KYSE410) derived from the poorly differentiated invasive esophageal squamous cell carcinoma, (LoVo) colon cancer, (PC3) prostate cancer, (L1CAM) L1 cell adhesion molecule, (CRC) Colorectal cancer

Chemotherapy is the most common cancer treatment. However, it still has many side effects. Because most drugs kill cancer cells and normal cells, they lack selectivity (Fan et al., 2023).

Aptamer, with its excellent specificity, has rapidly become a new type of targeted ligand used in drug delivery. Many aptamer-mediated DDS have been developed, including drug-aptamer conjugates, aptamer-siRNA, and aptamer-functionalized nanoparticle systems for effective cancer treatment (He et al., 2023). Aptamer has been used as a drug carrier that can conjugate with chemotherapeutic agents or potent toxins and deliver them precisely into tumors by targeting specific cell surface antigens, significantly improving the therapeutic effectiveness of drugs in cancer treatment (Yang et al., 2021).

Notably, the aptamer/drug ratio plays a crucial role in achieving optimal therapeutic efficacy (Gao et al., 2022). The aptamer-drug conjugate typically consists of three molecular components: the aptamer (ligand), linker, and drug. Aptamer serves as a ligand for targeting disease sites and guiding the delivery of therapeutic agents that modulate the biological function of the target biomarker (Kim et al., 2021). Table 3 displays recent research on targeted cancer therapy using aptamer-drug conjugates.

**Table 3.** Aptamer-drug conjugates for cancer therapy

| Aptamer | Target | Cell line | Drugs loaded | Cancer type | Ref |
|---|---|---|---|---|---|
| BGA | nucleolin | MCF-7 | DM1 | Breast cancer | (Jo et al., 2023) |
| PTK7 AP | PTK7 | 5637 | GEM | bladder cancer | (Xiang et al., 2022) |
| Sgc8 | PTK7 | A20 | dasatinib | Lymphoma | (Sicco et al., 2023) |
| E3 | (Tf 1). | LNCaP, DU145, PC3, 22RV1 | E (MMAE) F (MMAF) | prostate cancer | (Song et al., 2023) |
| CD71/CD44 dual-aptamer | CD71/CD44 | TCCSUP, UM-UC-3, EJ, and T24 | GEM | bladder cancer | (Liu et al., 2023) |
| CD133 RNA | CD133 | BAKP POT | trametinib mebendazole | melanoma cancer | (Haribabu et al., 2022) |
| anti-CD20 | CD20 | WM266-4, A375 | Adriamycin | Melanoma cancer | (Chen et al., 2023) |
| AS1411, FOXM1 | ---- | 4T1 | epirubicin | Breast cancer | Moradi et al., 2023) |
| EpCAM AP | EpCAM | Hep3B, Huh7 | Dox | Hepatocellular carcinoma | (Zhou et al., 2022) |
| apHAT610 | HAT1 | A549, SW900, H1650 | the aptamer is a potential drug | Lung cancer | (Klett-Mingo et al., 2022) |

(GEM) Gemcitabine, (PTK7) protein tyrosine kinase 7, (A20) Mus musculus B lymphoma A20 cell line, (Tf 1) transferrin receptor 1, (MMAE) monomethyl auristatin E, (MMAF) monomethyl auristatin F, (WM266-4 and A375) are human melanoma cell lines, (EpCAM) Epithelial cell adhesion molecule, (Hep3B, Huh7) HCC cell lines, (HAT1) Histone acetyltransferase 1, (5637) human bladder cancer cells, (DM1) drug has synergistic interaction with TOP1 inhibitors.

### Aptamers in Clinical Trials

After the discovery of aptamers, great efforts were made by researchers to achieve the clinical use of aptamers in treating diseases (Esawi et al., 2023). Many aptamers are currently at various stages in clinical trials. Figure 4 displays the number of Aptamer-based clinical trials by year, according to the official database of the US National Institutes of Health.

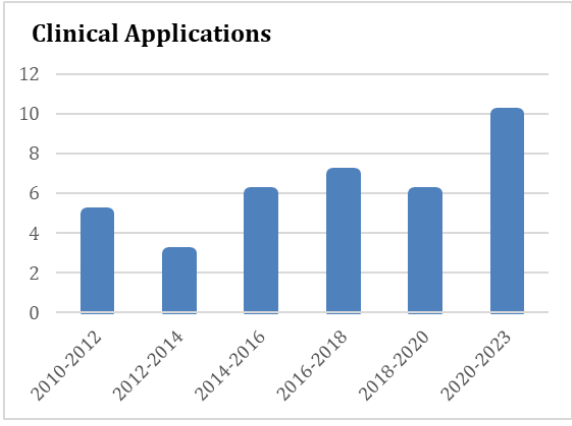


**Figure 4.** Number of aptamer-based clinical trials by year (www.clinicaltrials.gov)

Macugen® (pegaptanib) was the first aptamer-based drug approved by the US Food and Drug Administration (FDA) in December 2004 (Liu et al., 2022). Pegaptanib targets vascular endothelial growth factor (VEGF) for the treatment of neovascular age-related macular degeneration. It was also the first anti-angiogenic agent approved for this common eye disorder (Fine et al., 2005). VEGF plays an important role in tumors. Therefore, it was thought that Macugen might also have anti-cancer properties as well. Unfortunately, preclinical studies failed to support this hypothesis, so the drug has not been tested in clinical trials for oncology applications (Morita et al., 2018). To date, only a few therapeutic aptamers have progressed successfully into clinical trials for oncology (Table 4), but no aptamer has been approved by the FDA for cancer treatment (Shigdar et al., 2021).

#### - AS1411

The nucleolin-targeting DNA aptamer AS1411 is advanced in cancer therapy with great potential for clinical use due to its safety profile and ability to in-

duce strong response in a patient with intractable tumors (Yazdian-Robat et al., 2020). The unique G-rich quadruplex structure and pegylation support the pharmacokinetic (PK) profile of AS1411, resulting in nuclease evasion and an extended half-life (Morita et al., 2018).

Two clinical trials on AS1411 have been conducted to evaluate its efficacy in advanced solid tumors (NCT00881244) and renal cell carcinoma (RCC) (NCT00740441). Despite the trial (NCT00881244) being completed in 2007, no reports have been published about the outcomes. In a phase II trial (NCT00740441), the drug was found to have minimal activity in unselected patients with metastatic RCC (Rosenberg et al., 2014).

AS1411 is also being used in clinical trials to treat leukemia. A phase II clinical trial evaluated the efficacy of AS1411 in combination with cytarabine in patients with acute myeloid leukemia (AML), the results demonstrated that the combination therapy was superior to cytarabine alone (Stuart et al., 2009). Other phase II clinical trials (NCT00512083 and NCT01034410) aimed to assess the efficacy of AS1411 combined with cytarabine for the treatment of AML, but the results have not been published yet.

**- NOX-A12:**

NOX-A12 is an RNA-aptamer that targets CXCL12. NOX-A12 demonstrated improved overall response rates in chronic lymphocytic leukemia (CLL) patients in a phase I/II trial (NCT01486797) when combined with bendamustine and rituximab chemotherapy (Steurer et al., 2019). Clinical studies (NCT00976378 and NCT01194934) determined the safety profile of NOX-A12 in healthy volunteers. A second phase I clinical trial (NCT01194934) demonstrated that NOX-A12 is safe, well-tolerated, and effective in vivo by counteracting CXCL12 signaling (Vater et al., 2013).

Through a Phase I/II study (NCT03168139) in patients with metastatic microsatellite-stable colorectal and pancreatic cancer, with impaired immune systems, the combination of NOX-A12 and pembrolizumab was found to induce immune responses, stable disease in 25% of patients, and prolonged time on treatment (Halama et al., 2019).

A phase 2 study (NCT01521533) compared a single intravenous dose of NOX-A12 alone versus a combination with bortezomib and dexamethasone (VD) in previously treated multiple myeloma patients. The results indicated that NOX-A12 increased the efficacy of VD treatment without increasing treatment toxicity (Ludwig et al., 2017). Updated data from the expansion arm of the phase 1/2 GLORIA trial (NCT04121455) showed that the addition of NOX-A12 to standard-of-care radiotherapy and bevacizumab elicited a response when used as first-line treatment for patients with glioblastoma (Giordano et al., 2023).

**- SGC8:**

The Sgc8 single-stranded DNA aptamer (ssDNA) has been shown to accumulate more in PTK7-positive tumors and is currently under the early-phase I clinical trial (NCT03385148) to assess its diagnostic value in colorectal cancer.

**- EYE001:**

A phase I clinical trial (NCT00056199) aimed to test the ability of EYE001 to reduce retinal thickening, and improve vision in patients with Von Hippel-Lindau syndrome. Although this study was completed in 2005, no report of the results has been posted on (clinicaltrial.gov) or publicly published yet.

**Table 4.** Aptamers used in clinical trials for cancer diagnosis and therapy (www.clinicaltrials.gov)

| Aptamer | Type | Study Phase | Primary Purpose | Cancer | Last Update | Clinical Trial ID |
|---|---|---|---|---|---|---|
| AS1411 | DNA | Phase 1 | Treatment | Solid Tumours | 2009-04 | NCT00881244 |
| AS1411 | DNA | Phase 2 | Treatment | Renal Cell Carcinoma | 2009-09 | NCT00740441 |
| AS1411 | DNA | Phase 2 | Treatment | Acute Myeloid Leukemia | 2009-09 | NCT00512083 |
| AS1411 | DNA | Phase 2 | Treatment | Acute Myeloid Leukemia | 2017-12 | NCT01034410 |
| NOX A12 | RNA | Phase 2 | Treatment | chronic lymphocytic leukemia (CLL) | 2017-05 | NCT01486797 |
| NOX A12 | RNA | Phase 1/2 | Treatment | Metastatic Colorectal and Pancreatic Cancer | 2020-07 | NCT03168139 |
| NOX A12 | RNA | Phase 2 | Treatment | Relapsed Multiple Myeloma | 2015-10 | NCT01521533 |
| NOX A12 | RNA | Phase 1/2 | Treatment | Glioblastoma | 2023-06 | NCT04121455 |
| Sgc8 | ssDNA | Early Phase 1 | Diagnostic | Colorectal cancer (CRC) | 2011-02 | NCT03385148 |
| EYE001 | RNA | Phase 1 | Treatment | Retinal angioma | 2008-03 | NCT00056199 |

**Aptamer-Conjugated Nanoparticles:**

Nanotechnology-based drug delivery systems provide an advanced approach for precise and sustained drug delivery, ensuring optimal therapeutic outcomes over the desired timeframe and reducing the frequency of administration (Sheikh et al., 2022). A significant challenge in this field is to equip multifunctional polymeric nanoparticles with the ability to target specific molecules, evade the immune system, and control drug release to overcome biological barriers *in vivo* (Fang et al., 2020). Functionalizing nanoparticles using specific receptors has gained significant attention. Aptamers, known for their high specificity and affinity emerge as prime candidates for specific nanoparticle receptor functionalization (Kumar et al., 2023).

Although the aptamer can be directly conjugated to anticancer agents such as chemotherapeutic, the advantage of using nanoparticles is that they can deliver large quantities of drug payload or diverse treatments to cancer cells through delivery and recognition events (Fu et al., 2020). This combination is promising progress for targeted drug delivery (Gao et al., 2022). A schematic representation of aptamer-functionalized nanoparticles acting on a cancer cell is shown in Figure 5.

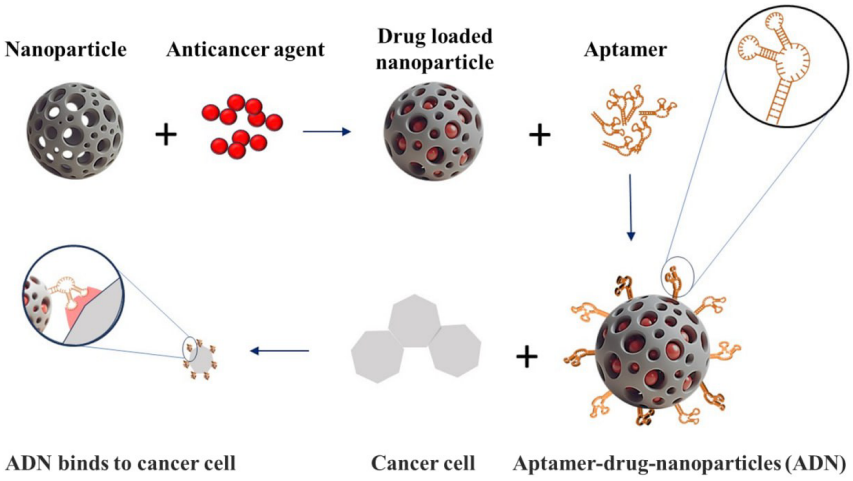


**Figure 5.** A schematic representation of aptamer-functionalized nanoparticles acting on a cancer cell

Recently, combinations of aptamers and nanoparticles have been widely used in the development of therapeutic platforms due to their unique potential in targeted drug delivery systems, diagnostics, and response monitoring (Khan et al. 2022). Some of the aptamer-NP structures used in anticancer drug delivery are listed in Table 5.

The concept of aptamer-conjugated nanoparticles was developed to overcome the drawbacks related to using each of them individually. To elucidate this idea, we compare three delivery systems developed to assess the effectiveness of Doxorubicin as a targeted treatment against MCF-7 cancer cells, including an aptamer-based delivery system, in which DOX was loaded between two complementary sequences of AS1411 (Rahimi et al., 2022), nanoparticle-based delivery system, in which DOX was loaded onto PEG-chitosan- mesoporous silica nanoparticles (MSN) (Moodley et al., 2020), and aptamer-conjugated nanoparticles- based delivery system, in which DOX was loaded into MSNs, chitosan was employed to cover the surface of MSNs, and AS1411 aptamers were electrostatically attached to the surface of the chitosan-coated MSNs (Khatam et al., 2021). By comparing the results of these reports (Figure 6), it can be observed that the aptamer-conjugated nanoparticles can combine the advantages of aptamer-drug conjugates and nanoparticle carriers, providing high target specificity, controlled release and increased toxicity to cancer cells while maintaining a high rate of viability for normal cells.

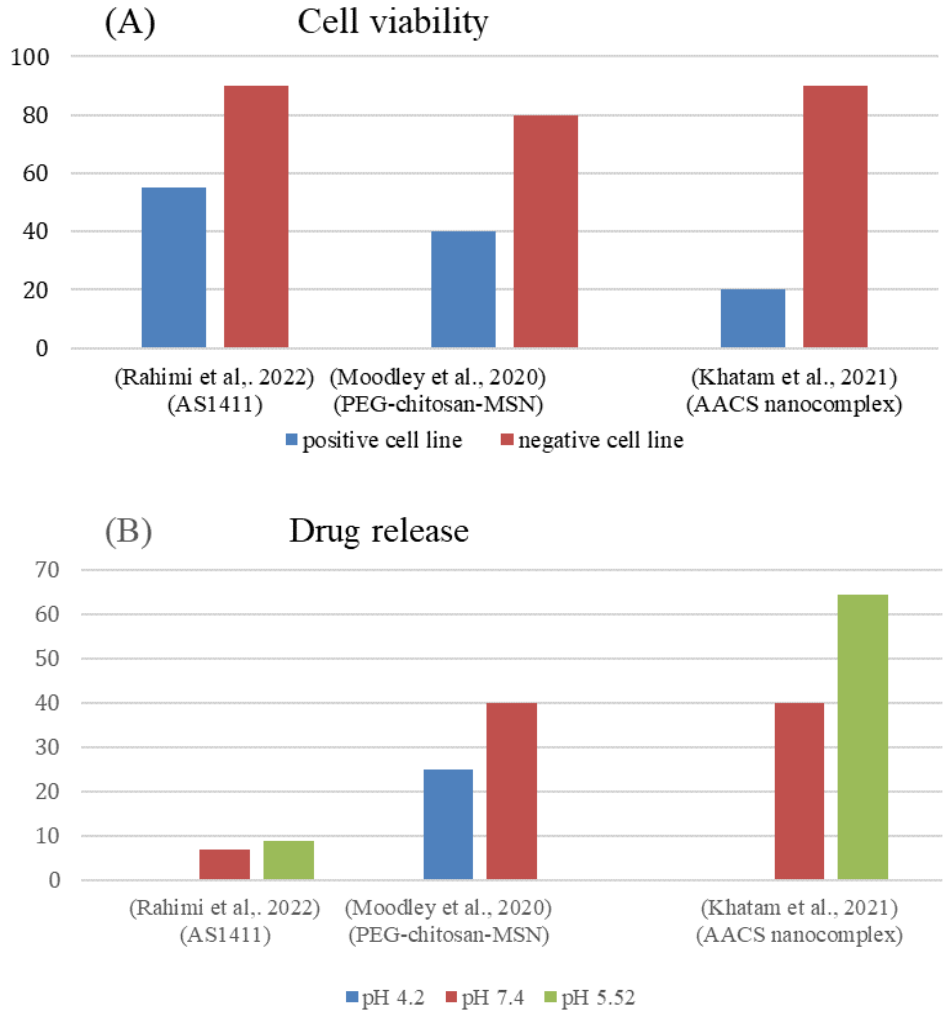


**Figure 6.** Comparison of cell viability (A) and drug release efficiency (B) between the results of previous reports

**Table 5**. Aptamer-NP formulations used for anti-cancer drug delivery

| Aptamer | NPs | Drug | Target | Cell line | Cancer Type | Ref |
|---|---|---|---|---|---|---|
| Aptamer | CS/COQ | FU-5, GA | Not specified | MCF-7 | Breast cancer | (Mazandarani et al., 2023) |
| MUC16 AP | MSNPs-PEG | SUN | mucin 16 | OVCAR-3, SK-OV-3 | Ovarian cancer | (Torabi et al., 2023) |
| AS1411 AP | CuFePBA@PEGMA | DOX | nucleolin | MCF-7 4T1 | Breast cancer | (Chen et al., 2023) |
| | CoFePBA@PEGMA | | | | | |
| Sgc8c AP | mSiO2-Au | AZD5363 | PTK-7 | CCRF-CEM | T-ALL | (Yang et al., 2023) |
| AP613-1 AP | (H-MnO2) | SRF | GPC3 | Huh7, HepG2 | (HCC) | (Wang et al., 2023) |
| MUC1 AP | PEG-Au | PTX | mucin 1 | MCF-7 | Breast cancer | (Kadkhoda et al., 2022) |
| AS1411 AP | ICG, TOA | DOX | nucleolin | 4T1 | Breast cancer | (Li et al., 2023) |
| MUC1 AP | Fe3O4@GO@Ce6 | PAC | mucin 1 | MCF-7 | Breast cancer | (Işıklan et al., 2022) |
| HB5 AP | cGO | Sili, DOX | HER2 | MCF-10A, MCF-7, SK-BR-3 | Breast cancer | (Shahidi et al., 2023) |
| AS1411 AP | CSSD | DOX | nucleolin | MDA-MB-231, 4T1 | Breast cancer | (Yu et al., 2023) |
| AS1411 AP | PEG-b-PVGLIG-PLA | SN38 | nucleolin | C26 | Colon cancer | (Ramezani et al., 2020) |
| ΔPSap4#5 AP | PLGA | ABR | PSMA | LNCaP,22Rv1 | Prostate cancer | (Al Hoque et al., 2023) |
| MUC1 AP | NHG-QDs | PTX/SO | mucin 1 | MCF-7 | Not specified | (Ranjbar-Navazi et al., 2021) |
| MUC-1 AP | nano barrel | PTX, DOX | mucin 1 | MCF-7 | Breast cancer | (Wang et al., 2023) |
| CD117 AP | PEG | Drn/Lut | Tf /CD117 | HL60 | Leukemia | (Zhu et al., 2023) |

(CS/COQ) Chitosan and carbon quantum dot, (GA) *Ganoderic acid*, (MSNPs) Mesoporous silica nanoparticles, (SUN) sunitini, (OVCAR-3 and SK-OV-3) Human ovarian cancer cell line, (CuFe) copper–iron, (CoFe) cobalt–iron, (PBA) Prussian blue analogs, (PEGMA) polyethyleneglycol methacrylate, (mSiO2) mesoporous silica, (AZD5363) is a selective Akt inhibitor with therapeutic potential for tumors, (CCRF-CEM) Human acute T lymphoblastic leukemia cell line, (T-ALL) T-cell acute lymphocytic leukemia, (H-$MnO_2$)The hollow mesoporous $MnO_2$ nanoparticles, (SRF) Sorafenib , (HCC) hepatocellular carcinoma, (GPC3) glypican-3 receptors, (HL60) The human leukocyte cell line, (Drn) daunorubicin,(Lut) luteolin, (Tf) transferrin receptor, (PSMA) prostate-specific membrane antigen, (ABR) Abiraterone, (TOA) DOX/ICG-loaded TOA, (ICG) indocyanine green, (cGO) carboxylated graphene oxide, (Sili) silibinin, (NHGs) nanohydrogels, (CSSD) chondroitin sulfate A-ss-deoxycholic acid, (QDs) quantum dots, (SO) sodium oxamate, (Ce6) photosensitizer, (PLA) polylactide, (PVGLIG) synthetic peptide

**Cytotoxicity:**

Cytotoxicity is often associated with the detrimental effects on a specific cell line. As a result, cytotoxicity is typically initially assessed through specific in vitro assays before proceeding to *in vivo* testing (Kus-Liśkiewicz et al., 2021). Visual examination of cells using bright-field microscopy serves as a fundamental means of assessing cytotoxicity. Commonly, colorimetric techniques are employed to assess plasma membrane integrity and metabolic activity in cytotoxicity assays. The LIVE/DEAD viability test is utilized to quantify the number of decreased cells. In the context of in vitro nanoparticle cytotoxicity assays, LDH, MTT, and MTS assays are extensively employed, with MTT and MTS being particularly useful for measuring the metabolic activity of viable cells (Nikzamir et al., 2021).

Chemotherapy is the most prevalent approach for cancer treatment. However, it comes with several challenges, such as low accumulation in tumor cells and limited target selectivity (Maghsoudi et al., 2019). Nanosized drug delivery systems often exhibit prolonged systemic circulation and lower accumulation in normal organs compared to tumor tissues. Nevertheless, one of their drawbacks is the potential toxicity to normal cells in addition to cancer cells (Hafeez et al., 2021). The key to cancer therapy is to improve the specific recognition of pathological. The interaction to aptamers with nanomaterials has helped to achieve this goal by increasing the effectiveness of anticancer drugs against their targets. The ability of aptamers to identify specific epitopes on the cell surface may lead to better drug accumulation in cancer cells (Khan et al 2021). Several studies have investigated the toxic effect of drug-nanoparticles-aptamer, nanosized drug delivery systems, and free drugs against cancer cell lines (target cells), and non-cancer cell lines (non-target cells) (Table 6). The results of some previous studies have shown that cell viability was lower (i.e., higher toxicity) with aptamer-conjugated nanoparticles compared to nanosized drug delivery systems, and free drugs, while the results of other research have shown opposite results. The same applies to the safety of non-cancer or non-targeted cells. Since the results are still conflicting, more studies are needed to determine the optimal drug delivery system.

**Table 6.** Effect on cell viability of drug-nanoparticles-aptamer, nanosized drug delivery systems, and free drugs on cancer and non-cancer cell lines

| Target & nontarget | cell line | Cell viability | | | Concentration | Time | Type of Therapy | Ref |
|---|---|---|---|---|---|---|---|---|
| | | Drug | Drugs & NPs | Drugs & NPs & AP | | | | |
| + | MCF-7 | 60% | 50% | 25% | 4.8 μg mL−1 of 5-FU | 48 h | Chemical | (Mazandarani et al., 2023) |
| + | OVCAR-3 | 54% | 21% | 12% | 20 μM of SUN | 24 h | Chemical | (Torabi et al., 2023) |
| - | SK-OV-3 | 80% | 75% | 74% | | | | |
| + | 4T1 | 50%, | - - - - | 62% | 20 μg mL−1 (CuFePBA@PEGMA@ AS1411/DOX) | 48 h | Chemical | (Chen et al., 2023) |
| - | L929 | 5% | - - - - | 40% | | | | |
| + | 4T1 | 50%, | - - - - | 62% | 20 μg mL−1 (CoFePBA@PEGMA@ AS1411/DOX) | 48 h | Chemical | |
| - | L929 | 5% | - - - - | 60% | | | | |
| + | CCRF-CEM | 8.3% | - - - - | 45.9% | 20 μM of AZD5363 | 24 h | Chemical | (Yang et al., 2023) |
| + | HepG2 | 50% | 45% | 18% | 8μg mL−1 of SRF | 24 h | Chemical | (Wang et al., 2023) |
| + | MCF-7 | 60% | 50% | 40% | 50 μM of PTX | 24 h | Chemical & photothermal | (Kadkhoda et al., 2023) |
| - | MDA-MB-231 | 55% | 65% | 85% | | | | |
| + | 4T1 | 80% | 45% | 20% | 20 μM of dox | 24 h | Chemical & photothermal | (Li et al., 2023) |
| + | MCF-7 | - - - - | 28% | 19% | 100 μg/ml | - - | Chemical & photothermal | (Işıklan et al., 2023) |
| + | C26 | 90% | 95% | 80% | 0.60 μg/mL of SN38 | 24 h | Chemical | (Ramezani et al., 2023) |
| - | CHO | 80% | 80% | 90% | | | | |
| + | LNCaP | 50% | 40% | 30% | 30μM | 48 h | Chemical | (Al Hoque et al., 2023) |
| - | PC3 | 70% | 60% | 60% | 30μM | 48 h | | |
| + | MCF-7 | 55% | 60% | 40% | 3μM (PTX) | 48 h | Chemical | (Ranjbar-Navazi et al., 2023) |
| + | HL60 | 48% | 42% | 30% | 5 μM | 48 h | Chemical | (Zhu et al., 2023) |

**Nanoparticle Triggering:**

Nanocarriers can be engineered to respond to both intrinsic and extrinsic triggers for drug release (Han et al., 2022). Internal triggers encompass variations in pH, temperature, enzyme activity, ATP levels, and hormonal responses, whether they occur intracellularly or extracellularly. External triggers, on the other hand, encompass factors such as light, ultrasound, magnetic fields, mechanical stress, and more (Virmani T et al., 2023). In practice, various nanoparticles that can change in size in response to different stimuli, including pH, UV light, temperature, and enzymatic activity, have been developed to achieve more uniform drug distribution within tumors and enhance their anti-tumor efficacy (Hu et al., 2018).

**PH-Responsive Drug Delivery Systems:**

PH-responsive nanoparticles have garnered significant research interest due to their ability to respond to changes in pH upon cellular internalization. Specifically, when nanoparticles are endocytosed into a cell, the pH decreases from approximately 7.4 in the bloodstream to about pH 6.5 in the early endosomal compartment and even lower - below pH 5- in the lysosomal compartment (Deirram et al., 2019). Furthermore -in nearly all solid tumors- there is a notable decrease in extracellular pH compared to normal tissues. This change is primarily attributed to anaerobic or aerobic glycolysis combined with a reduced removal of acidic metabolites (Thews et al., 2019). Tumor cells typically exhibit an extracellular pH of around 6.0, whereas normal cells maintain an extracellular pH of approximately 7.4. Additionally, while the intracellular pH of tumor cells is slightly higher than that of normal cells, the pH of lysosomes in tumor cells is lower (Shi et al., 2020).

PH-sensitive nanoparticles can exploit these varying environments for intracellular drug delivery. Upon cellular internalization through endocytosis, these nanoparticles can gradually swell or disassemble in response to the protonation of imidazole groups under acidic conditions, thereby triggering the release of loaded drugs (Guo et al., 2014). A previous study showed that altering the pH from 7.4 to 5.0 led to a 2.8-fold change in particle diameter (Hu et al., 2007).

Therefore, the efficiency of pH-dependent drug release has been a subject of investigation in many of the drug delivery studies. Most studies indicated that the most effective drug release occurred under acidic pH conditions. This aligns with the expected behavior upon nanoparticle entry into cells and the altered environment within tumors, as mentioned earlier. Table 7. presents recent studies on pH-responsive nanomaterials for anticancer drug delivery.

**Photodynamic Therapy (PDT) and Photothermal Therapy (PTT)**

PDT and PTT are effective cancer treatments, but complete eradication of cancer cells is not guaranteed, potentially leading to recurrence. Combinations of these therapies are explored to address limitations (Elbialy et al., 2019). In chemo-photothermal cancer therapy, researchers investigate the co-delivery of multiple agents using NPs for drug delivery and photothermal effects (Siddique et al., 2020). Controlled release mechanisms prevent premature drug release, targeting tumor cell necrosis and overcoming drug resistance (Zhang et al., 2020).

PDT is an emerging noninvasive treatment modality that relies on the use of a photosensitizer and light to generate reactive oxygen species (ROS) that are capable of killing cancer cells, offering a noninvasive cancer treatment with precise control (Zhen et al., 2019).

PTT represents a form of cancer therapy in which NPs are embedded within the tumor and generate heat in response to exogenously applied laser light. Using NPs as photothermal agents can lead to the release of heat, which can directly damage tumor cells (Siddique et al., 2020).

**Table 7.** Recent studies on pH-responsive drug delivery system

| drug-nanoparticle-aptamer | Sensitive | Release | Time | Ref |
|---|---|---|---|---|
| 5-FU-GA-Cs-CQD-Apt | pH 5.4 | 80% | 48 h | (Mazandarani et al., 2023) |
| | pH 7.4 | 56% | | |
| MSNP-PEG/SUN-MUC16 | pH 5.4 | 58.6% | 48 h | (Torabi et al., 2023) |
| | pH 7.4 | 14.1% | | |
| CuFePBA@PEGMA@AS1411/DOX | pH 5.0 | 56% | 48 h | (Chen et al., 2023) |
| | pH 7.4 | 23% | | |
| CoFePBA@PEGMA@AS1411/DOX | pH 5.0 | 75% | | |
| | pH 7.4 | 24% | | |
| mSiO2-Au-AZD5363 | pH 5.5 | 57.5% | 48 h | (Yang et al., 2023) |
| | pH 7.4 | 14.8% | | |
| H-MnO2-SRF-APT | pH 5.5 | 90% | 24 h | (Wang et al., 2023) |
| | pH 7.4 | 18% | | |
| ΔPSap4#5-ABR-NP | pH 5.0 | 92.4% | 672 h | (Al Hoque et al., 2023) |
| | pH 7.4 | 73% | | |
| Ap-NHG-QDs-PTX-SO | pH 5.8 | 70% | 168 h | (Ranjbar-Navazi et al., 2023) |
| | pH 7.4 | 45% | | |

This damage may be attributed to the fact that DNA repair processes, and cell membrane integrity, are severely affected by heat shock, enhancing permeability, and leading to the accumulation of a higher concentration of the drug at the tumor site (Faid et al., 2023). PTT-induced necrosis is the most traditional cell death pathway, which can lead to the release of large numbers of tumor fragments and many DAMPs "danger signals," such as heat shock proteins. These signals can be considered antigenic and immunostimulatory signals to activate the immune system (Han et al., 2022). Photosensitive nanoparticles offer a multitude of different applications, including controlled drug release resulting from physical/conformational changes in the delivery system in response to light of a specific wavelength (Pan et al., 2021).

Table 8 presents the results of previous studies in which high spatial and temporal on-demand drug release was achieved via phototriggerable. The results showed improved drug release when it was triggered by NIR laser irradiation/pH compared to pH-dependent release.

**Table 8.** Recent studies on drug release triggered by NIR laser irradiation/pH

| drug-nanoparticle-aptamer | Irradiation/pH | Release | Time | Ref |
|---|---|---|---|---|
| PTX/PEG-AuNPs-MUC1 | pH 5.4 | 65% | 60 h | (Kadkhoda et al., 2023) |
| | Under 810 nm NIR irradiation + pH 5.4 | 75% | | |
| TOADI | pH 5.0 | 30% | 24 h | (Li et al., 2023) |
| | NIR irradiation (808 nm, 1.0 W/cm2, 5 min) + pH 5.0 | 62% | | |
| Fe3O4-GO-Ce6-Apt-Pac | pH 5.5 | 35% | 72 h | (Işıklan et al., 2023) |
| | Under 808 nm NIR irradiation + pH 5.5 | 52% | | |

**Temperature-Sensitive Drug Delivery Systems**

Hyperthermia is an adjuvant therapy performed in combination with chemotherapy and radiotherapy to enhance cytotoxic effects. Increased cytotoxicity, and increased drug absorption through tumor vascular permeability, are the advantages of adding hyperthermia to chemotherapy (Mirrahimi et al., 2020). In addition, drug release may also depend on environmental temperature changes. Compared to healthy tissues (37°C), the tumor environment has a higher temperature (~ 40–42°C), which depends on its metabolic activity and vascularization (Amin et al., 2020).

Table 9 presents the results of previous studies in which drug release was triggered by temperature/pH. The results showed improved drug release at temperature 42, which was close to the tumor environment.

**Table 9.** Recent studies on drug release triggered by temperature/pH

| drug-nanoparticle-aptamer | pH/ temperature | Release | Time | Ref |
|---|---|---|---|---|
| Fe3O4-GO-Ce6-Apt-Pac | pH 5.5 & 45 °C | 47% | 72 h | (Işıklan et al., 2023) |
| | pH 5.5 & 37 °C | 36% | | |
| Apt-cGO-DOX-Sili | pH 5.5 & 42 °C | 70% | 72 h | (Shahidi et al., 2023) |
| | pH 5.5 & 37 °C | 50% | | |

**Oxidative- and Enzyme-Responsive Drug Release Systems**

Nanocarriers are precisely designed to be sensitive to various internal stimuli within the body, with particular emphasis on the redox response of enzymes.

This innovative approach ensures precise drug delivery, minimizes the risk of drug leakage into the bloodstream, and guarantees drug release specifically at the tumor site. Remarkably, this delivery system can even surpass expectations without the need for additional external stimuli (Li et al., 2020). One such critical factor in this context is glutathione (GSH), a thiol-containing tripeptide. GSH is found in significantly higher concentrations within the cell cytoplasm compared to its levels in the blood plasma. Notably, tumor cells exhibit much higher cytosolic GSH concentrations compared to normal cells (Sauraj et al., 2021).

Cancer cells exhibit distinctive enzymatic activity driven by their specific requirements for proliferation, growth, and metastatic invasion. Leveraging the heightened intracellular and extracellular enzyme expression in these cells, an enzyme-responsive drug-release system can be engineered (Yadav et al., 2021). Among these enzymes, matrix metalloproteinases (MMPs) stand out as overexpressed proteases in tumorous tissues across all stages of cancer (Vaghasiya et al., 2021).

Table 10 presents the results of previous studies in which drug release was investigated in the presence and absence of GSH and MMP-2 at pH 7.4. The results showed that MMP-2 significantly enhanced the release process. This is due to its role in dispersing the polymers carrying the drug and thus releasing it. GSH also enhanced drug release, but its role was to mimic body fluids in the presence of cancer cells.

**Table 10.** Recent studies on oxidative- and enzyme-responsive drug-release systems

| drug-nanoparticle-aptamer | Oxidative/ enzyme | Release | Time | Ref |
|---|---|---|---|---|
| D-ACS | (PBS +10 mM GSH) | 88.3% | 96 h | (Yu et al., 2023) |
| | PBS | 50% | | |
| SN38-pep-NPs | (PBS +10 mM MMP-2) | 80% | 100 h | (Ramezani et al., 2023) |
| | PBS | 11% | | |

(ACS) AS1411 aptamer-modified chondroitin sulfate A-ss-deoxycholic acid, (D-ACS) The ACS conjugation with Dox

**Aptamer-Controlled Release of Nanoparticle Cargo:**

Controlled drug release can also be achieved using an aptamer-gated mechanism. This mechanism relies on aptamer-target binding interactions as molecular stimuli to stimulate the release of the drug from nano-sized reservoirs. These systems use aptamers as guidance elements to direct drug nanocarriers to the targeted disease cells, but also use this biorecognition event as an open/close checkpoint to control drug release at specific sites (Thevendran et al., 2020). Two mechanisms of "aptamer-gated systems" have been described, the snap-top aptamer-based-gating systems (Zhu et al., 2011), and the nanovalve aptamer-based-gating systems (Abelow et al., 2010).

In the snap-top system, mesoporous silica nanoparticles (MSNs) were capped with gold (Au) nanoparticles modified with ATP aptamer. Through competitive displacement, gold particles were uncapped -in the presence of trigger molecule ATP- and the guest molecule was released (Zhu et al., 2011).

Likewise, nano valve systems also rely on aptamer-gated MSNs pores, but instead, utilize adsorption of the immobilized aptamer strand near the pore surface to block the openings, while converting to an open-state only in the presence of an aptamer-specific target molecule (Abelow et al., 2010; Thevendran et al., 2020). In addition, nano valves were developed to open and close pores in response to pH, light, temperature, and redox (Kavruk et al., 2015). However, both systems are limited by other factors such as being only applicable to materials that can form mesoporous structures, show sudden drug release, but decrease rapidly over time, or the desorption of the aptamer due to changes in surrounding pH or ionic strength that can indirectly cause aptamer-based gates to not close properly (Thevendran et al., 2020). MSN nanomaterials are suitable for controlled drug delivery, due to their unique physio-chemical properties such as large specific surface area and pore size, controllable particle size, high drug loading capacity, and remarkable biocompatibility and stability (Song et al., 2017). Most reports on aptamer-based gating silica nanoparticles (Pascual et al., 2017; Zhang et al., 2015) use aptamers that their target ligands present on the cell's surface, which causes the release of the drug close to the surface, but not into the cell. This reduces the efficacy and specificity of the therapy. To solve this problem, an effective strategy was developed in previous research in which bivalent aptamers consisting of ATP and AS1411 sequences were used to provide separate targeting and gating properties. Using this strategy, AS1411 targets the formulation toward nucleolin overexpressing cancer cells, and after penetration into the cells and facing high levels of ATP in the cancer cells cytoplasm, the drug releases by the interaction of ATP molecules with the second part of bivalent aptamer, the ATP aptamer (Charbgoo et al., 2021). Separated gating and targeting approach was also performed using hyaluronic acid-targeted nanocarrier based on silica nanoparticles gated with peptide nucleic acid and ATP aptamer and loaded with doxorubicin (Kazemi et al., 2022). However, the encouraging results of aptamer-based gating are expected to open great possibilities for future therapeutic applications in the field of drug delivery (Ozalp et al., 2011).

## CONCLUSION

The current review focused on recent achievements in the field of targeted anti-cancer drug delivery based on aptamers, nanoparticles, and aptamer-conjugated nanoparticles. The reviewed literature revealed that nanomaterials play a crucial role in targeted therapy, although they often lack specificity. Aptamers, on the other hand, offer a high degree of specificity towards cancer indicators. These two approaches have been utilized individually in the treatment of cancer, each having its strengths and limitations. However, when combined, the limitations of both approaches are mitigated, control is enhanced, and the results have shown great promise. We hope that this review will provide additional information that will facilitate advanced applications of nanoparticle/ aptamer-based drug delivery systems for cancer therapy.

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**AUTHOR CONTRIBUTION RATE STATEMENT**

Conceptualization, M.M.A. and D.J.; methodology, M.M.A. and D.J.; validation, M.M.A. and D.J.; formal analysis, M.M.A.; investigation, M.M.A. and D.J.; resources, M.M.A.; Data Curation, M.M.A. and D.J.; writing- original draft preparation, M.M.A.; writing-review & editing, D.J.; visualization, M.M.A.; Supervision, project administration, M.M.A. and D.J.; All authors have read and agreed to the published version of the manuscript.

**DECLARATION**

It is noteworthy that the visualization of the nanoparticle in Figure 5 presented in this article was generated using Bing.

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*REVIEW ARTICLE*

# Cyclic Imides Derivatives: A Potential Scaffold for the Synthesis of Anticonvulsant Agents

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***Cyclic Imides Derivatives: A Potential Scaffold for the Synthesis of Anticonvulsant Agents***

***SUMMARY***

*Cyclic non-aromatic nitrogen heterocycles such as phthalimides, maleimides, and succinimides are an intriguing class of compounds with an immense range of biological activities such as anti-convulsant, anti-inflammatory, anti-microbial, analgesic, and hypolipidemic activities as well as numerous pharmaceutical uses. Phthalimide, maleimide, and succinimide have an imide functional group and can be considered nitrogen analogs of anhydrides. The imide ring and general structure -CO-N(R)- CO- found in cyclic imides, including phthalimides, maleimides, succinimides, and their derivatives, imparts hydrophobicity and neutral properties onto the respective derivatives. The unsubstituted cyclic imide is a crucial feature that has been shown to retain considerable biological activity. In this article, we intend to investigate several phthalimides, maleimides, and succinimide derivatives, their vast biological activities, chemistry, structural activity relationship (SAR), and anticonvulsant activity.*

***Key Words:*** *Heterocyclic compounds, Imide ring, Phthalimides, Maleimides, Succinimides, Structural activity relationship, Anticonvulsant activity.*

***Halkalı İmid Türevleri: Antikonvülsan Ajanların Sentezi için Potansiyel Bir İskelet***

***ÖZ***

*Ftalimidler, maleimidler ve süksinimidler gibi aromatik olmayan azotlu heterohalkalar, anti-konvülsan, anti-inflamatuar, anti-mikrobiyal, analjezik ve hipolipidemik aktivite gibi çok çeşitli biyolojik aktivitelerin yanı sıra çok sayıda farmasötik kullanıma sahip ilgi çekici bir bileşik sınıfıdır. Ftalimid, maleimid ve süksinimid, bir imid fonksiyonel grubuna sahiptir ve anhidritlerin nitrojen analogları olarak kabul edilebilir. Ftalimidler, maleimidler, süksinimidler ve bunların türevleri dahil olmak üzere halkalı imidlerde bulunan imid halkası ve genel yapısı -CO-N(R)-CO-, ilgili türevlere hidrofobiklik ve nötr özellikler kazandırır. Sübstitüe edilmemiş halkalı imid, önemli ölçüde biyolojik aktiviteyi koruduğu gösterilen çok önemli bir karakteristiktir. Bu makalede çeşitli ftalimidler, maleimidler ve süksinimid türevlerini, bunların geniş biyolojik aktivitelerini, kimyasını, yapı aktivite ilişkisini (SAR) ve antikonvülsan aktiviteyi araştırmayı amaçlıyoruz.*

***Anahtar Kelimeler:*** *Heterosiklik bileşikler, İmid halkası, Ftalimidler, Maleimidler, Süksinimidler, Yapı aktivite ilişkisi, Antikonvülsan aktivite.*

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## INTRODUCTION

Organic chemists are particularly interested in the chemistry of heterocyclic molecules due to their strong coordination, high electron-donating abilities, and numerous applications(Sabir, Alhazza, & Ibrahim, 2016). Synthetic cyclic imides, including phthalimides, maleimides, succinimides, and their derivatives have a common structural similarity that has the potential for pharmaceutical and biological activity. Due to the presence of general structure -CO-N(R)-CO- and an imide ring, their derivatives become hydrophobic and neutral, quickly permeating through a biological membrane (Hargreaves et al., 1970). The amide (Figure 1) is the imide form with two carbonyl groups attached to the nitrogen atom. Any chemical that includes the divalent radical "-C(=O)-NH-C(=O)-" is referred to as an imide (Figure 2). These compounds are synthesized from ammonia or primary amine, replacing two hydrogen atoms with a bivalent acid group or two monovalent acid groups, leading to the emergence of two carboxylic acid groups or one dicarboxylic acid(Al-Azzawi et al., 2011). Despite the wide range of biological effects associated with cyclic imides, much of their biological and toxicological action mechanism at the molecular and cellular levels are currently unknown and need to be elucidated.

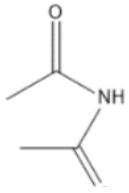



**Figure 1.** Amide

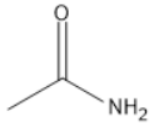



**Figure 2.** Imide

### Phthalimide

### Chemistry of Phthalimide

Phthalimide (1,3 isoindoline-dione) (Figure 3) is a white-colored, solid aromatic imide with an amine functional group and two carbonyl groups. It is critical to start synthon for organic synthesis to produce a wide range of physiologically active compounds. Its alkali metal salt is commonly employed in the Gabriel amine synthesis (Figure 4) (Gabriel, 1887). Most imides are cyclic compounds generated from dicarboxylic acids, and their names are derived from the parent acid. Such as phthalimide, which is derived from phthalic acid, and succinimide, which is derived from succinic acid, are two examples. Since imides have the formula NH and are strongly polar, they are soluble in polar fluids. The N-H core of ammonia-derived imides is acidic and can engage in hydrogen bonding(Kushwaha et al., 2016). Phthalimides are an intriguing type of bicyclic non-aromatic nitrogen heterocycles. Several alkaloids and pharmacophores have been synthesized using phthalimides as starting materials and intermediates.

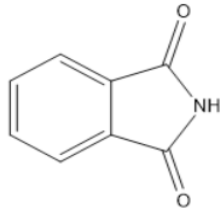



**Figure 3.** Phthalimide (1,3-isoindolinedione)

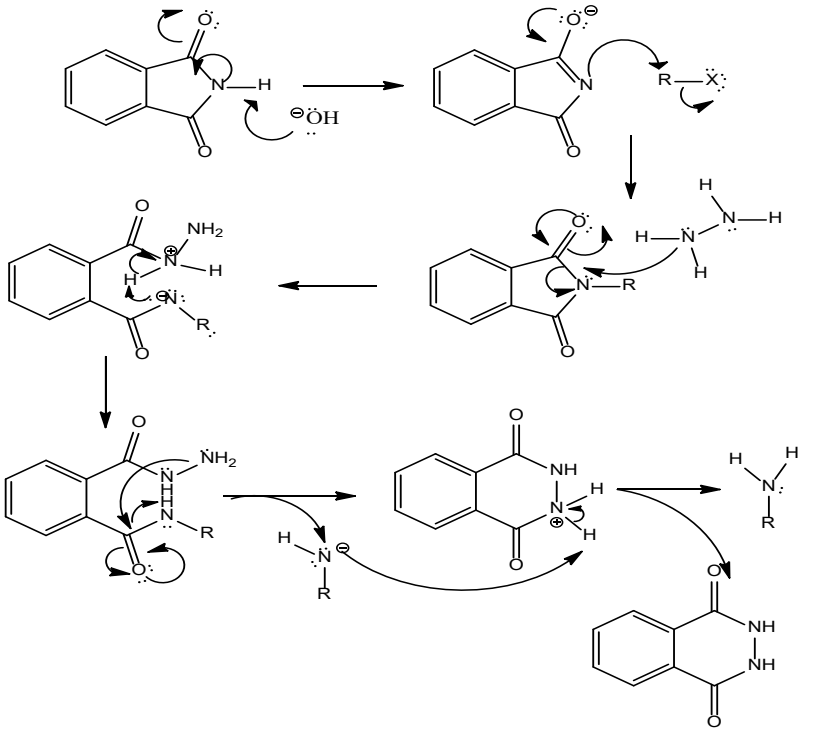


**Figure 4.** Gabriel amine synthesis

Phthalimide consists of two carbonyl groups surrounding a nitrogen atom. Because of the placement of the carbonyl group around the nitrogen atom, the molecules involved are mildly acidic. This effect is caused by neutral phthalimide molecules and their conjugate base (the anion generated by proton elimination) being resonance stabilized (Figure 5). As a result, for phthalimide, we may write multiple resonance forms that result in charge delocalization(Ginsburg, 1967).

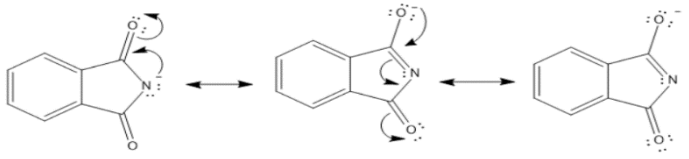
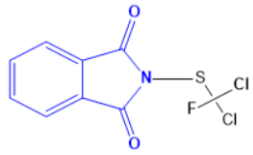
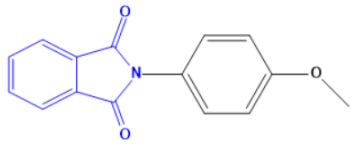
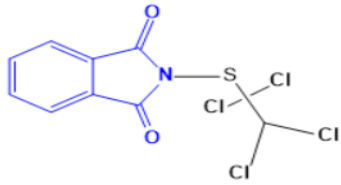
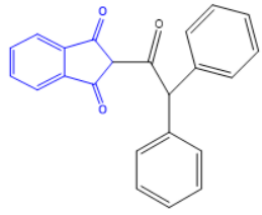
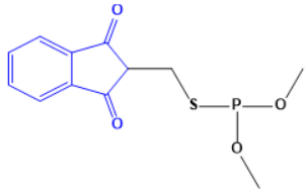
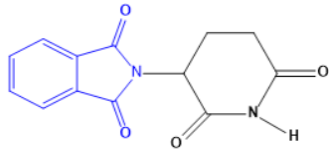
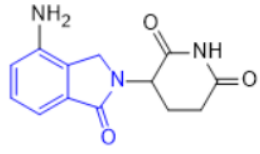
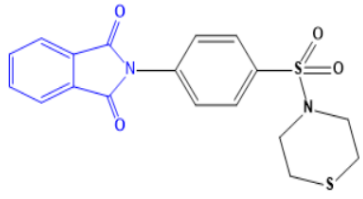


**Figure 5.** Resonance stabilization of Phthalimide

Due to their crucial biological effects, Phthalimide and N-substituted Phthalimides are an essential family of chemicals. Their action is distinguished by the following structural features: an electron-donor group, a hydrophobic aryl ring, a hydrogen bonding domain, and an additional distal hydrophobic location (Bhat & Al-Omar, 2011). Below is a list of some marketed phthalimido-containing drugs (Table 1.).

**Table 1.** Some biological active marketed Phthalimide derivatives

| S.No. | Name | Structure | Use | Reference |
|---|---|---|---|---|
| 1. | Fluorofolpet |  | Fungicide | (Paulus, 2004) |
| 2. | Anisindione |  | Anticoagulant | (Plesinac et al., 2006) |
| 3. | Captafol |  | Fungicide | (Schreurs, 1969) |
| 4. | Diphacinone |  | Anticoagulant | (Rattner et al., 2012) |
| 5. | Phosmet |  | Pesticide | (Shaw et al., 2002) |
| 6. | Thalidomide |  | Immunomodulatory and antineoplastic | (Gao et al., 2016) |
| 7. | Lenalidomide |  | Immunomodulatory and antineoplastic | (Segler and Tsimberidou, 2012) |
| 8. | LASSBio-468 |  | Anti-inflammatory and useful lead to treating rheumatoid arthritis | (De Castro Barbosa et al., 2012) |

**Anticonvulsant Activity of Phthalimide Derivatives**

Tabatabaei Rafiei *et al.,* in 2020, synthesized and evaluated a novel series of phthalimide-4,5-dihydrothiazole-amide derivatives for their anti-convulsant properties against seizures in mice generated by pentylenetetrazole (PTZ). Considering all of the chemical substances synthesized the 4-chloro derivative (Figure 6) was most potent with zero mortality during the PTZ examination. Docking of molecules was also executed to analyze the modes of interaction between $GABA_A$ receptors and synthesized compounds. Docking results showed that the 4-chloro derivative had the lowest binding energy and highest effective anti-convulsant properties. All the synthesized compounds were compared to thalidomide, used as a standard anti-epileptic agent (Tabatabaei Rafiei et al., 2020).

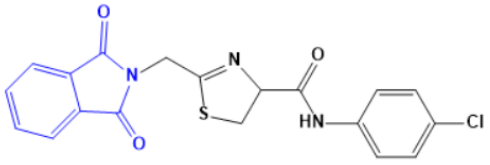



**Figure 6**

Asadollahi *et al.,* in 2019, synthesized a sequence of N-aryl-2-(1,3-dioxoisoindolin-2-yl)-3-phenylpropanamides derivatives under microwave radiation to create 2-(1,3-dioxoisoindolin-2-yl)-3-phenylpropanoic acid, that participated in the amination reaction with different anilines. The pentylenetetrazole at 70mg/kg induced seizure threshold technique was used to assess the anticonvulsant efficacy of the synthesized compounds in male mice (n=5) and compare it to thalidomide (70mg/kg) and aqueous DMSO (10% v/v), which served as positive and negative controls, respectively. The findings showed that the latency times for compound 7 (Figure 7) and thalidomide were substantially longer than those seen with aqueous DMSO ($P< 0.005$). Molecular docking studies were also performed to examine the interactions with the $GABA_A$ receptor compound 6 has the lowest binding energy and the highest interactions with the receptor's active site(Asadollahi et al., 2019).

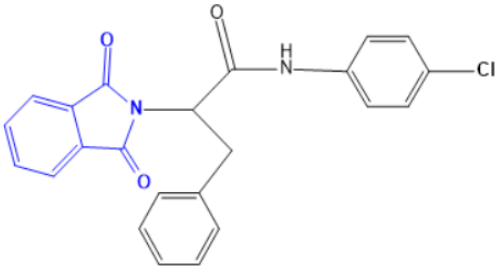



**Figure 7**

Ahuja *et al., 2*014 synthesized and investigated the anti-convulsant properties of novel N-(2-(benzylamino)-1-substituted-2-oxoethyl)-4-(1,3-dioxoisoindolin-2-yl)butanamide derivatives were created by combining the GABA-phthalimide moiety with an essential amino acid substituted on it. The intraperitoneal (i.p) maximal electroshock test and subcutaneous pentylenetetrazole (scPTZ) test investigated the anticonvulsant activity in Swiss male albino mice and adult Wistar rats. Neurotoxicity was also determined by the minimal motor impairment using the rota-rod test. The quantitative investigation in mice showed that the protective index (PI), essential for developing drugs with anticonvulsant efficacy, increased by 1.7, 2.3, and 4 times over phenytoin. On administration of the active compounds, the levels of gamma-aminobutyric acid in the various brain areas likewise elevated, with compound 8 (Figure 8) and it was the most effective derivative since it produced a substantial increase in the anticonvulsant activity (Ahuja et al., 2014).

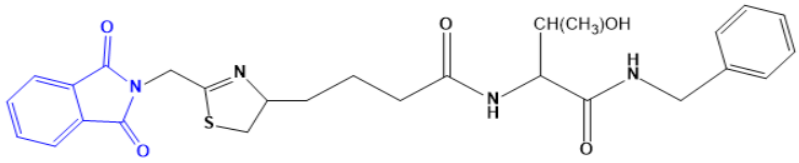



**Figure 8**

Fourteen analogs of 2-substituted phthalimide pharmacophore were synthesized by Davood *et al.,* in 2016 and also demonstrated their anticonvulsant activity using maximal electroshock seizure (MES) and pentylenetetrazole-induced seizures (PTZ) models. *In vivo* screening shows that all the synthesized compounds could show protection against PTZ and MES models. The maximum impact of these drugs was felt 30 minutes after administration. The two most potent analogs were compounds 9 and 10 (Figure 9 and Figure 10), while the substances with 100% protection in MES were compounds 9, 10, 11, 12, 13, and 14 (Figure 11- Figure 14). Molecular docking results demonstrated that the ligands mainly formed hydrogen bonds with the NAV 1.2 residues II-S6 and had additional hydrophobic interactions with other domains in the inner pore of the channel. The most effective analog for treating tonic and clonic seizures is compound 8, which has a robust lipophilic property and is more effective than phenytoin as a standard treatment and can be considered for further investigation (Davood et al., 2017).

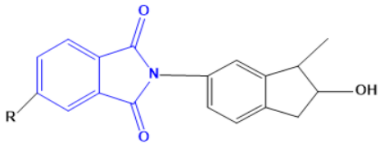



**Figure 9**

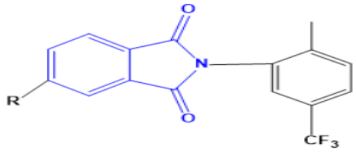



**Figure 10**

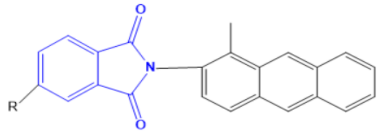



**Figure 11**

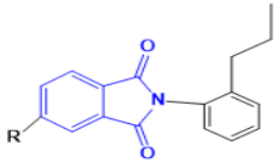



**Figure 12**

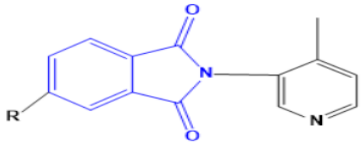



**Figure 13**

**R=H**

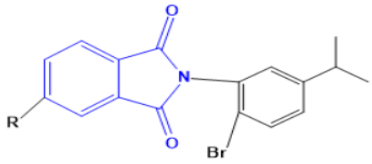



**Figure 14**

Mashooq *et al.,* synthesized novel sequences of 1,3,4-oxadiazole derivatives of phthalimides, and their anticonvulsants and neurotoxicity were also performed. The SAR studies show that the methoxy group is present in ring B, which makes the molecules more lipophilic. The production of the alkoxy group at the distal aryl ring boosted the lipophilic nature. During metabolism, alkoxy groups were thought to have been dealkylated and replaced with hydrogen in these molecules. Paramethoxy substituent compound 15 (Figure 15) in the MES test showed that it was possible to make the distal hydrophobic core more lipophilic than the phenyl ring. The distal hydrophobic center modifies the bioavailability of drugs. The current findings suggest that several phthalimide derivatives display a spectrum of efficacy in anticonvulsant screens, with compound 15 exhibiting anti-MES activity equivalent to phenytoin (Bhat & Al-Omar, 2011).

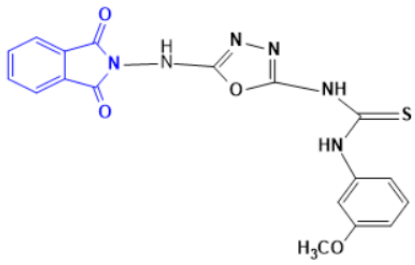


**Figure 15**

Two series of pharmacophoric hybrids of phthalimide-GABA-anilids/hydrazones i.e. 4-(1,3-dioxo-1,3-dihydro-2H-isoindol-2yl)-N-(substituted phenyl) butanamides (Figure 16) and N-aryl/alkylidene-4-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)butanoyl (Figure 17) hydrazides were designed, synthesized, and evaluated by Jegadeesan et al., 2006 their anticonvulsant and neurotoxic studies were also performed. *In vivo,* screening was performed using sc strychnine (scSTY) and intraperitoneal picrotoxin (ipPIC)- induced seizure threshold tests. The synthesized compounds were inactive against the MES test. The majority of the compound were active in scSTY and ipPIC animal models however, only a few compounds demonstrated protection in the scPTZ model (Ragavendran et al., 2007).

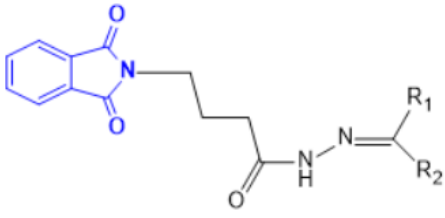


**Figure 16**

| $R_1$ | $R_2$ |
|---|---|
| H | $C_6H_5$ |
| H | 3-$NO_2$-$C_6H_4$ |
| H | 4-$NO_2$-$C_6H_4$ |
| H | 2-OH-$C_6H_4$ |
| H | 4-OH, 3-$OCH_3$-$C_6H_3$ |
| $CH_3$ | $C_6H_5$ |
| $CH_3$ | 4-$NO_2$-$C_6H_4$ |
| $CH_3$ | 2-OH-$C_6H_4$ |
| $CH_3$ | 4-OH-$C_6H_4$ |
| $CH_3$ | 3-$NH_2$-$C_6H_4$ |
| $CH_3$ | 4-$CH_3$-$C_6H_4$ |
| $C_2H_5$ | $CH_3$ |
| $C_6H_5$ | $C_6H_5$ |

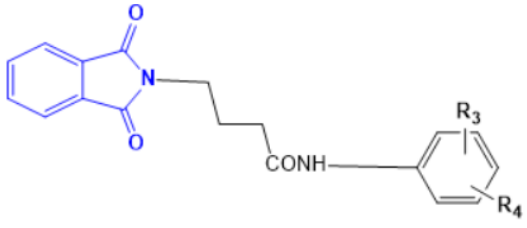


**Figure 17**

| $R_1$ | $R_2$ |
|---|---|
| H | 4-Cl |
| H | 2-$CF_3$ |
| H | 3-F |
| 2-$CH_3$ | 6-$CH_3$ |
| 2-$CH_3$ | 5-$CH_3$ |
| 4-Br | 3-$CH_3$ |
| 2-$CH_3$ | 4-$CH_3$ |
| H | 4-$CH_3$ |
| H | 2-Br |
| 3-Cl | 2-$CH_3$ |

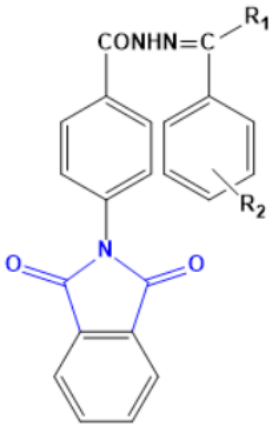



**Figure 18**

| R1 | R2 |
|---|---|
| H | 4-OH |
| H | $3,4(OCH_3)_2$ |
| H | $3\text{-}NO_2$ |
| $CH_3$ | 2-OH |
| $CH_3$ | 4-OH |
| $CH_3$ | $4\text{-}CH_3$ |
| $CH_3$ | 4-Cl |
| $CH_3$ | $4\text{-}NO_2$ |
| $CH_3$ | $4\text{-}OCH_3$ |
| $CH_3$ | $2,4\text{-}(Cl)_2$ |
| $CH_3$ | 2-OH |
| $CH_3$ | $2\text{-}NO_2$ |

Bhat *et al.* 2011 synthesized a series of Schiff bases of Phthalimide and evaluated them for their anticonvulsant and neurotoxicity activity. *In vivo,* screening was performed using the MES. The anticonvulsant evaluation was performed using the MES test at three doses (30, 100, 300 mg/kg). Compound 18 (Figure 18) was the most promising anticonvulsant drug with little neurotoxicity and nitro substitution at the ortho position of the distal aryl ring. All the reported compounds were less neurotoxic than phenytoin (Bhat and Al-Omar, 2011).

**Structure-Activity Relationship (SAR) of Phthalimide Derivatives As An Anticonvulsant**

The review paper presents a summary of the work on the structure-activity relationship of phthalimide derivatives as anticonvulsants. The imide group at N2 position acts as a hydrogen bond donor which is essential for anticonvulsant activity. Furthermore, the substitution of a methoxy group at a distant phenyl ring shows a highly potent derivative. The SAR is shown in (Figure 19).

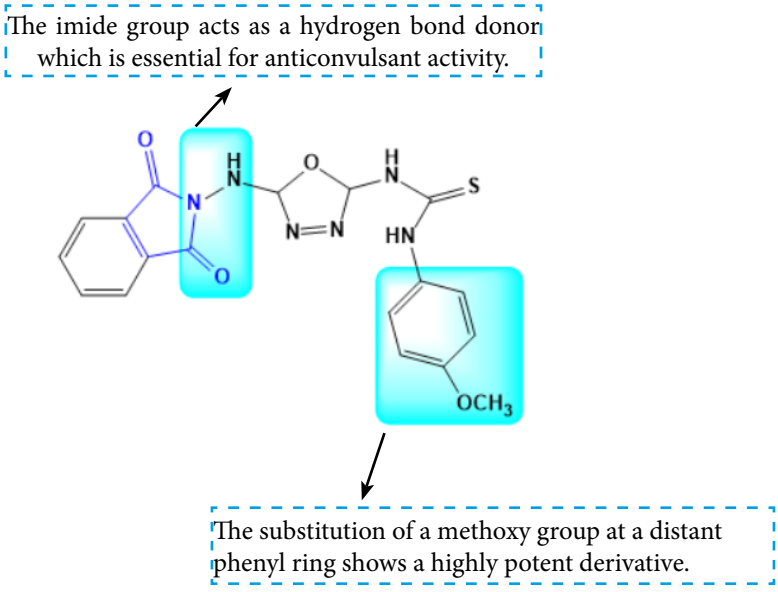



**Figure 19.** SAR of Phthalimide bearing derivative.

**Succinimides**

**Chemistry of Succinimides**

Succinimide (pyrrolidine-2,5-dione) (Figure 20) is a pyrrolidine dicarboximide with oxo groups substituting positions 2 and 5. It is a dicarboximide and a pyrrolidinone. Its chemical formula is $(CH_2)_2(CO)_2HN$.

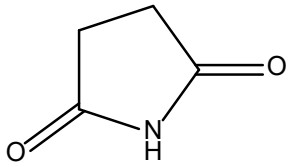
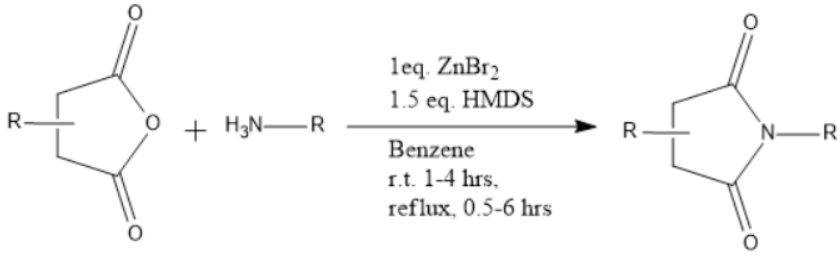


**Figure 20.** Succinimide (pyrrolidine-2,5-dione)

There have been several vital practical ways for synthesizing succinimides, which are listed below.

The easy technique is described in Scheme 1, for the direct synthesis of substituted succinimides 23, in which succinic anhydride 21 was reacted with amine 22 utilizing a Lewis acid catalyst, in the presence of Hexamethyldisilazane



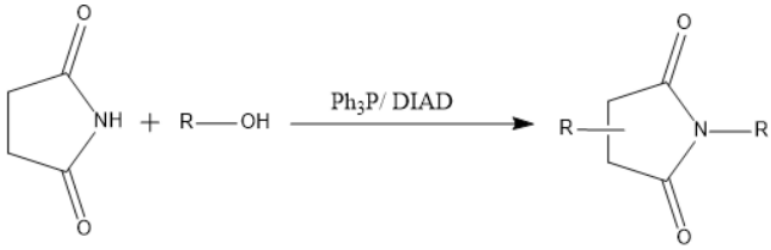

**21** **22** **Scheme 1** **23**

The newly established method for producing succinimide derivative 24 from succinic anhydride 25 by solvent-free synthesis. $TaCl_5$ and Lewis acid catalyze the reaction Scheme 2 (Chandrasekhar et al., 1997).


**24** **25**

**Scheme 2**

The synthesis of N-substituted succinimide 28 was accomplished by a modified Mitsunobu reaction that involved the interaction of succinimide 26 with alcohol 27 in the presence of triphenylphosphine and the reagent diisopropyl azodicarboxylate (DIAD) Scheme 3 (Kosinska et al., 2021).




**26** **27** **28**

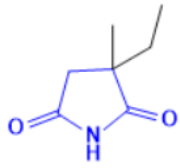
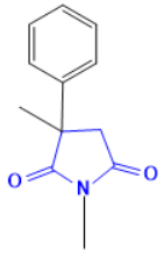
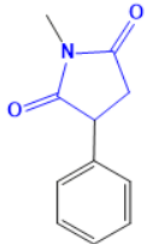
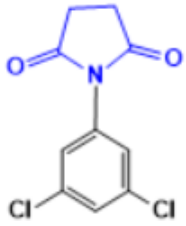
**Scheme 3**

Reacting succinic anhydrides 27 and amine 28 using microwave-assisted reactions, substituted succinimides 29 were prepared. The reaction was carried out in DMF, acetic anhydride, or water as a solvent. Compared to the conventional approach, the yield obtained by the microwave-assisted reaction was outstanding (Mandal's Sip et al., 2014).


**29** **30** **31**

**R**= benzyl; R= (CH2)6; **R**=4-methyl phenyl; **R**= 4,4'-methanediphenyl; **R**= 1-naphthyl **Scheme 4**

**Table 2.** Some biological active marketed Succinimide derivatives

| S.No. | Name | Structure | Use | Reference |
|---|---|---|---|---|
| 1. | Ethosuximide |  | Anticonvulsant (treat petit mal seizures) | (Battino et al., 1982) |
| 2. | Methsuximide |  | Anticonvulsant (treat petit mal seizures) | (Teschendorf and Kretzschmar, 1985) |
| 3. | Phensuximide |  | Anticonvulsant (treat petit mal seizures) | (Teschendorf and Kretzschmar, 1985) |
| 4. | N-(3,5-Dichlorophenyl) succinimide (NDPS) |  | Antifungal | (Li et al., 2015) |

**Anticonvulsant Activity of Succinimide Derivatives**

A series of 27 novel 1-(4-phenylpiperazine-1-yl)- or 1-(morpholin-4-yl)-2,5-dioxopyrrolidin-1-yl)propanamides and (2,5-dioxopyrolidin-1-yl) butanamides as potential anticonvulsants were synthesized by Kamiński *et al.,* in 2015. Antiepileptic drugs (AEDs) such as lacosamide, levetiracetam, and ethosuximide were utilized to join the fragments of the novel hybrid molecule. In the MES test, the subcutaneous pentylenetetrazole (scPTZ) test, and the six-hertz (6Hz) model of pharmacoresistant limbic seizures, compound **32, 33, 34** and **35** (Figure 32-35) showed a wide range of action. Compound 30 provides the best protection (ED MES=88.4 mg/kg, ED50 scPTZ= 59.9mg/kg, and ED50 6Hz= 21.0 mg/kg). Even at higher doses (TD50>1500 mg/kg), this molecule produced excellent protective indices (PI MES> 16.97, PI PTZ> 25.04, PI 6 Hz> 71.43), this compound did not affect the animal's ability to coordinate their movements during the chimney test. Although ethosuximide, lacosamide, and valproic acid are therapeutically significant AEDs, compound 32 showed a noticeably superior safety profile than those drugs (Kamiński et al., 2015).

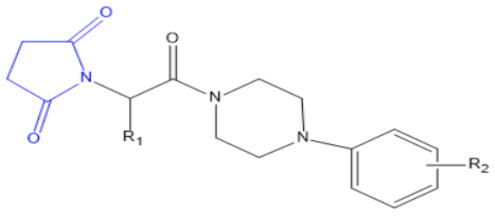


**Figure 32-35**

| Compound | R1 | R2 |
|---|---|---|
| 32 | $CH_3$ | 4-Cl |
| 33 | $CH_3$ | 2-$CF_3$ |
| 34 | $CH_3$ | 3- $CF_3$ |
| 35 | $C_2H_5$ | 3- $CF_3$ |

In 2016, Kamiński *et al* synthesized a series of 21 novel N-phenyl-2-(2,5-dioxopyrrolidin-1-yl) propanamides, 2-(3-methyl-2,5-dioxopyrrolidin-1-yl)propanamides, and 2-(2,5-dioxopyrrolidin-1- yl) butanamides as potential hybrid anticonvulsant agents. The "classical" MES and scPTZ tests were used for the initial anticonvulsant screening in mice (i.p), as well as in the 6 Hz model of pharmacoresistant limbic seizures. The rotarod test was used to detect acute neurological toxicity. All preclinical seizure models demonstrated a broad spectrum of efficacy for compounds 36, 37, 38, and 39 (Figure 36-39). In the 6 Hz test, the butanamide derivatives 38 and 39 was the most effective. The best level of protection and a significant safety profile were shown in the rotarod test for derivative 36 by quantitative pharmacological tests in mice administered intraperitoneally. This substance was identified as the most promising with positive protective indexes (PI MES = 3.5, PI scPTZ = 4.4, PI 6 Hz = 7.6). The *in vitro* binding experiments demonstrated that the influence on the voltage-sensitive sodium channel and the diltiazem site of the L-type calcium channel in neurons was the most likely mechanism of action for compound 36 (Kamiński et al., 2016a).

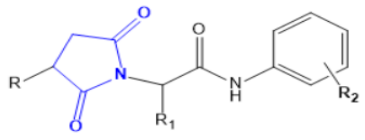


**Figure 36-39**

| Compound | R | R1 | R2 |
|---|---|---|---|
| 36 | H | $CH_3$ | H |
| 37 | H | $CH_3$ | 3-Cl |
| 38 | H | $C_2H_5$ | H |
| 39 | $CH_3$ | $CH_3$ | H |

A series of 34 novel 3-methyl- and 3,3-dimethyl-(2,5- dioxopyrrolidin-1-yl)propanamides or butanamides hybrid anticonvulsant agents were synthesized. The "classical" MES and scPTZ test and the 6 Hz model of pharmacoresistant limbic seizure were used in the initial anticonvulsant screening in mice (i.p). The chimney test was used to assess acute neurological toxicity. Compounds **40**, **41**, **42**, and **43** (Figure 40-43) demonstrated a broad spectrum of efficacy in all preclinical seizure models. The quantitative pharmacological tests in mice (i.p) indicated the best protection and a satisfactory safety profile in the chimney test for compound 41. Compared to traditional AEDs, this chemical emerged as the most promising molecule with favorable protective indexes. In addition, six anticonvulsants demonstrated decisive antinociceptive action in mice's formalin model of tonic pain. The *in vitro* binding tests for compound 41 shows that the impact on neuronal voltage-sensitive sodium and L-type calcium channels was the most probable molecular mechanism of anticonvulsant and antinociceptive activity(Kamiński et al., 2016b).

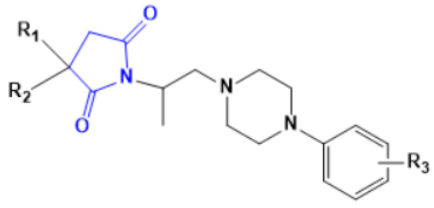



**Figure 40-42**

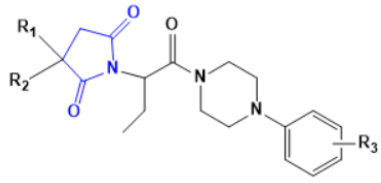



**Figure 43**

| Compound | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| 40 | $CH_3$ | H | H |
| 41 | $CH_3$ | H | 3-$CF_3$ |

**$R_1$**= $CH_3$, **$R_2$**= H, **$R_3$**= 3-$CF_3$

The series of N-Mannich bases of 3-phenyl-, 3-(2-chloro-phenyl)-, 3-(3-chlorophenyl)- and 3-(4-chlorophenyl)-pyrrolidine-2,5-diones were synthesized and investigated for their anticonvulsant properties by Kaminski *et al.,* in 2013. The primary synthetic techniques involve the synthesis of 3-substituted pyrrolidine-2,5-diones followed by an amino alkylation reaction (Mannich-type) with formaldehyde and corresponding secondary amines, yielding the final compounds. The findings demonstrated that most compounds protected MES. Several substances were also active in seizures induced by pentylenetetrazole (scPTZ) and psychomotor (6Hz). The most active 1-(morpholinomethyl)-3-phenyl-pyrrolidine-2,5-dione (Figure 44) was effective in the MES, scPTZ, 6-Hz, and pilocarpine-induced status prevention (PISP) tests, indicating its potential utility in tonic-clonic, absence, and refractory epilepsy, as well as *status epilepticus*. This compound, as the most promising in the series, inhibited CYP3A4 activity very little *in vitro* and is unlikely to interact with other CYP3A4 metabolized medicines *in vivo*(Kamiński et al., 2013).

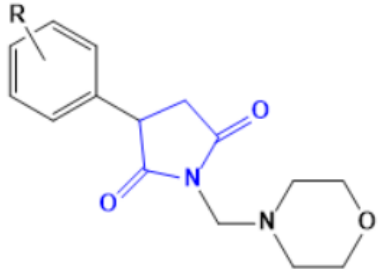



**Figure 44**

The library of 28 novel 1,3-substituted pyrrolidine-2,5-dione as potential anticonvulsant agents was synthesized and evaluated by Rybka *et al* in 2017. Anticonvulsant action was assessed in mice using three acute seizure models MES, subcutaneous pentylenetetrazole (scPTZ), and psychomotor seizure tests (6-Hz). The rotarod test was used to evaluate neurotoxicity. N-[morpholin-1-yl-methyl]-3-benhydryl-pyrrolidine-2,5-dione (Figure 45) was determined to be the most promising chemical, since it was active in the MES (ED50= 41.0 mg/kg), scPTZ (ED50= 101.6 kg/mg), and 6Hz (ED50= 45.42 mg/kg) assays. This compound outperformed antiepileptic medications like ethosuximide, lacosamide, and valproic acid regarding a favorable protection index (PI). Furthermore, *in vitro* tests revealed that for compound 45 the most likely mechanism of action is the inhibition of neuronal volage-sensitive sodium and L-type calcium channels (Rybka et al., 2017).

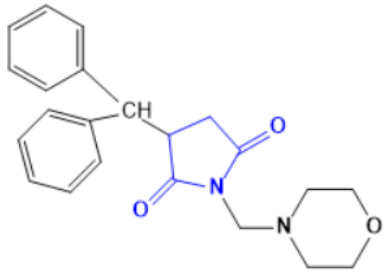



**Figure 45**

A series of sixteen novel 1-[(4-arylpiperazin-1-yl)-propyl]-3-methyl-3-phenyl- and 3-ethyl-3-methylpyrrolidine-2,5-dione derivatives as possible anticonvulsant drugs were synthesized by Obniska *et al.,* in 2012. MES, subcutaneous pentylenetetrazole (scPTZ), and psychomotor seizure tests (6-Hz) tests were used for the evaluation of anticonvulsant properties. The rotarod screening was also performed to investigate acute neurological toxicity. The 1-{3-[4-(3-chlorophenyl)-piperazin-1-yl]-propyl}-3-methyl-3-phenyl-pyrrolidine-2,5-dione (Figure 46) was the most active compound from whole series with the ED50 value of 28.2mg/kg, TD50 value of 268.5mg/kg, and protective index (PI) of 9.52 after *po* administration in rats (Obniska et al., 2012).

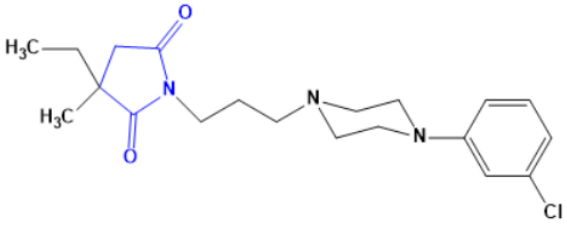



**Figure 46**

A series of N-[(4-arylpiperazin-1-yl)-methyl] derivatives of 3-arylpyrrolidine-2,5-dione and 2-aza-spiro[4,4]nonane-1,3-dione were synthesized by Obniska *et al.,* in 2003 and also anticonvulsant activity was assessed in the maximum electroshock seizure (MES) and pentylenetetrazole- induced seizures (scPTZ) tests. The N-[{4-(3-chlorophenyl)-piperazin-1-yl}-methyl]-3-(2-chlorophenyl)-pyrrolidine-2,5-dione (Figure 47) (ED50= 14.18 mg/kg) and N-[{4-(2-methoxyphenyl)-piperazin-1-yl}-methyl]-3-(3-bromophenyl)-pyrrolidine-2,5-dione (Figure 48) (ED50= 33.64 mg/kg) was the most potent compound amongst the whole series (Obniska and Zagorska, 2003).

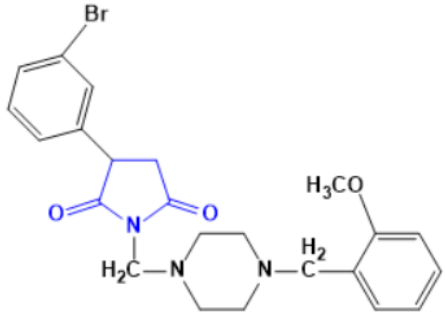



**Figure 47**

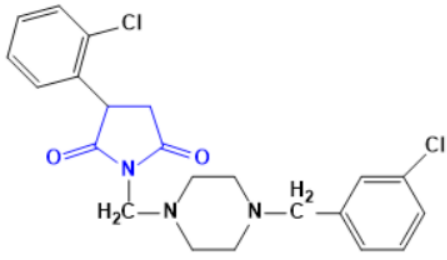



**Figure 48**

Obniska *et al.,* 1999 synthesized new N-pyridyl derivatives of 3-phenyl and 3,3-diphenyl succinimides and described their physicochemical properties. The synthesized compounds were evaluated for anticonvulsant activity. The N-pyridyl derivatives of 3-phenylsuccinimides (Figure 49-54) were ineffective in protecting against MES and scMET-induced seizures. The Molecular electrostatic potential (MEP) was also performed, and it was found that the active compounds differ significantly from the inactive ones (Obniska et al., 1999).

**Structure-Activity Relationship (SAR) of Succinimide Derivatives As An Anticonvulsant**

The review paper presents a summary of the work on the structure-activity relationship of succinimide derivatives as anticonvulsants. The substitution of the pyridyl ring at the imide group at the N2 position is a characteristic property of anticonvulsants. The pyridyl ring can be further substituted with the methyl group which aids in the establishment of the hydrogen bond with the receptor. Also, the substitution of the aromatic ring at the C5 position of succinimide moiety gives an active compound (Figure 49-54). The SAR of succinimide moiety is shown in (Figure 55) (Obniska, Zejc, & Karolak-Wojciechowska, 1999).

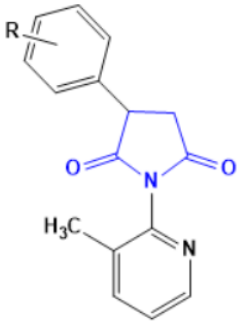



**Figure 49-54**

| Compound | R |
|---|---|
| 49 | 4-F |
| 50 | 3-F |
| 51 | 2-Cl |
| 52 | 3-Br |
| 53 | 2-$OCH_3$ |
| 54 | 3-$OCH_3$ |

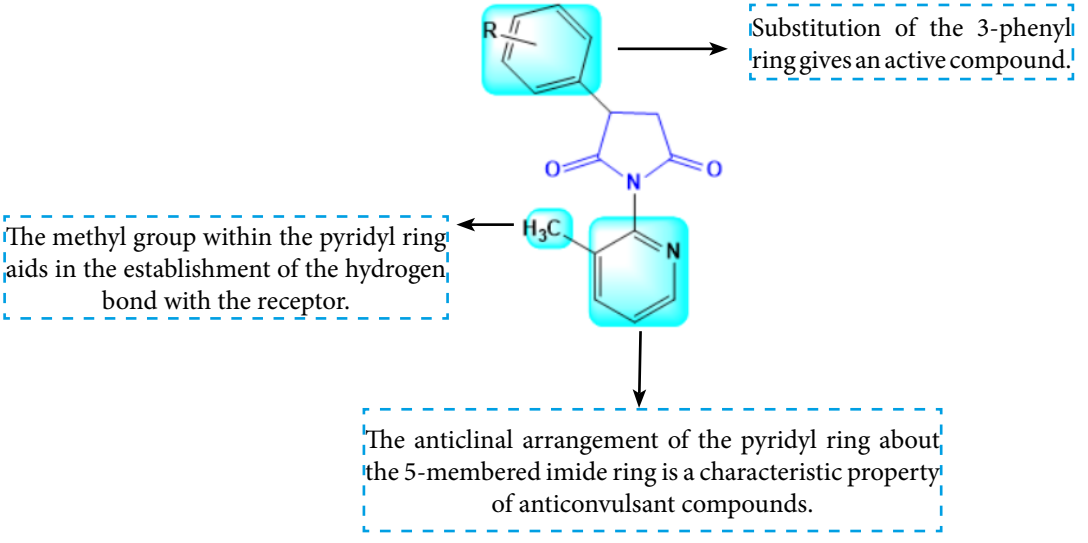



**Figure 55.** SAR of Succinimide bearing derivative(Obniska et al., 1999)

**Maleimide**

**Chemistry of Maleimide**

Maleimide (Pyrrole-2,5-dione) (Figure 56) is an example of cyclic dicarboximide in which the two carbonyl groups on nitrogen combine to generate a 1H-pyrrole-2,5-dione structure. Maleimide is an effective moiety, wherein the NH group is replaced by an alkyl or aryl group, such as a methyl or phenyl, as suitable. Meanwhile, a maleimide moiety with a 1.2-disubstituted ethylene structure can have its vinylene group polymerized using radical or anionic initiators to produce a material with high thermostability or heat resistance, which can then be copolymerized with vinyl (Jarzyński et al., 2023).

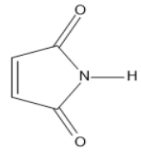
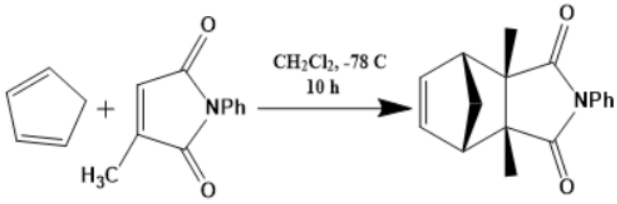



**Figure 56.** Pyrrole-2,5-dione

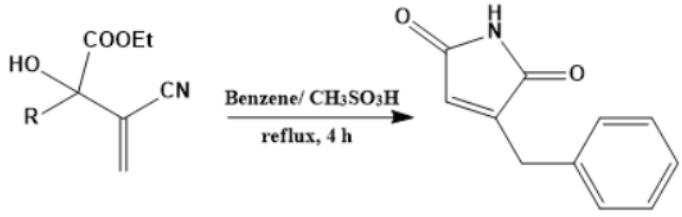
In terms of chemical reactivity, maleimide derivatives are particularly appealing compounds. They catalyze several reactions, such as the Diels-Alder reaction with dienes(Mukherjee and Corey, 2010) and the nucleophilic Michael-type addition of thiols or amines to the vinylene molecules (Abel and McCormick, 2016).




(Cyclopentadiene) (N-Phenylmaleimide) (Exo/endo diels product)

**Figure 57**

Friedel-Crafts reaction of Baylis-Hillman adducts maleimide: Synthesis of 4-substituted 3-benzyl-1H-2,5-dione derivatives (Basavaiah et al., 2011).




(3-ethoxycarbonyl-3 -hydroxy-3aryl(alkyl)-2-Methylenepropanenitrile)

(4-substituted 3-benzyl-1H-2,5-dione derivative)

**R**= aryl/alkyl

**Figure 58**

A unique modification of the Mitsunobu reaction allows for a high-yield synthesis of N-alkyl maleimides (Walker, 1995).

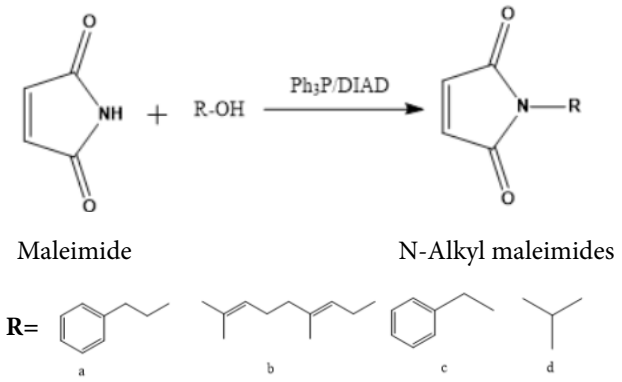



**Figure 59**

**Anticonvulsant Activity of Maleimide Derivatives**

Abram M *et al.* 2021 generated a series of water-soluble hydrochlorides of pyrrolidine-2,5-dione derivatives developed as possible anticonvulsants with added antinociceptive characteristics. Preclinical results in mice demonstrated that these drugs provided strong protection and broad-spectrum efficacy in many animal models of seizures, including the MES, 6 Hz (32/44 mA), and, scPTZ tests. Compound 60 demonstrated the most favorable anticonvulsant effects [ED50 MES= 49.6 mg/kg, ED50 Hz (32 mg/kg), ED50 scPTZ= 67.4 mg/kg] and safety profile(Abram et al., 2021).

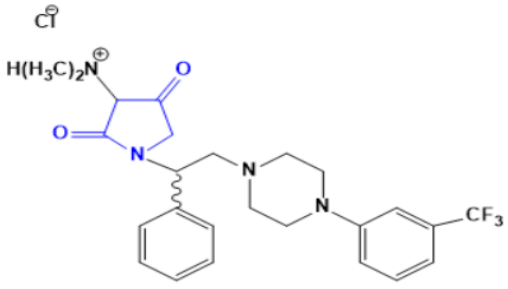
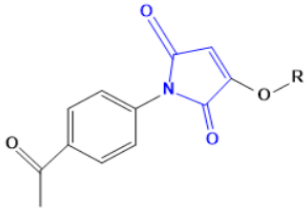



**Figure 60**

A novel series of Michael adducts as a 1-(4-acetyl-phenyl)-3-aryloxypyrrolidine-2,5-dione derivatives utilizing cellulose sulfuric acid catalyst to obtain lead compounds for future development as anti-convulsants were evaluated by Jigarkumar *et al.*, in 2013. Among the fourteen Michael adducts of 1-(4-acetylphenyl)-pyrrole2,5-dione, 1-(4-acetyl-phenyl)-3-(4-Bromophenyloxy)-pyrrolidine-2,5-di-one (Figure 61a) and 1-(4-acetylphenyl)-3-(salicylde-hydroxy)-pyrrolidine-2,5-dione (Figure 61b) *in vitro* brain GABA-transaminase activity was higher with $IC_{50}$ values of (100.5, 5.2mM) and IC50 (160.4, 6.2 mM) respectively. The investigation utilized fluoro-metric analytical assessment data compared to vigab-atrin, a reference standard (Patel et al., 2013a).




| Compound | R |
|---|---|
| 61a | p-Br-$C_6H_4$ |
| 61b | o-CHO-$C_6H_4$ |

**Figure 61**

A series of 4-dialkyl amino-2-butynyl-maleimide and hydantoin derivatives were designed for dual-acting anticonvulsant and antimuscarinic activity by Roberto *et al.,* in 1997, and compound 62 (Figure 62) derivatives were found to be the most potent (Hudkins et al., 1997).

**Structure-Activity Relationship (SAR) of Maleimide Derivatives As An Anticonvulsant**

The review paper presents a summary of the work on the structure-activity relationship of maleimide derivatives as anticonvulsants. Substituting the bromine atom which is an electron-donating group to a phenyl ring at position gives Michael adduct with maleic amide moiety results in considerable GABA-T activity. Substituting CHO (aldehyde group) at the ortho position of the same phenyl ring gives potent derivatives (Figure 61). The SAR of maleimide moiety is shown in (Figure 63)(Patel, Dholakiya, & Mishra, 2013).

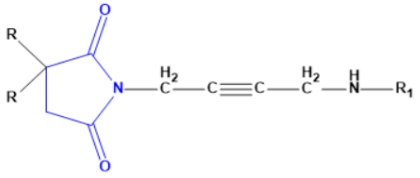


**Figure 62**

| Compound | R | R1 |
|---|---|---|
| 62a | Ph | $C_2H_5$ |
| 62b | 2-dinitrosubrane | $C_2H_5$ |

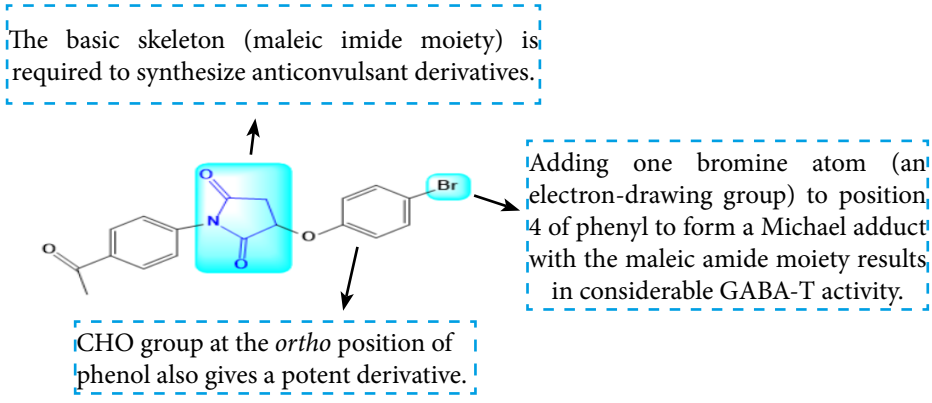
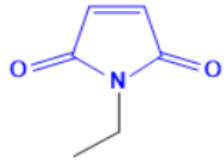
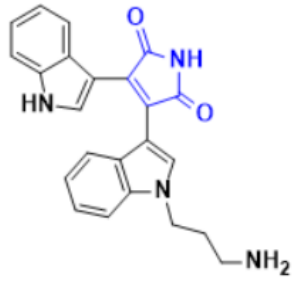
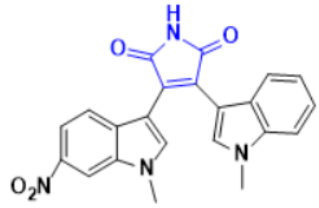
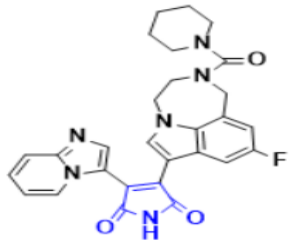



**Figure 63.** SAR of Maleimide bearing derivatives (Patel et al., 2013b)

**Table 3.** Some biologically active experimental maleimide derivatives

| S.No. | Name | Structure | Use | Reference |
|---|---|---|---|---|
| 1 | N-ethylmaleimide |  | Anti-coronaviral agent | (Wenge and Bönisch, 2008) |
| 2 | Bisindolylmaleimide III |  | Protein kinase C inhibitor | (Brehmer et al., 2004) |
| 3 | MKC-1 |  | Antineoplastic activity | (Faris et al., 2012) |
| 4 | LY2090314 |  | Antineoplastic activity | (Magnus et al., 2010) |

## CONCLUSION

Based on prior studies, we concluded that the cyclic imide derivatives had significant biological activity. Cyclic imide derivatives are considered an important class of medications used as anticancer, antibacterial, anti-inflammatory, analgesic, and antituberculosis agents. Diverse phthalimide, maleimide, and succinimide derivatives could be further engineered and developed to produce unique and more effective therapeutically active compounds by including new pharmacophores at various places. The structure-activity relationship of different cyclic imide derivatives reveals that substituting a methoxy group at a distant phenyl ring shows a highly potent derivative in the case of Phthalimide derivatives e.g. **Figure 19**. Furthermore, for succinimide derivative's structure-activity relationship divulges that the anticlinal arrangement of the pyridyl ring about the 5-membered imide ring is a characteristic property of anticonvulsant compounds. Also, the methyl group within the pyridyl ring helps in the formation of the H bond with the receptor e.g., **Figure 55**. Also, the SAR study of maleimide reveals that the introduction of one Bromine atom (Electron withdrawing group)

in position 4 of phenyl to form Michael adduct with maleic amide moiety offers high GABA-T action; additionally, the presence of the basic skeleton (maleic imide moiety) is crucial for the development of the anticonvulsant derivatives e.g. **Figure 63**. From the overall study, we may thus conclude that the numerous synthetic cyclic imide compounds help treat epilepsy.

**CONFLICTS OF INTERESTS**

The authors declare that there is no conflict of interest.

**AUTHOR CONTRIBUTION STATEMENT**

Conception & design were given by S Singh, Acquisition of the data was done by A J Rana, drafting of the article was seen by M Rana, Collection and assembly of data was done by S Singh, A V Pargaein, Administrative, technical, or logistic support was provided by M Rana and H Joshi, Plagiarism was checked by H K Awasthi.

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*REVIEW ARTICLE*

# The Effects of Topical Applications of *Momordica charantia* Extract on Wound Healing: A Comprehensive Review

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***The Effects of Topical Applications of Momordica charantia Extract on Wound Healing: A Comprehensive Review***

***SUMMARY***

*A wound is the event of deterioration of the integrity of soft tissue. The intricate tissue restoration process during wound healing involves cellular and molecular activities. Wound healing includes three phases: hemostasis and inflammatory phase, proliferation phase, and remodeling phase. Diabetes mellitus (DM), an endocrine system disease, occurs due to the absence of insulin or resistance to the effect of insulin. Problems with angiogenesis, immunity, and risk of infection make diabetic wound care difficult. Various studies have been carried out to increase wound healing in diabetes and skin wounds, and the use of herbal products in this direction is increasing. Momordica charantia (MC) is well-known for its ability to treat diabetes and repair wounds. MC is a plant widely grown in the world and in our country. It is a used both in the pharmaceutical industry and as a nutrient. MC, a member of the Cucurbitaceae family, is the scientific name for bitter gourd. Sterols, triterpenoids, polypeptides, saponins, flavonoids, and alkaloids are present in MC. Phytochemical studies have shown that bioactive components have antidiabetic, antioxidant, antiviral, antimicrobial, anti-inflammatory, antitumor, hypolipidemic, immunomodulatory, and wound-healing activities. Studies have shown that MC extract increases epithelialization, neovascularization and fibroblast proliferation in wounds and accelerates wound healing by suppressing inflammation. It is thought that the topical application of the extract of the MC plant can accelerate wound healing in skin and diabetic wounds and help treatment.*

***Key Words:*** *Momordica charantia, skin wound, diabetic wound, topical application.*

***Momordica charantia Ekstraktının Topikal Uygulamalarının Yara İyileşmesi Üzerindeki Etkileri: Kapsamlı Bir Derleme***

***ÖZ***

*Yara, yumuşak dokunun bütünlüğünün bozulması olayıdır. Yara iyileşmesi, dokunun onarılması için hücresel ve biyokimyasal olayları içeren karmaşık bir süreçtir. Yara iyileşmesi, hemostaz ve inflamatuar faz, proliferasyon fazı ve yeniden olgunlaşma fazı olmak üzere üç evreden oluşmaktadır. Bir endokrin sistem hastalığı olan diabetes mellitus (DM) insülin yokluğuna veya insülinin etkisine olan dirence bağlı olarak ortaya çıkmaktadır. Anjiyogenez, bağışıklık ve enfeksiyon riski ile ilgili sorunlar diyabetik yara bakımını zorlaştırmaktadır. Diyabette ve deri yaralarında yara iyileşmesini arttırmak için çeşitli çalışmalar yapılmakta ve bu yönde bitkisel ürünlerin kullanımı giderek artmaktadır. Momordica charantia (MC), diyabeti tedavi etme ve yaraları onarma becerisiyle tanınır. MC dünyada ve ülkemizde yaygın olarak yetiştirilen bir bitkidir. Hem ilaç sektöründe hem de besin maddesi olarak kullanılmaktadır. Cucurbitaceae familyasının bir üyesi olan MC, acı kabakların bilimsel adıdır. MC'de steroller, triterpenoitler, polipeptitler, saponinler, flavonoitler ve alkaloitler bulunur. Yapılan fitokimyasal araştırmalar, bu bitkideki biyoaktif bileşenlerin; antidiyabetik, antioksidan, antiviral, antimikrobiyal, antiinflamatuar, antitümör, hipolipidemik, immunomodülatör ve yara iyileştirici aktivitelerinin olduğunu göstermiştir. Yapılan çalışmalar MC ekstresi uygulanan yaralarda epitelizasyonun, neovaskülerizasyonun ve fibroblast proliferasyonunun arttığını ve inflamasyonun baskılanarak yara iyileşmesinin hızlandığını göstermiştir. MC bitkisinin ekstresinin topikal uygulamasının deri yaralarında ve diyabetik yaralarda yara iyileşmesini hızlandırabileceği, tedaviye yardımcı olabileceği düşünülmektedir.*

***Anahtar Kelimeler:*** *Momordica charantia, deri yarası, diyabetik yara, topikal uygulama.*

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## INTRODUCTION

A wound is an event in which the integrity of soft tissue is disrupted (Pekbilir, 1990). Accidental trauma is a frequent cause of skin wounds. The complex wound-healing process involves biochemical mechanisms (Sagástegui-Guarniz et al., 2021). Hemostasis, inflammatory response, proliferation, and remodeling are the stages of wound healing. The healing period for acute wounds is usually 5-10 days but can take up to a month. These types of wounds are generally skin, muscle, nerve, and tendon injuries caused by surgical incisions, aseptic wounds, and open or closed fractures (Durmuş & Başa, 2018). Infections lead to complications during the recovery period. The tissue's capacity to heal itself, the size and type of the wound, and the general condition of the tissue are important factors in wound healing (Sagástegui-Guarniz et al., 2021). If wound healing is disturbed during the inflammatory phase, healing is delayed. Oxygen plays an important role in wound healing as it is necessary for collagen synthesis and fibroblast migration. Hypoxia, diabetes, arterial disease, malnutrition, and steroid use cause chronic wound healing. Chronic wounds pose serious problems in veterinary medicine (Durmuş & Başa, 2018).

The most crucial complication of diabetes is the deterioration of wound healing processes (Hussan, Teoh, Muhamad, Mazlan, & Latiff, 2014). The cause of non-healing wounds in diabetes depends on many factors. The first metabolic event that occurs in diabetes is decreased oxygenation of the scar tissue and a delay in tissue mend and regeneration due to constriction of the arteries in the wound from hyperglycemia. In the later stages, perception of sensation is lost, and defense against infections is weakened as the peripheral nerves and immune system are damaged by chronic hyperglycemia (Singh, Garcia-Gomez, Gudehithlu, & Singh, 2017). Numerous studies are being done to enhance wound healing in diabetics, and more herbal medications are being used (Hussan et al, 2014).

Because of the limited efficacy, low availability, high cost, and some side effects of wound-healing agents, plants are widely used in the general population. According to numerous researchers, various plant extracts are effective in the wound healing (Sagástegui-Guarniz et al., 2021). Today, phytotherapy, which benefits from the healing effects of plants, has become widespread, and the use of natural remedies has come to the fore (Prisăcaru et al., 2013). Herbal medicines can be used for wound healing. Numerous herbal preparations such as MC, have been proven in studies to have wound-healing effects (Hussan et al., 2014). Additional names for MC include "bitter melon" or "bitter gourd" due to its bitter flavor, which becomes more apparent as the fruit ripens. MC grows widely in Asia, India, East Africa and South America, and in the west of the Marmara Region and the Aegean Region in Turkey. It is a climbing plant from the Cucurbitaceae family that can generally grow up to 5 meters tall. It is a tropical perennial plant (Joseph & Jini, 2013; Kısacık & Güneş, 2017).

It is thought that MC plant can be used in veterinary medicine to accelerate wound healing in skin and diabetic wounds.

### *Momordica charantia*

MC is a plant grown both for consumption as food and for use in the pharmaceutical industry. Researches based on its pharmacological properties date back to the 16th century (Kısacık & Güneş, 2017).

#### Botanical Features of *Momordica charantia*

A member of the Cucurbitaceae family is the bitter gourd and is called MC (Kısacık & Güneş, 2017). It is known as 'Bitter Melon' in English (Ekizce, 2019). It is an annual, tropical plant whose thin leaves grow in the form of ivy. Momordica means 'bite' in Latin. It has various names like bitter melon, balsam pear, and bitter gourd. It is known that the plant, whose homeland is India, grows in the tropical regions of Asia, South America, and Africa, and in our country, in the west of the Marmara Region and the Aegean Region (Kısacık & Güneş, 2017). The leaves of the plant are

4-12 cm in size and have toothed edges. Its fruit is oval and rough. While immature fruits are green, this color becomes orange-yellow as they mature. The fruit is fleshy and has 3 parts, and as the fruit matures, it folds back and opens. When a fruit is opened, red seeds are seen within. It bears bitter fruit (Ekizce, 2019).

**Chemical Composition and Biological Activities of *Momordica charantia***

Despite the bitter taste of the MC fruit, it is popular many locations around the globe. In many regions it is also used as herbal medicine. Fruits, leaves, roots of the plant; it is used by people for diseases such as toothache, diarrhea, boils, constipation, gout, jaundice and rheumatism. It is also known to be used as an anthelmintic. It is known that bitter gourd tea made from dried plant slices is quite common in some regions. Triterpenoids, alkaloids, polypeptides, flavonoids, saponins, and sterols are all present in MC. Phytochemical studies have shown that bioactive components have anti-inflammatory, antidiabetic, antioxidant, antiviral, antimicrobial, antitumor, hypolipidemic, immunomodulatory, and wound healing activities (Jia, Shen, Zhang, & Xie, 2017).

Bitter melon plant contains; momordicin, momorcharin, momordin, charantin, polypeptide-p, cucurbitacin B, linolenic acid (constitutes 60% of the seed oil of the plant), iron, calcium, magnesium, phosphorus, β-carotene, potassium, magnesium and vitamin A (Kısacık & Güneş, 2017). Except for methionine, lysine and cysteine, MC contains most of the essential amino acids (Yuwai, Rao, Kaluwin, Jones, & Rivett, 1991).

Phenolic compounds like gentisic acid, caffeic acid, benzoic acid, chlorogenic acid, tannic acid, and gallic acid are found in the fruits of MC. Tocopherol, catechin, and epicatechin in MC exhibit antioxidant properties. The seed membranes in ripe fruits are a source of lycopene (Ekizce, 2019). Phenolic compounds are metabolic aids that have a structure similar to benzene. They are tannins, coumarins, lignins, lignans, flavonoids, common phenols, and phenolic acids. They have antioxidant, antimicrobial, and anticancer effects (Faith Oyelere et al., 2022).

The predominant terpenoids in MC are momordicin, and charantin. Terpenoids, the largest group of secondary metabolites, are found mostly in lower invertebrates and plants. They have anti-cancer, anti-inflammatory, plant growth-promoting and cardiovascular disease-reducing effects (Faith Oyelere et al., 2022).

A combination of 5,25-stigmadiene-3β-ol-glycoside and β-sitosterol-β-D-glucoside makes up charantin (Sharma, Sharma, & Kohli, 2010). Chloroform and dichloromethane are less polar solvents in which charantin is readily soluble, due to its steroidal structure in the aglycone part. Due to charantin's glucoside structure, it can also dissolve in solvents such as ethanol and methanol (Türkoğlu, 2018). Active substances with hypoglycemic characteristics include charantin and polypeptide-p. Additionally, soluble pectins, 5-hydroxytryptamine, saponins, and steroidal glycosides are present in the MC plant (Hussan et al., 2014). MC's leaves, stems, fruits, and roots contain saponins. Cucurbitacins, alkyl-type saponins, are a group of mainly tetracyclic, triterpenic plant substances with a bitter taste and high oxygen content. Studies have shown that saponins have antidiabetic and hypoglycemic activities (Jia et al., 2017).

Several phytosterols have been identified in MC, such as β-sitosterol, campesterol, 25,26-dihydroesterol, stigmasterol, daucosterol, clerosterol, and diosgenin. Low amounts of a group of sterols called phytosterols can be found in plants. Phytosterols, which may contain up to 30 carbon atoms in their structure, have various effects such as anticholesterol, anticancer, immunomodulator, skin protector, antioxidant, hypocholesterolemic, and anti-inflammatory (Faith Oyelere et al., 2022).

Polysaccharides are classified as heteropolysaccharides and consist of glucose, galactose, arabinose, mannose, and rhamnose but their polysaccharide content can be affected by different conditions. By

preventing the nuclear factor kappa B (NF-κB) signaling pathway, polysaccharides, one of the key bioactive components of MC fruit, are known to reduce oxidative stress, inflammation, apoptosis, and hyperlipidemia during myocardial infarction. They also have several other bioactivities, including antioxidant, immune-boosting, antidiabetic, neuroprotective, antitumor, and antimicrobial properties. Additionally, investigations have demonstrated that it boosts the synthesis of volatile fatty acids, modifies the rumen fermentation pathway, and impacts the population of cellulolytic bacteria (Jia et al., 2017).

Proteins and peptides the primarily functional compounds, are found in the fruits and seeds of MC. Many protein and peptide species such as lectin, anti-HIV protein, and α- β- γ- δ- ε-momorcharin have been extracted from different parts of MC and have a cancer-fighting, immune-suppressing, and antimicrobial effect. It is also reported to have properties such as RNA N-glycosidase activity, DNAase-like activity, polyalkylene glycol (PAG) activity, superoxide dismutase activity and phospholipase activity. Plant cells release a specific kind of hypoglycemic peptide that binds to carbohydrates called polypeptide-p, which is crucial for cell adhesion and recognition processes (Jia et al., 2017).

**Wound Healing**

The skin is the most elastic organ surrounding the body of all vertebrates and occupies the most significant space. Derived from the Latin word "cutis," the skin is referred to in the literature as "cutaneous." Histologically, three layers comprise the skin: the epidermis, dermis, and subcutis. The epidermis is the skin's topmost layer and consists of the stratum corneum, stratum basale, stratum spinosum, stratum granulosum, and stratum lucidum, in that order (Yumuşak, 2012). The epidermis acts as a waterproof barrier. Keratinocytes are the most abundant cells in the epidermis (about 90%). In addition, there are melanocytes, Langerhans cells, and Merkel cells (Nourian Dehkordi, Mirahmadi Babaheydari, Chehelgerdi, & Raeisi Dehkordi, 2019). The dermis layer is under the epidermis layer and provides elasticity to the skin. This layer, which contains blood and hair follicles, sweat glands, sebaceous glands, lymphatic vessels, and sensory nerves, is also responsible for supplying cells in the epidermal layer by diffusion through capillaries. Two layers, the stratum papillare and the stratum reticulare, comprise the dermis. The stratum papillare is a layer of connective tissue rich in fibrocytes and fibroblasts and contains sweat glands, sebaceous glands, and hair follicles. The stratum reticulare is the layer where the sweat glands and hair follicles are interwoven with the stratum papillare. The subcutis, which lies beneath the dermis, is the lowest layer. Subcutis is the subcutaneous connective tissue and contains fat cells, fibrocytes, fibroblasts, and dense collagen bundles. In this layer, which includes dense vascular and nervous structures, connective tissue cells are arranged parallel (Yumuşak, 2012).

The term "wound" refers to the deterioration of the skin's or mucosa's tissue integrity for various causes, such as illnesses or physical damage. Wounds in which the integrity of the skin tissue is not completely disrupted due to crush, sprain or dislocation are called 'closed wounds'. Wounds in which the integrity of the skin is completely disrupted due to cuts, stings, and punctures are called 'open wounds' (Baktır, 2019).

Normalization of cellular, biochemical, and systemic processes defines wound healing and the deterioration caused by trauma with the formation of new tissue. Fibroblast growth factor (FGF), transforming growth factor β (TGF-β), epidermal growth factor (EGF), granulocyte-macrophage colony-stimulating killing factor (GM-CSF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), tumor necrosis factor-alpha (TNF- α) and interleukins (IL-1 and IL-2), are a few examples of cytokines and growth factors that support the healing of wounds (Baktır, 2019).

Neutrophil granulocytes are the first cells to attack the area during the wound healing, and they play a

role in both antimicrobial protection and the production of free oxygen radicals. The two main variables that slow the healing of wounds are inflammation and oxidative stress. Therefore, local antioxidant activity may be effective in the healing process (Chen, Liou, Tzeng, Lee, & Liu, 2013; Yadav, Singh, Yadav, & Verma, 2018).

During wound healing, excessive collagen or granulation tissue development may result in an atypical scar. TGF-β regulates scar development on its own. The three TGF-β isoforms are responsible for this. TGF-β3, which has been demonstrated to have anti-scar effects, balances out TGF-β2, which is implicated in producing scars. Thus, abnormal scar formation is prevented (Hussan et al., 2014).

**Stages of Wound Healing**

There are three stages of wound healing: Hemostasis and inflammation, proliferation, remodeling and maturation (Baktır, 2019).

**Phase 1: Hemostasis and inflammation**

As a result of the injury, bleeding occurs due to the destruction of vessels and tissues (Çiçek, 2015). Inflammation happens right after an injury and protects against bacterial contamination by cell growth. The coagulation system is initially activated to stop the bleeding (hemostasis) in the wound region (Yazıcıoğlu, 2009). The activation, adhesion, and aggregation process begins with platelets. Platelets bind to selectin and integrin receptors on extracellular matrix proteins. Later, with platelet degranulation, many factors (thromboxanes, prostaglandins, serotonin, fibrinogen, fibronectin, Von Willebrand factor, PDGF, EGF, and TGF-β1) in the granules are released into the environment (Çiçek, 2015). These factors provide both the formation of fibrin clots and the regulation of cellular response. Neutrophils are the first cells to arrive at the site of a wound (Yazıcıoğlu, 2009). Neutrophils stick to the endothelium once adhesive components close to the wounded tissue on the vascular endothelial surface are activated. Then, neutrophils reach the tissue space by passing through damaged capillaries or the spaces between endothelial cells. Neutrophils are crucial for tissue debridement and the prevention of infections. Since neutrophils produce various growth factors and proteases, they also show their effects by participating in the healing of wounds. Circulating monocytes quickly transform into adult macrophages as they enter the tissue area (Baltzis, Eleftheriadou, & Veves, 2014). After 2-3 days, the number of neutrophils decreases and macrophages become the most abundant cell population in the wound. Macrophages are responsible for clearing the impacted area of dead neutrophils, foreign objects, and injured tissue (Yazıcıoğlu, 2009). T-lymphocytes enter the location of the wound during at the late stages of inflammation and have a modulatory effect on tissue remodeling (Baltzis et al., 2014).

**Phase 2: Proliferation**

This stage starts the second day following the injury and lasts up to three weeks. Fibroblasts begin to produce new extracellular matrix and immature type III collagen when they are stimulated by cytokines and growth factors generated by inflammatory cells in the wound area. As collagen accumulates, the wound's resistance to stretching increases rapidly. Epithelial cells derived from the basal layer form a new surface at the edges of the wound. Wound contraction occurs due to the transformation of some of the fibroblasts into myofibroblasts (Baktır, 2019).

**Phase 3: Remodeling and Maturation**

This stage starts in the third week when the amount of fibroblasts in the wound region declines and collagen production approaches equilibrium. Epithelialization is complete. Remodeling stage is the remodeling of collagen fibers. Type III collagen, which is soft and gelatinous, transforms into tighter type I collagen. Some of the contractions also occur at this stage. Thus, the wound regains 80-95% of its initial strength at the end of 6 weeks. This phase, in which scar tissue is formed, lasts between 6 and 24 months (Baktır, 2019).

Studies have revealed that the typical wound

healing phases are not entirely followed by diabetic wounds; diabetes causes ulcers and prolongs the healing process (Liu, Liu, Deng, Li, & Nie, 2021).

**Diabetic Wound**

DM, an endocrine system disease, occurs due to the absence of insulin or resistance to the effect of insulin. DM characterized by carbohydrate, protein, and fat metabolism disorders, is frequently observed in cats and dogs. DM can be asymptomatic, but usually clinical signs of DM, such as polyphagia, weight loss, polyuria, polydipsia, and weakness, are typically present. Due to metabolic abnormalities, the disease has a wide range of organ and systemic effects (Şimşek & İçen, 2008).

According to the latest classification system, there are four types of diabetes (Şahinduran & Vurkaç, 2018).

Hyperglycemia and the death of pancreatic beta cells are characteristics of type I diabetes (Wang, Bai, Wu, Li, Xie, Ji, & Gao, 2023). In this type of diabetes, the cells are destroyed by antibodies against ß-cells in the pancreatic islets of Langerhans. Type I diabetes is most common in dogs. In mild cases, the number and size of ß-cells are reduced. In severe cases, the damage is so severe that the cells are unrecognizable. It was observed that antibodies against ß-cells developed in half of the dogs with diabetes, and autoimmune damage occurred (Şahinduran & Vurkaç, 2018).

While the pancreas is macroscopically normal in type II diabetes, microscopic degenerative lesions are observed ß-cells in the pancreatic islets of Langerhans. II diabetes is most common in cats. Amyloid deposition in the islets of Langerhans with microscopic changes is observed in most cats with diabetes and is considered a characteristic finding for type II diabetes. Insulin resistance develops in target tissues with pancreatic secretion of abnormal insulin. Although insulin secretion is very high in the early stages of the disease, blood sugar is among the reference values. The failure to secrete insulin to maintain a healthy blood glucose level results in hyperglycemia in the later phases. As a result of hyperglycemia, loss of function in ß-cells occurs and an adequate amount of inability to secrete insulin. Thus, insulin deficiency and an increase in insulin release can be observed in this type of diabetes (Şahinduran & Vurkaç, 2018).

Type III gestational diabetes is typically seen in middle-aged dogs and during the second half of pregnancy. If diabetes continues to be observed after pregnancy or diestrus has ended, another disease other than gestational diabetes should be suspected and diagnosed. Gestational diabetes in dogs is rare, and a study has shown that diabetes cases are higher in pregnancies in winter (Şahinduran & Vurkaç, 2018).

Type IV secondary diabetes mellitus is a form of secondary diabetes that arises from other conditions. Endocrine abnormalities such as hyperadrenocorticism and progesterone-acting growth hormone problems are the most common causes of type IV diabetes in dogs. In addition, type IV diabetes is encountered in approximately 15% of acute pancreatitis cases (Şahinduran & Vurkaç, 2018).

The wound healing process is more extended in diabetic patients as many factors are negatively affected compared to routine wound healing. In routine wound healing, TNF-α level reaches its maximum level by macrophages 12-24 hours after wound formation. After the completion of the proliferative stage, TNF-α decreases to the basal level. Due to the increased TNF-α level in diabetic wounds, fibroblast proliferation, angiogenesis, cell proliferation, and cell migration are impaired. As the cytokines TNF-α and IL-6 increase, the amount of IL-10 decreases, which leads to a prolongation of the leukocyte infiltration time (Ekizce, 2019). There is a decrease in resistance to microorganisms in diabetic animals. Diseases such as pus cystitis, prostatitis, bronchopneumonia, and dermatitis can be observed chronically and recurrently due to dysfunctions in leukocytes. Hepatomegaly may develop due to fatty liver. Chronic kidney diseases, gangrene, and blindness may develop due to microangiopathy (Özmen & Topsakal, 2019).

In diabetic wounds, blood vessels are damaged, and their angiogenic capacity decreases, resulting in inadequate nutrition and low oxygenation in wounds. Intense use of oxygen by cells during inflammation causes hypoxia. Due to hypoxia, the concentrations of free oxygen radicals increase further, delaying the wound healing time. The first affected cells in DM are neurons (Dong, Wu, & Tian, 2023). Due to hyperglycemia, loss of function occurs in the schwann cells and myelin sheaths in the nervous system, resulting in neuropathy in the form of numbness, pain or atrophy. The loss of function observed in the sebaceous and sweat glands due to autonomic neuropathy causes the development of anhidrosis, drying, peeling, and hyperkeratosis on the plantar surface of the foot as a result of the deterioration of thermoregulation in the foot. Deformities in the foot and restricted joint movements result in excessive pressure on the plantar surface of the foot. If such long-term forces are applied continuously to a particular area, ulceration develops due to focal tissue ischemia, local inflammatory response, and tissue destruction. Foot ulcers, which are a severe diabetic complication, can grow at a rate of 15% throughout the life of diabetic patients and are essential because they may require amputation in 7-20% of cases (Ekim & Ekim, 2016).

Continuous hyperglycemia in diabetic patients affects wound-healing processes in various ways. Hypercoagulation and decreased skin functions are observed in the hemostasis phase. While some growth factors and irregularities in inflammatory factors in the inflammatory phase cause chronic inflammatory reactions, a predisposition to diabetic scar formation occurs due to the decrease in neutrophil concentration. Decreased migration and proliferation of keratinocytes due to prolonged hyperglycemia causes insufficient wound re-epithelialization. One of the reasons for poor wound healing in diabetic wounds is the different expression of the extracellular matrix formed by fibroblasts (Liu et al., 2021).

Problems in angiogenesis, immunity, and risk of infection make diabetic wound care difficult (Hussan et al., 2014). In addition, any wound in the lower extremities can become ulcers as they become susceptible to infections (Liu et al., 2021). Wounds usually require debridement, in some cases amputation may be performed. Various studies are being conducted to improve wound healing in diabetics, and the usage of herbal medications in this direction is growing. MC fruit extract is also known for its wound healing and antidiabetic properties (Hussan et al., 2014).

**Topical Applications in Wound Healing**

Vascular endothelial growth factor (VEGF), TGF-β, PDGF, IGF, and cytokines regulate the wound healing. In response to injury, platelets, fibroblasts, and macrophages produce TGF- β. This factor, which participates in every step of wound healing, speeds up the healing process by enhancing monosaccharide conversion to macrophages and promoting fibroblast contraction. TGF- β affects the chemotaxis of granulation tissue, activates fibroblasts and stimulates collagen, fibronectin synthesis, and extracellular matrix accumulation. A crucial component of the extracellular matrix is collagen. Collagen helps wound healing by binding to fibronectin (creates the environment for fibroblast proliferation and increases the elasticity of granulation tissue) and platelets. Excessive collagen or granulation tissue production, on the other hand, might result in aberrant scar formation. TGF- β regulates not normal scar formation by producing tissue inhibitors of metalloprotease and promoting matrix metalloproteinase production. Because of the three TGF- β isoforms' activity. TGF- β may also speed up collagen and total protein depositing, which could explain why MC-treated animals heal faster (Hussan et al., 2014).

Fibroblasts produce an extracellular matrix (ECM) at the time wound healing that meets the edges of the wounds. Wound contraction, re-epithelialization, and angiogenesis are required for complete wound closure. Angiogenesis ensures that nutrients reach the granulation tissue constituents (Pişkin et al., 2014). Protein content of injured tissue represents

protein levels and cellular proliferation (Teoh et al., 2009). Any protein can affect the intracellular mechanism of tropocollagen synthesis or the extracellular deposition mechanism and organization of collagen fibers, thereby altering the repair process (Prashanthi, Mohan, & Siva, 2012).

Wound contraction happens due to a centripetal move of the wound margins to close a full-thickness wound. The increased pace of wound contraction due to the activation of IL-8, α-chemokine, and various growth factors, or the inhibition of proinflammatory indicators such as IL-1β and TNF-α, that influencing the activity and recruiting of different inflammatory cells. Increased quantities of hydroxyproline, the primary component of the collagen protein, are observed in the granulation tissue during the wound healing process. Proline and hydroxyproline are crucial for the integrity of collagen. The primary element of extracellular tissue, which provides strength and support, is collagen. Collagen deterioration results in the release of hydroxyproline and related peptides. It is possible to use the measurement of hydroxyproline as a collagen turnover index. Hexosamine is an essential substrate for collagen synthesis. At the beginning of wound healing, it is known to rise, then fall. Similar to previous results, a rise in uronic acid concentrations in animals suggests enhanced glycosaminoglycan production. The maximum force a material can withstand per unit of area is known as its 'tensile strength'. The higher collagen concentration and fiber stability could both be contribute factors to the rise in tensile strength. The collagen components that were produced at the site of the wound area cross-linked to create fibers. Tensile strength results from collagen remodeling and the formation of enduring intra- and intermolecular cross-links (Prashanthi et al., 2012).

Free radicals can be defined as unstable, reactive molecules. Free radicals occur as a result of biochemical reactions in the metabolism of cells, produced by endogenous and exogenous sources. When the production of free radicals increases, and antioxidants are insufficient or deactivated, 'oxidative stress' occurs. One of the important lipid peroxidation markers is malondialdehyde (MDA) and is an indicator of oxidative damage in tissues. MDA is formed by the breakdown of unsaturated fatty acids containing three or more double bonds. Superoxide dismutase (SOD) is the most effective antioxidant and is known to be found in all cells that use oxygen. Glutathione reductase (the antioxidant defense system enzyme) catalyzes the reduction of oxidized glutathione to decreased glutathione in its presence of Nicotinamide adenine dinucleotide phosphate (NADPH). With the decrease of glutathione reductase activity, the protection of cells from oxidative damage decreases (Ekizce, 2019).

The MC antioxidant activity has been linked to a faster rate of wound healing. Low reactive oxygen species and oxidative stress are necessary for wound healing physiology. Thus, excessive exposure to oxidative stress results in inadequate wound healing (Sagástegui-Guarniz et al., 2021).

Pişkin et al. (2014) conducted a study has shown that on 28 New Zealand rabbits (in the group in which MC was applied twice a day) compared to the other groups (dexpanthenol, nitrofurazone applied and control group), the rate of macroscopic wound healing and re-epithelialization rate was higher at the end of the 28th day. Under a microscope, the average ratio of epidermis to papillary dermis, the average number of fibroblasts, and the fraction of fibroblasts and collagen fibers in the reticular dermis were higher than the other groups. The MC group also had a thicker epidermis, more collagen bundles, capillaries, and macrophages than the other groups. They demonstrated that the MC oil extract increased epithelialization, neovascularization and fibroblast proliferation and accelerated wound healing by suppressing inflammation. In addition, the normal epithelium of the rabbit skin after treatment indicates that MC extract protects the skin against oxidative damage.

Teoh et al. (2009), observed that MC application shorter wound healing time in the group in which they induced DM, while MC application didn't make

a significant difference in the group in which they didn't induce DM. On the 5th day, they observed a rise in the quantity of infiltrating cells and a well-formed epidermis layer in the MC-applied group compared to the other groups. They showed that the protein content was higher in the group with DM and MC extract was used compared to the different groups. High protein concentration, MC extract might encourage cellular migration and proliferation. Although there was no visible difference in wound healing, the presence of more granulation tissue in the non-DM MC extract group than in the control group raises the possibility that it may aid the healing process. The study showed that MC extract was beneficial in the diabetic wound healing process.

Prasad et al. (2006) showed that in the MC powder ointment group supported wound healing activity by inducing epithelialization, fibrosis, and angiogenesis. In addition, they found a higher level of hydroxyproline in the group using MC powder ointment than in the other groups.

Hussan et al. (2014) showed that in diabetic groups compared to the control group, incomplete epithelialization and epidermis and dermis layers did not intertwine. However, it was observed that these abnormalities were less in the MC ointment-applied group than in the other diabetic groups. In addition, the epidermis in the MC ointment applied group exhibited full-thickness epithelialization with regular collagen fibers, like the control group. On day 5, diabetic groups had a slower rate of wound closure than the control group. The group that received no treatment had the lowest closure rate. Compared to the other diabetes groups, the group receiving MC ointment on day 10 had the highest wound closure rate. They claimed increased TGF-β expression was related to a faster wound closure rate. The diabetic group who had not received treatment had low TGF-β expression and slow wound healing. Since excessive scar formation was not observed in the MC ointment group, all isoforms of TGF-β might play a role in maintaining its function. The fact that MC-treated animals had higher total protein levels shows that MC can increase the number of cells in scar tissues through migration or proliferation. Through increased TGF-β expression, topical administration of MC fruit extract sped up wound healing in animals with DM. The MC ointment-treated group has a higher wound closure rate, TGF-β expression, and better wound histological characteristics, compared to the MC powder-treated group.

Satar et al. (2013), in their research on New Zealand rabbits, showed that the oily extraction form of MC caused a decrease in the epithelialization time in the wound area. During their study, they performed complete blood counts in rabbits and did not detect any pathology. They argued that this data indicates that topical application of MC does not cause any systemic abnormalities. Animals treated with the oily extraction form of MC had a significant reduction in wound area and healing time compared to the other groups (MC powder, extra virgin olive oil, and control groups).

Prashanthi et al. (2012), in their study on albino rats, showed that wound contraction was faster in groups where MC extract was applied compared to the control group, thus inducing early healing. It was revealed in their study that the MC outer layer extract had higher hydroxyproline and protein content in the granulation tissue compared to other groups (seed extract and the control group). Hexosamine and uronic acid concentration was higher than the control group in the granulation tissue. Skin tissues healed in the MC-treated groups had considerably more tensile strength than those in the control group.

Ekizce (2019), according to his study in rats, found the most severe inflammation findings and changes characterized by fibroblastic activity and neovascularization related to delayed healing in the diabetic group without MC. Significant wound healing was observed compared to the group that did not receive MC. While there was no significantly differ from the wound group and the groups in which MC was ap-

plied to the wound in terms of MDA levels, a significant increase was detected in the diabetes group. It was determined that the MDA value, which increased in the group treated with MC by creating diabetes, decreased significantly at the end of the treatment compared to the diabetic group. Still, the MDA value was higher than in the non-diabetic groups. There was no discernible difference between the groups that had MC treatment and the wound group in terms of SOD, activities. There was a finding that SOD activity decreased considerably in the diabetic group and an increase was observed in the diabetic group in which potency pomegranate was applied, compared to the diabetic group. Still, the SOD value was discovered to be less than the non-diabetic groups. Regarding glutathione reductase value, when the group treated with MC and the group with diabetes were compared, it was determined that the glutathione reductase value increased considerably in the group treated with MC. It has been shown that there is no effect on blood glucose levels in external application on rats with diabetes and wounds on the skin.

Sagástegui-Guarniz et al. (2021), in their study, demonstrated that MC gel and cream shortened the time of skin wound healing in mice and 1% cream formulation was the most effective. Phytocomponents like steroids, flavonoids, and cucurbit triterpenes, which exhibit antimicrobial and antioxidant activities, have reportedly been linked to the healing process of MC. It has been suggested that the ingredients in MC acetonic extract are more effective than those in the cream formulation. Because of the formulation of these chemicals being lipid-soluble. Aglycone flavonoids are often extracted using less polar solvents like acetone. Acetone is thought to be the optimum solvent for extracting flavonoids from MC.

## CONCLUSION

There are no standards in preparing MC fruit extraction, and studies on short-term diabetes are limiting. Although studies show that MC plant has a wound healing effect, more studies are needed on its wound healing mechanism. The MC plant can be used in veterinary medicine for wound healing in skin wounds and diabetic wounds due to its availability, ease of extraction, and lack of any side effects in topical application. Investigations should be conducted into how the active phytochemicals in MC affect the healing of wounds. More research is needed on the mechanism of action of growth factors and cytokines on wound healing. More studies should be on the effects on wound healing among powder, gel and cream formulations.

## CONFLICTS OF INTEREST

The authors declare that there is no conflicts of interest.

## AUTHOR CONTRIBUTION STATEMENT

The authors SA and MP contributed equally

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*REVIEW ARTICLE*

# Molecularly Targeted Therapies in Breast Cancer: A Traditional Review

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***Molecularly Targeted Therapies in Breast Cancer: A Traditional Review***

***SUMMARY***

*Globally, breast cancer stands as the most frequently diagnosed cancer among women, with its incidence continually on the rise. Conventional treatment methods for breast cancer have inherent limitations. Consequently, the development of innovative treatment approaches is imperative. Among these, targeted therapy strategies have emerged as a critical point in contemporary clinical research, significantly diversifying the treatment landscape for breast cancer in recent years. The goals of these new treatment strategies are evolving toward a future that can overcome the limitations of traditional treatment methods, protect patients from side effects, and improve patients' survival rates and life quality. Nowadays, therapeutic agents that bind to preferred targets are used based specifically on various subtypes of breast cancer. The FDA-approved targeted therapeutic agents—such as monoclonal antibodies, gene therapies, immunotherapeutic cancer vaccines, and small molecule inhibitors, are reviewed in this article.*

***Key Words:*** *Molecular targeted therapies, breast cancer, small molecule drugs, monoclonal antibodies, immunotherapeutic cancer vaccines, gene therapy.*

***Meme Kanserinde Moleküler Hedefe Yönelik Tedaviler: Geleneksel Bir Derleme***

***ÖZ***

*Küresel olarak, meme kanseri, kadınlar arasında insidansı sürekli artan ve en sık teşhis edilen kanserlerden biridir. Meme kanserinin konvansiyonel tedavi yöntemlerinin doğal sınırlamaları vardır. Sonuç olarak, yenilikçi tedavi yaklaşımlarının geliştirilmesi zorunludur. Bunlar arasında, hedefe yönelik tedavi stratejileri, çağdaş klinik araştırmalarda kilit bir nokta olarak ortaya çıkmış ve son yıllarda meme kanseri için tedavi ortamını büyük ölçüde çeşitlendirmiştir. Bu yeni tedavi stratejilerinin hedefleri, geleneksel tedavi yöntemlerinin sınırlamalarının üstesinden gelebilen, hastaları yan etkilerden koruyabilen ve hastaların hayatta kalma oranlarını ve yaşam kalitesini iyileştirebilen bir geleceğe doğru evrilmektedir. Günümüzde, özellikle meme kanserinin çeşitli alt tiplerine göre tercih edilen hedeflere bağlanan terapötik ajanlar kullanılmaktadır. Bu makalede monoklonal antikorlar, gen tedavileri, immünoterapötik kanser aşıları ve küçük molekül inhibitörleri gibi FDA onaylı hedefe yönelik terapötik ajanlar gözden geçirilmiştir.*

***Anahtar Kelimeler:*** *Moleküler hedefe yönelik tedaviler, meme kanseri, küçük moleküllü ilaçlar, monoklonal antikorlar, immünoterapötik kanser aşıları, gen tedavisi.*

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## INTRODUCTION

Lowering the likelihood of disease and preventing fatalities is the primary goal of all cancer research and innovations (Akram, Iqbal, Daniyal, & Khan, 2017; Engin, 2005). Following a cancer diagnosis, the primary treatment modalities often involve surgery, immunotherapy, radiation, endocrine therapy, traditional cytotoxic chemotherapy, or a combination of them (Kimiz-Gebologlu, Gulce-Iz, & Biray-Avci, 2018; Waarts, Stonestrom, Park, & Levine, 2022). These traditional treatments are subject to challenges such as multi-drug resistance, a lack of tumor cell selectivity in comparison to normal cells, insufficient drug concentrations in tumors, and systemic toxicity (Seledtsov, Goncharov, & Seledtsova, 2015; Xu & Mcleod, 2001). These challenges make the design of innovative and potent treatments for cancer necessary.

Over the past two decades, significant initiatives such as the Cancer Genome Atlas (NCI/NIH) have combined next-generation sequencing with cutting-edge computational data analysis methods to develop molecularly targeted pharmacological therapeutics (Bedard, Hyman, Davids, & Siu, 2020; M. F. Berger & Mardis, 2018; Jacobs, Martinez Castaneda-Cruz, Rose, & Connelly, 2022). These therapeutics can complement or even eventually replace broad-spectrum cytotoxic drugs (Inal et al., 2013). For instance, the term "magic bullet" was first used for these therapeutics by Paul Rich in 1906 to emphasize their capacity to target microorganisms selectively and promote the targeted release of therapeutics at the site of the disease, while minimizing off-target side effects in healthy tissues (Adams & Weiner, 2005).

The U.S. Food and Drug Administration (FDA) has authorized various molecularly targeted medicines that have demonstrated great clinical effectiveness in treating a variety of malignancies including breast, colorectal, lung, ovarian cancers and leukemia as well as other non-cancer illnesses (Akçay, 2002; Brodsky, 1988; Gerber, 2008; Gray et al., 2020; Lee, Tan, & Oon, 2018). Numerous studies have demonstrated that hybrid treatment of cancer using a variety of medications (conventional + targeted) produces better synergistic anti-tumor benefits than single-drug therapy (Fisusi & Akala, 2019; Kay et al., 2021).

The characteristics and effects of molecularly targeted therapeutic medicines used to treat cancer may vary. Depending on the tumor type, cell surface antigens, telomeres, or telomerase as well as substances in the tumor microenvironment can be targeted to activate the immune system. These medicines can control the cell cycle progression, apoptosis, metastasis, and angiogenesis. They also act on growth factors, receptors, or signal transmission pathways. More specifically, they can limit metastasis, assist cell cycle control, promote apoptosis or autophagy, and disrupt signals that aid in the proliferation of cancer cells. As such, they can deliver toxic substances to cancer cells' particular molecules in a targeted manner to destroy them (Amer, 2014; Gerber, 2008; Lee et al., 2018; Padma, 2015; Saijo, 2010).

### Breast cancer

Breast cancer, the most common type of cancer in women, is a heterogeneous neoplasm with many hereditary and clinical subtypes (Cadirci & Sengül, 2023). The classification of clinical subtypes has evolved. Classifications include treatment decisions and prognosis, tumor size, lymph node involvement, histological grading, age of the patient, ki67 (a marker of proliferation index) proliferation index, estrogen receptors (ER) expression, and progesterone receptors (PR) expression. Traditional classification systems for biological and immunohistochemical (IHC) markers such as human epidermal growth factor-2 (Her-2 or c-erbB2) expression are carried out (Gao & Swain, 2018; Gholikhani et al., 2022; Yersal & Barutca, 2014). Immunohistochemical classification of breast cancer is the most commonly used classification system. This classification system includes the expression of hormone receptors (HR) ER and PR and Her-2 (Erasmo

Orrantia-Borunda, Patricia Anchondo-Nuñez, Lucero Evelia Acuña-Aguilar, Francisco Octavio Gómez-Valles, & Claudia Adriana Ramírez-Valdespino, 2022). Positive HR indicates the presence of estrogen or progesterone receptors, which can stimulate the development of tumor cells. ER-positive tumors express ER and PR, ER-responsive genes, and other genes that encode ER and PR, which are typical proteins of lumen epithelial cells, so they are called lumen groups. It is defined in two subtypes Lumen-a and Lumen-b (Yersal & Barutca, 2014). This classification is divided into four subgroups according to NCI SEER data: HR+/Her-2-, HR-/Her-2-, HR+/Her-2+, and HR-/Her-2+ (Kaplan et al., 2015; National Cancer Institute, 2020). Table 1 lists the molecular subtypes of breast cancer.

**Table 1.** Molecular subtypes of breast cancer.

| **Molecular Subtype** | **Hormone Receptor Status** | **Characteristic** |
|---|---|---|
| Luminal A | ER and, or PR Positive<br>Her-2 Positive | 30-45% of the patients<br>Low proliferation (Ki67<14)<br>Better prognosis<br>Longer survival rates |
| Luminal B | ER and, or PR Positive<br>Her-2 Positive or Negative | 10-20% of the patients<br>High proliferation rate (Ki67>14)<br>Grade II, III, or IV<br>Poorer prognosis |
| Her-2-enriched | HR and, or PR Negative<br>Her-2 Positive | 15-20% of the patients<br>High proliferation (Ki67>14)<br>Poorer prognosis |
| Basal-like/Triple Negative | ER, PR, and Her-2 Negative | 15-20% of the patients<br>High proliferation rate (Ki67>14)<br>Poorer prognosis |

**Hormone Receptors**

Approximately 75% of breast cancers are positive for ER and, or PR. HR is divided into two subtypes: lumen-a and lumen-b. Lumen-a is the most common subtype and represents 50-60% of all breast cancers. These tumors often have low histological grade, low nuclear pleomorphism, low mitotic activity, and a good prognosis. Lumen-α is characterized by higher levels of ER and lower levels of proliferation-related genes. Lumen-a serotype is defined by immunohistochemistry as ER-positive and, or PR-positive tumors with low Her-2 and low Ki67 (proliferative cell nuclear antigen) index. Lumen-b tumors constitute 15-20% of breast cancers and have a more aggressive phenotype, higher histological grade, proliferative index, and worse prognosis. Lumen-b subtype is defined as ER-positive, Her-2 negative, and Ki67 high or ER and Her-2 positive tumors or ER-negative PR-negative, Her-2 negative (Yersal & Barutca, 2014).

Endocrine therapy is the first step in HR-positive breast cancers (Corti et al., 2023). Tamoxifen, an anti-estrogen treatment for ER-positive breast cancers, was approved by the FDA in 1970 and is considered the first targeted therapy in the history of cancer (Debien, de Azambuja, & Piccart-Gebhart, 2023). There are approaches such as suppression of ovarian function, inhibition of aromatase enzyme, and inhibition of selective estrogen receptors in the treatment of ER-positive breast cancers (Debien et al., 2023). At the same time, small molecule inhibitors such as CDK4/CDK6 and PI3k/AKT/mTOR pathway inhibitors approved by the FDA are listed in Table 2.

**Her-2**

An over-expression of Her-2 is observed in 20% of all breast tumors. Her-2 is an oncogene that regulates angiogenesis, invasion, proliferation, survival, differentiation, and metastasis, and is the main target in targeted therapeutic methods (Gutierrez & Rachel Schiff, 2011; Kunte, Abraham, & Montero, 2020; Moasser, 2007; Tarantino, Morganti, & Curigliano, 2021). Tyrosine kinase receptors, or type I transmembrane growth factors, comprise the Her family of proteins. When these receptors receive signals from outside the cell, intracellular signaling pathways are activated. The extracellular ligand-binding layer, the transmembrane layer, and the intracellular tyrosine kinase layer are the three levels of the complex biological network that comprise Her-2-Neu receptors.

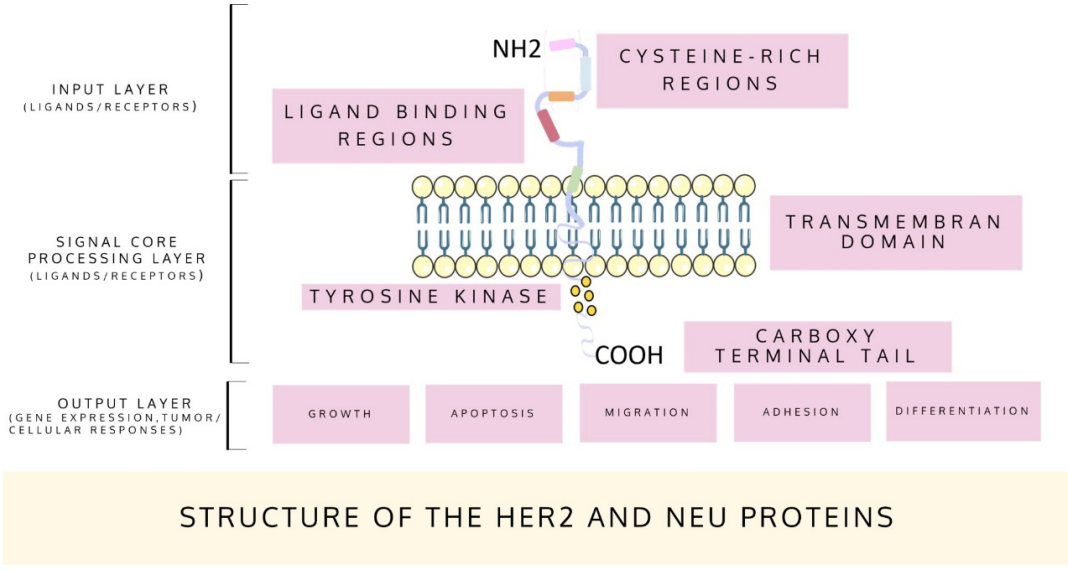


**Figure 1.** Structure of the Her-2 and Neu proteins.

As more specifically shown in (Figure 1.), the first layer of the Her-2 protein receptors is the extracellular ligand binding layer in contrast the second layer is the core processing layer of the kinases that transmit the signals to the nucleus. The third layer is the intracellular outlet layer, which is the tyrosine kinase field of transcription factors that control genes that affect various cellular functions (Gutierrez & Rachel Schiff, 2011; Moasser, 2007; Swain, Shastry, & Hamilton, 2023). Homodimerization or heterodimerization of receptors which is a result of ligand binding to Her proteins activates the signaling pathways that prevent apoptosis, cell growth, and division (Swain et al., 2023). The invention of medications against Her-2, also known as ERBB2, is an essential advancement for targeted therapy in breast cancer treatment. For instance, as shown in (Figure 2.), four alternative therapies have been established since the formulation of the drug Trastuzumab: 1) Her-2 antibody-drug conjugates, 2) anti-Her-2 monoclonal antibodies, 3) tyrosine kinase inhibitors for Her2+ subtypes, and 4) immunotherapy and therapeutic cancer vaccines (Loibl & Gianni, 2017). When we checked most monoclonal antibodies and other immunotherapies, most approvals for breast cancer were for Her-2 positive subtypes. The response for monoclonal antibody treatment is higher in Her-2 positive types is higher than in other types.

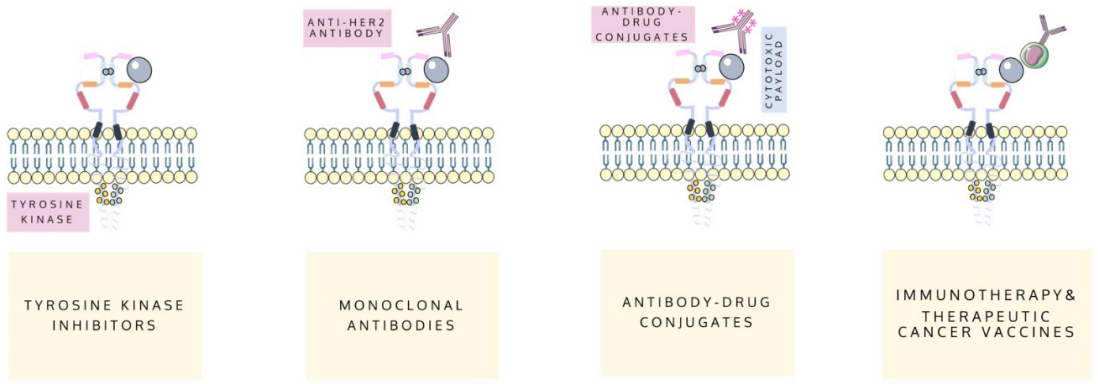


**Figure 2.** Basic mechanisms of dimerization of Her-2 receptor family in breast cancer

**Types of Molecularly Targeted Therapy**

Molecularly targeted therapeutic agents used in cancer treatment may exhibit different functions and properties. Depending on the targets, it acts on cell surface antigens, apoptosis, telomeres or telomerase, growth factors, receptors, or signal transduction pathways that regulate cell cycle progression, cell death, metastasis, and angiogenesis (Saijo, 2010). Agents used in molecular targeted therapy are classified as small molecules, monoclonal antibodies, immune therapeutic cancer vaccines, and gene therapy (National Cancer Institute, 2017; Padma, 2015). Drugs used in molecular targeted therapy can target cancer cells and components of the tumor microenvironment to activate the immune system, as well as block signals that promote cancer cell growth, interfere with cell cycle regulation, and, or induce cell death to kill cancer cells. (Amer, 2014; Padma, 2015). These are further explored in the subsequent subsections.

**Small molecule inhibitors**

Small molecule inhibitors are substances with a relatively low molecular weight (900 <Da) which enables them to enter the cells and target a particular protein (Joo, Visintin, & Mor, 2013). The majority of monoclonal antibodies are designed to attack targets outside of cells or on the cell surface because they cannot cross the plasma membrane of cells (Joo et al., 2013). Owing to their smaller size relative to monoclonal antibodies, small molecule inhibitors can bind to a broader array of extracellular and intracellular targets. Moreover, specific small-molecule inhibitors can diminish tumor-related brain lesions by penetrating the blood-brain barrier (Berger & Mardis, 2018; Gharwan & Groninger, 2016; Martínez-Reyes & Chandel, 2021; Shi et al., 2022). Table 2 lists the FDA-approved small molecule inhibitors that are utilized in the treatment of breast cancer. Tyrosine kinase inhibitor is one of the small chemical inhibitors that targets the intracellular catalytic kinase domain of Her-2 and inhibits phosphorylation while also triggering the downstream signaling cascades (Swain et al., 2023). Small molecule inhibitors work by targeting and neutralizing key enzymes like cyclin-dependent kinases (CDKs) and poly ADP-ribose polymerase (PARP) inhibitors. These agents disrupt signaling pathways involved in carcinogenesis, activate cell cycle checkpoints, induce apoptosis, and inhibit the activity of target proteins by binding to specific "pockets" on cell membranes (Berger & Mardis, 2018; Gharwan & Groninger, 2016; Joo et al., 2013; Lee et al., 2018; Martínez-Reyes & Chandel, 2021; Shi et al., 2022). For example, Lapatinib and Neratinib are dual-targeted inhibitors that block the actions of both EGFR and Her-2 (Paul, Trovato, & Thompson, 2008).

**Table 2.** List of FDA-approved therapeutic small molecule drugs used in clinic.

| SMALL MOLECULE DRUGS | TARGET | CANCER TYPE | YEAR APPROVED | REFERENCES |
|---|---|---|---|---|
| Abemaciclib (Ly2835219) Verzenio | CDK4, CDK6 | Combination Therapy With An Aromatase İnhibitor Or With Fulvestrant Or As A Monotherapy For Breast Cancers | 2017 | (Fassl, Geng, & Sicinski, 2022; Pandey et al., 2019; Roskoski, 2021, 2023; Royce et al., 2022) |
| Palbociclib (Pd-0332991) Ibrance | CDK4, CDK6 | Estrogen Receptor- And Her-2 Positive Breast Cancers | 2019 | (Fassl et al., 2022; Gharwan & Groninger, 2016; Pandey et al., 2019; Rocca et al., 2017; Roskoski, 2021, 2023; Wedam, Fashoyin-aje, et al., 2020) |
| Ribociclib (Lee011) Kisqali | CDK4, CDK6 | Combination Therapy With An Aromatase İnhibitor For Breast Cancers | 2017 | (Fassl et al., 2022; Pandey et al., 2019; Rascon et al., 2018; Roskoski, 2021, 2023) |
| Lapatinib (Gw572016) Tykerb | EGFR, ERBB2, Her-2 | Her-2 Positive Breast Cancer | 2007 | (Gharwan & Groninger, 2016; Mcarthur, 2009; Paul et al., 2008; Roskoski, 2021, 2023) |
| Tucatinib (Ont-380) Tukysa | ERB2, Her-2 | Combination Second-Line Treatment For Her-2 Positive Breast Cancers | 2020 | (Roskoski, 2021, 2023; Sirhan, Thyagarajan, & Sahu, 2022) |
| Neratinib (Hkı-272) Nerlynx | ERB2, Her-2 | Her-2 Positive Breast Cancer | 2017 | (Roskoski, 2021, 2023) |
| Everolimus (Rad001) Afinitör | FKBP12, mTOR | Her-2 Negative Breast Cancer | 2009 | (Finn, Linnartz, Chen, & Slamon, 2015; Houghton, 2010; Roskoski, 2021, 2023) |
| Alpelisib | PI3K-α | | 2019 | (Narayan, Prowell, et al., 2021) |
| Olaparib | PARP's | | 2014 | (Le & Gelmon, 2018) |

**Therapeutic monoclonal antibodies**

The body's protection is provided by the immune system which makes antibodies against foreign substances (Canpolat, Avcı, & Çadırcı, 2023)specific proteins known as antigens. Monoclonal antibodies are specialized antibodies that target a particular antigen and are widely used in clinical settings in cancer immunotherapy. They exhibit many modes of action stemming from their natural features, their binding of cytotoxic T cells, and their delivery of cytotoxic loads. (Canpolat et al., 2023; Goydel & Rader, 2021). In addition to their direct biological effect, antibodies can deliver chemicals such as radioactive isotopes, poisons, and anti-cancer medicines to specific cells. Five monoclonal antibodies are used in clinical settings: rituximab, trastuzumab, gemtuzumab ozogamicin, alemtuzumab, and ibritumomab tiuxetan (Cersosimo, 2003). For instance, the antibody-drug conjugate trastuzumab emtansin combines trastuzumab's Her-2 targeted antitumor properties with the cytotoxic activity of the microtubule-inhibiting drug imptansin (a derivative of maytansin), enabling intracellular drug delivery to cells that overexpress Her-2 while minimizing exposure to the healthy tissue (Loibl & Gianni, 2017).

FDA-approved therapeutic monoclonal antibodies used and monoclonal antibody-drug conjugates used in the treatment of breast cancer are listed in (Table 3).

**Table 3.** List of FDA-approved therapeutic monoclonal antibodies and antibody-drug conjugates used in the clinic.

| THERAPEUTIC MONOCLONAL ANTIBODY | TARGET | CANCER TYPE | YEAR APPROVED | REFERENCES |
|---|---|---|---|---|
| Adotrastuzumab Emtansine (Kadcylavr) | Her-2 | Her-2 Positive Breast Cancer | 2013 | (Wedam, Fashoyin-Aje, et al., 2020) |
| Fam-Trastuzumab Deruxtecan (Daiichi Sankyo) | Her-2 | Her-2 Positive Breast Cancer | 2019 | (Narayan, Osgood, et al., 2021) |
| Sacituzumab Govitecan (Immunomedics) | Trop-2 | Trıble Negative Breast Cancer | 2020 | (Spring et al., 2021) |
| Margetuximab | Her-2 | Her-2 Positive Breast Cancer | 2020 | (Markham, 2021) |
| Pertuzumab | Her-2 | Her-2 Positive Breast Cancer | 2017 | (Amiri-Kordestani et al., 2014) |
| Trastuzumab Emtansin (T-Dm1) | | Her-2 Positive Breast Cancer | 2019 | (Abelman, Medford, Spring, & Bardia, 2022; Bahçeci et al., 2021) |
| Trastuzumab Deruxtecan (Ds-8201) | | Her-2 Positive Breast Cancer | 2022 | (Abelman et al., 2022) |

**Therapeutic cancer vaccines**

Vaccines used in cancer treatment are different from vaccines developed against infections. Vaccines against infections prevent the disease from occurring. Unlike these vaccines, cancer vaccines create an immune defense against the existing disease. cancer vaccines, comprising cancer cells, cell fragments, or specific antigens, are often enhanced with adjuvants to amplify immune system response. These vaccines are crafted to provoke tumor-specific immune reactivity within the body. The most prominent type among therapeutic cancer vaccines is the peptide-based variety, typically containing immunogenic epitopes from either tumor-specific or tumor-associated antigens. Broadly, cancer vaccines are categorized into five types: whole cell-based vaccines, multi-peptide vaccines, DNA/RNA-based vaccines, dendritic cell-based vaccines, and in situ vaccination methods (Corti, Giachetti, Eggermont, Delaloge, & Curigliano, 2022; Zhang, Zhou, Sha, Xie, & Liu, 2022).

Among the treatment strategies, peptide-based, whole protein-based, cell-based, allogeneic cell-based, viral vector-based, and gene-based vaccine studies are carried out, generally targeting Her-2. In addition, peptide-based and gene-based vaccine studies targeting HR are being carried out (Corti et al., 2022). Although different types of breast cancer vaccines have been evaluated in clinical studies, none of them have provided significant benefits and among the vaccines in phase studies, there is not yet a therapeutic cancer vaccine that has received FDA approval (Corti et al., 2022; Zhu & Yu, 2022).

Active vaccination therapy for breast cancer has several theoretical advantages over conventional chemotherapy and anti-HER2 immunotherapy via monoclonal antibodies: better tolerance, lower toxicity, and prolonged immune response with tumor specificity. Additionally, some vaccines may provide immunity against tumors lacking HER2 expression if the vaccine target is derived from non-HER2-related antigens (Zhu & Yu, 2022).

A significant drawback of cancer vaccines is that patients might not respond as expected due to immune suppression caused by chemotherapy. Consequently, cancer patients with advanced tumors often may not be suitable candidates for immunotherapy (Jain, 2021).

**Gene therapy**

Gene therapy is the insertion, repair, or alteration of faulty genes with functional counterparts to therapeutically address the absence or lowered levels of gene expression activity. It was first used to cure hereditary illnesses. Some examples of gene therapy techniques that are employed in the treatment of cancer are gene editing, suicide gene therapy, gene suppression/silence, the use of entrapment oligodeoxynucleotides to target transcription factors, miRNA targeting, and vaccination with DNA. To prevent the side effects of traditional treatments, gene therapy targets solely the tumor cells and the gene seeks to convey the genetic material to target cells via a vector and cure the gene flaw (Lundstrom, 2015; Zhou et al., 2017). This makes developing a safe, reliable, and controlled gene delivery device a crucial stage in the gene therapy process. Through a vector, genetic material is transferred from the source cell to the target cell. Today, both viral and non-viral vectors are used to transfer genes (Lundstrom, 2015; Sun et al., 2019). Viral vectors are frequently utilized since they can infiltrate into the cells. The retrovirus, lentivirus, alphavirus, adenovirus, adeno-associated virus, pox virus, and baculovirus are the most often employed viral vectors (Lundstrom, 2015; Zaimy et al., 2017). In addition to their nature, viral vectors are thought to be harmful due to their toxicity and immunogenicity. Non-viral vectors based on polymers and liposomes have been created as a result of this drawback. These vectors could be safer, convey more genetic information, and elicit a milder immune response (Sun et al., 2019). For the efficient transmission of genetic material to cells, non-viral vectors need chemical, physical, or biological delivery methods. The biological delivery system's primary function is to transfer genetic material to cancer cells and the blood circulation starts this process. The gene therapy agent travels through the blood to the tumor and builds up there. The substance that has gathered around the tumor cells enters the tumor tissue and is taken up by the tumor cells. It then starts working by releasing the genetic material into the cytosol and cell nucleus. The oscillation in the cell nucleus allows DNA replication and the oscillation in the cytosol affects the RNA (Zhou et al., 2017). An example of an FDA-approved gene therapy agent used in breast cancer is doxorubicin, which was approved in 1993 (Barenholz, 2012).

**Targeted Therapies for Triple-Negative Breast Cancer**

Triple-negative breast cancer (TNBC) is a type of breast cancer that is particularly aggressive and has high recurrence rates, typically with a worse prognosis compared to other types of breast cancer, accounting for 20% of all breast cancer cases (Berger et al., 2021; Camorani et al., 2020; Newman, Reis-Filho, Morrow, Carey, & King, 2015; Won & Spruck, 2020)and in the metastatic setting, the 5-year overall survival is 12%. Due to the lack of receptor expression, there has been a paucity of targeted therapeutics available, with chemotherapy being the primary option for systemic treatment in both the neoadjuvant and metastatic setting. More recently, immunotherapy has revolutionized the landscape of cancer treatment, particularly immune checkpoint inhibitor (ICI). TNBC describes a group of mammary tumors that lack ER and PR receptors and express a heterogeneous group of tumors with low expression of Her-2 receptors (Camorani et al., 2020; James, Quinn, Mullan, Johnston, & Harkin, 2007; Newman et al., 2015; Won & Spruck, 2020)progesterone receptor, and HER2. These tumors account for 12–17 % of all breast cancers, preferentially affect young women, are more frequent in women of African and Hispanic descent, and are enriched in the population of patients diagnosed with "interval cancers." TNBCs account for the majority of breast cancers arising

in BRCA1 germline mutation carriers (approximately 80 %. ER limits targeted therapeutic options due to the lack of PR and Her-2 expression, and TNBC does not respond to hormonal or anti-Her-2 therapies for which no treatment is yet approved by the FDA (Erasmo Orrantia-Borunda et al., 2022; Newman et al., 2015; Seiffert, Schmalfeldt, & Müller, 2017; Van Swearingen et al., 2017; Won & Spruck, 2020)lacking expression of hormone and human epidermal growth factor receptor 2 receptors, is an aggressive subtype that frequently metastasizes to the brain and has no FDAapproved systemic therapies. Previous literature demonstrates mitogen-Activated protein kinase (MEK). TNBC is sensitive to chemotherapy, so the current standard of treatment includes chemotherapy and surgery. Commonly used chemotherapy agents include anthracycline, alkylating agents, and the anti-metabolite fluorouracil. Responses to treatment are usually short-lived and recur rapidly. There is no standard chemotherapy regimen for patients with relapsing TNBC (Caswell-Jin et al., 2018; O'Shaughnessy et al., 2014; Plevritis et al., 2018). Therefore, there is a dire need to identify novel agents for specific TNBC targeting and treatment (Camorani et al., 2020)the lack of any known targetable proteins has not allowed a specific anti-tumor treatment. Therefore, the identification of novel agents for specific TNBC targeting and treatment is desperately needed. Here, by integrating cell-SELEX (Systematic Evolution of Ligands by EXponential enrichment).

## CONCLUSION

Molecular targeted therapy can be used alone or in conjunction with conventional chemotherapy drugs to treat cancer. Biomarkers for every form of cancer can now be found and predicted, thanks to the advancements in molecular medicine. The efficacy of treatment may be increased by combining molecularly targeted therapy with cytotoxic chemotherapy medications or other molecularly targeted therapy agents that co-inhibit two or more targets in a single or complementary pathway. Numerous studies have demonstrated that hybrid treatment of cancer using a variety of medications produces better synergistic anti-tumor benefits than single-drug therapy. However, the main obstacle to developing effective cancer therapies and biomarkers is tumor heterogeneity. Tumor heterogeneity can lead to the formation of diverse cell populations with various genomes. Inconclusive biopsy results due to the existence of tumor subclonal populations may have negative clinical effects. Additionally, a high degree of cancer clonal heterogeneity, intratumor genetic heterogeneity, epigenetic editing, and cell signaling complexity may contribute to drug resistance associated with molecularly targeted therapy for cancer treatment. These factors can cause the cancer cells to adapt to the selective impact of therapeutic regimens. Therefore, to design medications that can effectively stop the spreading of cancer cells that are already resistant to conventional therapies, it is necessary to identify the prognostic marker for intratumor heterogeneity and identify novel therapeutic targets. The therapeutic outcomes rely on discovering tumor-specific protein expression patterns and interaction signaling networks that function stand-alone or in concert to prevent drug resistance before the treatment. In addition, the success of molecularly targeted therapeutics is limited by the one-size-fits-all pharmacological strategy because different patients do not react the same to the same medications. Therefore, the transition from the existing strategy to the precision medicine paradigm holds considerable potential for the treatment of cancer. As such, precision medicine is developed using molecularly focused therapy and takes into account the unique genetic profile of each patient.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHORS CONTRIBUTIONS STATEMENT

B.I.S. and H.H. organisation of paper and collection of data. E.C. Supervision

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*RESEARCH ARTICLE SUPPLEMENT*

**SURVEYFORM**

**Demographic & General Information**

1. Birthyear:

2. Gender:

☐ Woman
☐ Male
☐ Other……..

3. Branch:

☐ Psychiatry
☐ Infection

4. Academictitle:

☐ Specialist(Yearof specialization:)
☐ Research assistant (Year you started your specialization education:)

5. Have you examined an HIV positive patientwith mental problems within the last 6months?Ifso,howmanypeopleperweekonaverage?

6. Typeof hospital:

☐ Education–Research
☐ University
☐ Public Hospital
Other……

**Approaches About Drug-Drug Interactions(DDIs)**

1. Who do you use when you want to know more about potential DDI s? (You can choose more than one)

☐ Prospectus ☐ Written material (book, brochure, monograph)
☐ Pharmacist ☐ Internet
☐ Smart device applications ☐ senior physician
☐ Other …… ……. ☐ medical pharmacologist

2. How often does a potential DDI affect your decision when prescribing medication?

☐ Never ☐ Rarely ☐ Sometimes ☐ Often ☐ Always

3. How often do you inform patients about potential DDI s?

☐ Never ☐ Rarely ☐ Sometimes ☐ Often ☐ Always

4. How sufficient do you think your DDI knowledge is to intervene in medical treatment?

☐ None ☐ Little ☐ Middle ☐ A lot ☐ Full

5. Please choose the appropriate one(s) for you regarding DDI s between antiretroviral and psychiatric drugs for your patients. (You can choose more than one)

☐ I check it at every meeting.
☐ I only check when starting a new psychiatric medication.
☐ I check when an antiretroviral drug is added to psychiatric medication.
☐ I never check.
☐ Other ……

6. Have you ever had a patient who was adversely affected by the combined use of antiretroviral and psychiatric drugs?

☐ Yes
☐ No

7. What approach do you take when you encounter a DDI?

☐ Changing all medications,
☐ Changing all antiretroviral medications
☐ interacting antiretroviral medications
☐ Changing all psychiatric medications
☐ Changing interacting psychiatric medications
☐ None
☐ Other ……

**CASES**

**CASE 1**

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**patient history**

OA 48 years old, male

Height: 166 cm, Weight: 75 kg.

Cigarettes: 52 packs/year; No alcohol or substance use.

He was diagnosed with schizophrenia at the age of 28. He was started on paliperidone 3 years ago and his delusions and hallucinations improved subsequently. The patient does not describe any side effects. He complains of difficulty falling asleep.

HIV infection was detected in tests performed 2 years ago due to oral thrush and weight loss, and treatment was started. After ART, the thrush lesions in his mouth resolved and he gained weight.

---

**Laboratory findings**

CD4 cell count: 215/mm 3, HIV viral load: negative.

ALT:8 U/L (<35 U/L), AST:18 U/L (<35 U/L), GGT:26 U/L (<35 U/L)

BUN: 8.5 mg/dl (6-20 mg/dl), Uric acid: 5.67 mg/dl (2.6-6 mg/dl), Creatinine: 0.81 mg/dl (0.5-0.95 mg/dl) LDH:188 U/L (<248 U/L), CK:134 U/L (<171 U/L)

---

**Medicines**

**Antiretroviral Drugs:**

- Tenofovir / Emtricitabine (245/200 mg)
- Darunavir / ritonavir (800/100 mg)

**Psychiatric Drugs:**

- Quetiapine (100 mg)
- Paliperidone (6 mg)

---

**1st question;**

In this patient Who do you use when you want to know more about potential DDIs? (You can choose more than one)

☐ Prospectus
☐ Written material (book, brochure, monograph)
☐ Pharmacist
☐ Internet
☐ Smart device applications
☐ senior physician
☐ Other …… …….
☐ medical pharmacologist

**2nd question;**

If you think there is a DDI, which of the following would you do? Circle the drug(s) you intend to intervene with.

- ☐ Dose change of interacting drug(s)
- ☐ Change in interacting drug(s)
- ☐ Discontinuation of interacting drug(s)
- ☐ Extra monitoring regarding interaction (ECG, plasma level)
- ☐ Clinical monitoring (continue routine monitoring)
- ☐ I don't think there is a DDI

a. Tenofovir / Emtricitabine (245/200 mg)
b. Darunavir / ritonavir (800/100 mg)
c. Quetiapine (100 mg)
d. Paliperidone (6 mg)

_____

**DDI databases (1st case, 1st interaction)**

- ritonavir + quetiapine
  - Using quetiapine and ritonavir simultaneously quetiapine may result in increased exposure; There is an increased risk of QT prolongation.
  - Ritonavir It will increase the level and effect of quetiapine by affecting CYP3A4, one of the hepatic/intestinal enzymes.

**3rd question;**

In light of the information given above, if you think there is a DDI, which of the following would you do? Tick the box(es). Write the code of the drug(s) you want to intervene in parentheses.

- ☐ Dose change of interacting drug/drugs ( )
- ☐ Change of interacting drug(s) ( )
- ☐ Discontinuation of interacting drug/drugs ( )
- ☐ Extra monitoring related to interaction (ECG, plasma level)
- ☐ Clinical monitoring (continue the same routine monitoring)
- ☐ I don't think there is a DDI

a. Tenofovir / Emtricitabine (245/200 mg)
b. Darunavir / ritonavir (800/100 mg)
c. Quetiapine (100 mg)
d. Paliperidone (6 mg)

_____

**CASE 2**

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**patient history**

AE 23 years old, male

Height: 175 cm, Weight: 55 kg.

Cigarettes: 5 packs/year, Alcohol: 2 double raki per week; Substance: marijuana, bonsai, heroin (last 1 year ago)

Bipolar for 5 years due to substance abuse He has been followed for 3 years due to Affective Disorder Type 1, and while it was stable, escitalopram was added for depression after the diagnosis was made.

He presented with withdrawal symptoms 1 year ago and was started on buprenorphine / naloxone. He has been followed up with HIV infection for 3 months and is receiving antiretroviral treatment.

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**Laboratory findings**

CD4 cell count: 199, HIV viral load 800 copies/ml.

ALT:40 U/L (<35 U/L), AST:71 U/L (<35 U/L), ALP:91 U/L (30-120 U/L), GGT:24 U/L ( <35 U/L),

BUN: 49.66 mg/dl (6-20 mg/dl), Uric acid: 9.02 mg/dl (2.6-6 mg/dl), Creatinine: 0.84 mg/dl (0.5-0.95 mg/dl)

LDH:199 U/L (<248 U/L), CK:80 U/L (<171 U/L)

Li plasma level: 0.85 mEq /L (0.6-1 mEq /L)

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**Medicines**

**Antiretroviral Drugs:**

- Tenofovir / emtricitabine / elvitegravir / cobicistat (245/200/150/100 mg)

**Psychiatric Drugs:**

- Lithium (900 mg)
- Buprenorphine / naloxone (4/1 mg)
- Escitalopram (10 mg)

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**1st question;**

In this patient Who do you use when you want to know more about potential DDI s? (You can choose more than one)

☐ Prospectus
☐ Pharmacist
☐ Smart device applications
☐ Other …… …….
☐ Written material (book, brochure, monograph)
☐ Internet
☐ senior physician
☐ medical pharmacologist

**2nd question;**

If you think there is a DDI, which of the following would you do? Write the drug (s) you intend to intervene with in parentheses.

☐ Dose change of interacting drug/drugs ( )
☐ Change in interacting drug (s) ( )
☐ Discontinuation of interacting drug (s) ( )
☐ Extra monitoring related to interaction (ECG, plasma level)
☐ Clinical monitoring (continue the same routine monitoring)
☐ I don't think there is a DDI

a. Tenofovir / emtricitabine / elvitegravir / cobicistat (245/200/150/100 mg)
b. Lithium (900 mg)
c. Buprenorphine / naloxone (4/1 mg)
d. Escitalopram (10 mg)

_____

**Interaction information according to DDI databases;**

- ***elvitegravir / cobicistat / emtricitabine / tenofovir + buprenorphine (2nd case, 1st interaction)***
  - Cobicistat will increase the level and effect of buprenorphine by affecting CYP3A4, one of the hepatic/intestinal enzymes.
  - Cobicistat is a CYP3A4 inhibitor, therefore concomitant use with CYP3A4 substrates is contraindicated because serious and life-threatening events may occur due to increased plasma concentration.

***3rd question;***

In light of the information given above, if you think there is a DDI, which of the following would you do?

☐ Dose change of interacting drug/drugs ( )
☐ Change in interacting drug (s) ( )
☐ Discontinuation of interacting drug (s) ( )
☐ Extra monitoring related to interaction (ECG, plasma level)
☐ Clinical monitoring (continue routine monitoring)
☐ I don't think there is a DDI

a. Tenofovir / emtricitabine / elvitegravir / cobicistat (245/200/150/100 mg)
b. Lithium (900 mg)
c. Buprenorphine / naloxone (4/1 mg)
d. Escitalopram (10 mg)

_____

**DDI databases (2nd case 2nd interaction);**

- ***elvitegravir / cobicistat / emtricitabine / tenofovir + escitalopram***
  - Cobicistat will increase the level and effect of escitalopram by affecting CYP3A4, one of the hepatic/intestinal enzymes.
  - Cobicistat is a CYP3A4 inhibitor, therefore concomitant use with CYP3A4 substrates is contraindicated because serious and life- threatening events may occur due to increased plasma concentration.

**4th question;**

In light of the information given above, if you think there is a DDI, which of the following would you do?

☐ Dose change of interacting drug/drugs ( )

☐ Change in interacting drug (s) ( )

☐ Discontinuation of interacting drug (s) ( )

☐ Extra monitoring related to interaction (ECG, plasma level)

☐ Clinical monitoring (continue routine monitoring)

☐ I don't think there is a DDI

a. Tenofovir / emtricitabine / elvitegravir / cobicistat (245/200/150/100 mg)

b. Lithium (900 mg)

c. Buprenorphine / naloxone (4/1 mg)

d. Escitalopram (10 mg)

_____

**CASE 3**

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**patient history**

NY 68 years old, female Height: 155 cm, Weight: 76 kg.

No smoking, alcohol or substance use.

He has been followed up for HIV infection since the age of 48 and has been receiving antiretroviral treatment since the age of 52.

He lost his wife 6 months ago and was diagnosed with depression.

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**Laboratory findings**

CD4 cell count: 392/mm 3, HIV viral load: negative

ALT:21 U/L (<35 U/L), AST:35 U/L (<35 U/L), ALP:44 U/L (30-120 U/L), GGT:28 U/L ( <35 U/L)

BUN: 25.46 mg/dl (6-20 mg/dl), Uric acid: 4.42 mg/dl (2.6-6 mg/dl), Creatinine: 0.65 mg/dl (0.5-0.95 mg/dl)

LDH:140 U/L (<248 U/L), CK:95 U/L (<171 U/L)

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**Medicines**

**Antiretroviral Drugs:**

- Tenofovir / emtricitabine (245/200 mg)
- Rilpivirine (25 mg)

**Psychiatric Drugs:**

- Venlafaxine (150 mg)
- Trazodone (50 mg)

**1st question;**

In this patient Who do you use when you want to know more about potential DDI s? (You can choose more than one)

☐ Prospectus
☐ Pharmacist
☐ Smart device applications
☐ Other …… …….
☐ Written material (book, brochure, monograph)
☐ Internet
☐ senior physician
☐ medical pharmacologist

**2nd question;**

If you think there is a DDI, which of the following would you do?

☐ Dose change of interacting drug/drugs ( )
☐ Change in interacting drug (s) ( )
☐ Discontinuation of interacting drug (s) ( )
☐ Extra monitoring related to interaction (ECG, plasma level)
☐ Clinical monitoring (continue the same routine monitoring)
☐ I don't think there is a DDI

a. Tenofovir / emtricitabine (245/200 mg)
b. Rilpivirine (25 mg)
c. Venlafaxine (150 mg)
d. Trazodone (50 mg)

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**DDI databases (3rd case, 1st interaction)**

- efavirenz + trazodone
  - o Efairenz affects the CYP3A4 enzyme, causing trazadone to reduces its effectiveness. It should be used with caution and clinical monitoring.
- rilpivirine + trazodone
  - o Potential weak interaction. There are not enough studies. However, the possibility of interaction is low based on metabolism and excretion. Rilpivirine Its interaction with trazodone at therapeutic doses (25 mg/day) does not appear to be clinically significant. However, it is known that at supratherapeutic doses (75-300 mg/day), there is an increase in the risk of Torsades de Pointes by prolonging the QTc interval.

**3rd question;**

In light of the information given above, which of the following would you do if you think there is a DDI?

☐ Dose change of interacting drug/drugs ( )
☐ Change in interacting drug (s) ( )
☐ Discontinuation of interacting drug (s) ( )
☐ Extra monitoring related to interaction (ECG, plasma level)
☐ Clinical monitoring (continue the same routine monitoring)
☐ I don't think there is a DDI

e. Tenofovir / emtricitabine (245/200 mg)
f. Rilpivirine (25 mg)
g. Venlafaxine (150 mg)
h. Trazodone (50 mg)

**CASE 4**

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**patient history**

KO is 41 years old. Male Height: 185 cm, Weight: 63 kg.

Smoking 32 packs/year, alcohol social drinker, no substance use.

He has been under follow-up for HIV infection for 9 months and has been receiving ART for 8 months. There is no resistance transferred.

Posttraumatic stress disorder was diagnosed 7 months ago and fluoxetine 40 mg was started. Carbamazepine 500 mg was prescribed to regulate mood , and lorazepam 1 mg was prescribed if necessary for anxiety accompanied by sleep disturbance .

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**Laboratory findings**

CD4 cell count: 342/mm 3 , HIV viral load: negative

ALT:17 U/L (<35 U/L), AST:19 U/L (<35 U/L), ALP:117 U/L (30-120 U/L), GGT:13 U/L ( <35 U/L)

BUN: 13.11 mg/dl (6-20 mg/dl), Uric acid: 5.44 mg/dl (2.6-6 mg/dl), Creatinine: 0.74 mg/dl (0.5-0.95 mg/dl)

LDH:194 U/L (<248 U/L), CK:84 U/L (<171 U/L)

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**Medicines**

**Antiretroviral Drugs:**

- Tenofovir / emtricitabine (245/200 mg)
- Darunavir / ritonavir (800/100 mg)

**Psychiatric Drugs:**

- Lorazepam (1mg)
- Fluoxetine (40 mg)
- Carbamazepine (500 mg)

**1st question;**

In this patient Who do you use when you want to know more about potential DDI s? (You can choose more than one)

☐ Prospectus
☐ Pharmacist
☐ Smart device applications
☐ Other …… …….
☐ Written material (book, brochure, monograph)
☐ Internet
☐ senior physician
☐ medical pharmacologist

**2nd question;**

If you think there is a DDI, which of the following would you do?

☐ Dose change of interacting drug(s)
☐ Change in interacting drug(s)
☐ Discontinuation of interacting drug(s)
☐ Extra monitoring related to interaction (ECG, plasma level)
☐ Clinical monitoring (continue routine monitoring)
☐ I don't think there is a DDI.

a. Tenofovir / emtricitabine (245/200 mg)
b. Darunavir / ritonavir (800/100 mg)
c. Lorazepam (1mg)
d. Fluoxetine (40 mg)
e. Carbamazepine (500 mg)

_____

**DDI databases (4th case 1st interaction)**

- carbamazepine – darunavir
  - o Concurrent use of CARBAMAZEPINE and DARUNAVIR may result in potential for reduced darunavir concentrations, loss of therapeutic effect, and development of resistance.
  - o carbamazepine decreases levels of darunavir by increasing metabolism contraindicated.
  - o carbamazepine will decrease the level or effect of darunavir by affecting hepatic / intestinal enzyme CYP3A4 metabolism. Avoid or Use Alternate Drug.
  - o darunavir will increase the level or effect of carbamazepine by affecting hepatic / intestinal enzyme CYP3A4 metabolism. Use Caution / Monitor plasma levels when used concomitantly
- carbamazepine – ritonavir
  - o Concurrent use of CARBAMAZEPINE and RITONAVIR may result increased carbamazepine exposure; decreased ritonavir exposure.
  - o carbamazepine will decrease the level or effect of ritonavir by affecting hepatic / intestinal enzyme CYP3A4 metabolism. Avoid or Use Alternate Drug.

**3rd question;**

In light of the information given above, if you think there is a DDI, which of the following would you do?

□ Dose change of interacting drug/drugs ( )
□ Change in interacting drug (s) ( )
□ Discontinuation of interacting drug (s) ( )
□ I make extra monitoring regarding the interaction (ECG, plasma level)
□ Clinical monitoring (continue the same routine monitoring)
□ I don't think there is a DDI

a. Tenofovir / emtricitabine (245/200 mg)
b. Darunavir / ritonavir (800/100 mg)
c. Lorazepam (1mg)
d. Fluoxetine (40 mg)
e. Carbamazepine (500 mg)

_____

**drug-DDI databases (4th case 2nd interaction)**

- fluoxetine - ritonavir
  - o Concurrent use of FLUOXETINE and RITONAVIR may result increased fluoxetine exposure; increased risk of QT- interval prolongation
  - o Ritonavir will increase the level or effect of fluoxetine by affecting hepatic enzyme CYP2D6 metabolism. Minor / Significance Unknown.

**4th question;**

In light of the information given above, which of the following would you do if you think there is a DDI?

- ☐ Dose change of interacting drug/drugs ( )
- ☐ Change in interacting drug (s) ( )
- ☐ Discontinuation of interacting drug (s) ( )
- ☐ I make extra monitoring regarding the interaction (ECG, plasma level, etc.)
- ☐ Clinical monitoring (continue the same routine monitoring)
- ☐ I don't think there is a DDI

a. Tenofovir / emtricitabine (245/200 mg)
b. Darunavir / ritonavir (800/100 mg)
c. Lorazepam (1mg)
d. Fluoxetine (40 mg)
e. Carbamazepine (500 mg)

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*****The survey was completed. Thank you for your participation*****

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*RESEARCH ARTICLE SUPPLEMENT*

**Table S1.** Common genes between TCDD and cardiovascular diseases via MyVenn CTD tools.

| | |
|---|---|
| **Common 1,438 genes between TCDD and cardiovascular diseases** | ABCA1, ABCA3, ABCB1, ABCB1A, ABCC1, ABCC2, ABCC6, ABCC9, ABCG2, ABCG5, ABCG8, ABL1, ABO, ABRA, ACAA1B, ACACA, ACAD9, ACADS, ACE, ACLY, ACOT2, ACTA1, ACTA2, ACTB, ACTC1, ACTG1, ACTN2, ACVR2B, ACVRL1, ADA, ADAM17, ADAMTS2, ADAMTS7, ADCY3, ADCY5, ADCY8, ADCYAP1, ADD1, ADD3, ADH1B, ADIPOQ, ADORA1, ADORA2A, ADORA2B, ADORA3, ADRA1B, ADRA1D, ADRA2A, ADRA2C, ADRB2, ADRB3, ADTRP, AFF4, AGER, AGK, AGT, AGTR1, AGTR1A, AGTR1B, AGTR2, AHR, AHR2, AIF1, AK1, AKAP5, AKAP6, AKAP9, AKIP1, AKT1, AKT1S1, ALAD, ALB, ALDH1A7, ALDH2, ALDH6A1, ALDOA, ALG10B, ALKBH5, ALMS1, ALOX15, ALOX5, ALOX5AP, ANGPTL4, ANK2, ANKRD23, ANKS6, ANXA1, AOC1, AOC3, AOPEP, APCS, APEX1, APLN, APLP2, APOA1, APOA4, APOA5, APOB, APOC1, APOC3, APOE, APP, AQP1, AQP2, AR, AREG, ARG2, ARHGAP10, ARHGAP9, ARHGDIB, ARMH3, ARNT2, ASAH1, ASIC1, ASS1, ATF3, ATM, ATOX1, ATP1A1, ATP1A2, ATP1A3, ATP1B1, ATP2A1, ATP2A2, ATP2B1, ATP2B3, ATP5ME, ATP5PF, ATP7A, ATXN1, AVP, AXIN2, B2M, B3GALT6, B3GAT3, B4GALT7, BAD, BAG3, BAK1, BAMBI, BAX, BAZ1B, BBC3, BCHE, BCL2, BCL2L1, BCL3, BCOR, BDKRB1, BDKRB2, BDNF, BECN1, BEST3, BGLAP, BGN, BHLHE40, BLK, BMAL1, BMP10, BMP2, BMP8A, BMPR2, BNIP3, BRAF, BRAP, BRD4, BSN, BTG1, C1QB, C1R, C1S, C3, C6, CA9, CABIN1, CACNA1C, CACNA1D, CACNA2D1, CACNB2, CALCA, CALM1, CALM2, CALR, CALU, CALY, CAMK2D, CAND2, CANX, CAPN2, CAPZB, CARF, CASP1, CASP12, CASP3, CASP7, CASP8, CASP9, CASQ2, CAST, CAT, CAV1, CAV2, CAV3, CBFB, CBL, CBLN2, CBR1, CBS, CBX7, CCDC103, CCDC22, CCDC40, CCDC92, CCL1, CCL11, CCL12, CCL2, CCL3, CCL4, CCL6, CCL7, CCL9, CCM2, CCN2, CCND1, CCND2, CCR1, CCR4, CD163, CD2, CD27, CD36, CD38, CD40, CD40LG, CD70, CD86, CDC25B, CDH2, CDK13, CDK6, CDKN1A, CDKN2A, CDKN2B, CEBPB, CEBPD, CELA2A, CELSR2, CEP68, CES3, CFAP53, CFC1, CFD, CFL2, CGA, CHAT, CHDH, CHRM2, CHST14, CHST3, CIDEA, CIRBP, CITED2, CKB, CKM, CLCNKA, CLEC10A, CLEC11A, CMA1, CNNM2, CNR1, CNR2, CNTF, COA5, COA6, COBLL1, COL18A1, COL1A1, COL1A2, COL3A1, COL4A1, COL4A2, COL5A1, COL6A1, COL8A1, COLEC10, COLEC11, COMT, COX1, COX15, COX2, COX3, COX5B, CPT1B, CRB1, CRBN, CREB1, CREB5, CREG1, CRELD1, CREM, CRH, CRHR2, CRK, CRKL, CRP, CRYAB, CRYM, CS, CSF1, CSF2, CSF3, CSRNP1, CSRP3, CST3, CTC1, CTF1, CTNNA3, CTNNB1, CTSC, CTTNBP2, CUL4A, CX3CL1, CX3CR1, CXCL1, CXCL10, CXCL12, CXCL2, CXCL3, CXCL8, CXCL9, CXCR2, CXCR3, CYBA, CYBB, CYCS, CYP17A1, CYP1A1, CYP1B1, CYP27A1, CYP2C11, CYP2C19, CYP2C9, CYP2E1, CYP2U1, CYP3A4, CYP3A5, CYP3A62, CYP3A9, CYP4A10, CYP4A3, CYP4F2, CYRIB, CYTB, CYTH1, DAB2IP, DCAF17, DCHS1, DCN, DDAH2, DDIT3, DES, DGCR2, DGCR6, DGCR8, DGKB, DGKZ, DIABLO, DLAT, DLG4, DMD, DNAAF4, DNAH11, DNAH5, DNAI1, DNAI2, DNAJC19, DNM1L, DPF3, DPT, DRC1, DRD1, DRD2, DSC2, DSE, DSG2, DSP, DSTN, DTNA, DUSP1, DUSP5, DUSP6, DYNLL1, ECE1, EDN1, EDN3, EDNRA, EDNRB, EFNA5, EFNB2, EGF, EGFR, EGR1, EGR2, EHMT1, EIF2A, EIF2AK3, EIF2AK4, ELK3, ELMO2, ELN, ELOVL6, ENG, ENO1, ENSA, EPHA2, EPHA3, EPHB4, EPHX1, EPHX2, EPN3, ERAP1, ERBB4, ESR1, ESR2, ESS2, ETS1, EYA4, F10, F11, F12, F13A1, F13B, F2, F2RL1, F2RL3, F3, F5, F7, F8, FABP5, FADD, FANCB, FAS, FASL, FASLG, FASN, FBLN5, FBN1, FBRSL1, FBXO32, FCGR1A, FCGR2A, FCGR3A, FES, FEV, FEZ2, FFAR2, FGA, FGD1, FGF1, FGF2, FGF21, FGF5, FGF7, FGF8, FGFR1, FGFR3, FHL1, FHL2, FIBIN, FIP1L1, FKBP14, FKTN, FLNA, FLNC, FLT1, FLT3, FLT4, FN1, FNDC5, FOLR1, FOS, FOXC1, FOXC2, FOXE3, FOXF2, FOXH1, FOXO1, FRMD4B, FSHB, FSTL1, FURIN, FUT4, FXYD2, FXYD3, GAA, GABRA6, GAD1, GAD2, GAL, GAPDH, GAS5, GAS6, GATA2, GATA4, GATA6, GATAD1, GATM, GBA1, GBP2, GCH1, GCLC, GCLM, GCOM1, GDF1, GDF15, GDF2, GDF3, GFOD1, GGCX, GGT1, GH1, GHR, GHRL, GJA1, GJA4, GJB1, GJC1, GK, GLA, GLP1R, GLRX3, GNA11, GNA12, GNAI2, GNAQ, GNAS, GNB3, GNB4, GNB5, GNRH1, GOPC, GOSR2, GP1BB, GPC3, GPC4, GPR17, GPT, GPX1, GPX4, GRIA1, GRIN2B, GRK2, GRK5, GSK3B, GSN, GSTA1, GSTA4, GSTA5, GSTM1, GSTM2, GSTP1, GSTT1, GSTT2, GTF2I, GTF2IRD1, GUCY1A1, GYG1, H19, H2-T23, HADHA, HADHB, HAMP, HAND2, HBA1, HBEGF, HCAR3, HCN4, HCP5, HDAC1, HDAC2, HDAC3, HDAC6, HDAC9, HECTD4, HERC1, HEY2, HGF, HHIPL1, HIF1A, HIP1R, HIVEP2, HK1, HK2, HLA-A, HLA-B, HLA-DPB1, HLA-DRB1, HMGB1, HMGCS2, HMOX1, HMOX2, HNF1A, HOXA1, HOXA3, HOXD13, HP, HPX, HRAS, HRC, HRH1, HRH2, HRH3, HSD11B1, HSD11B2, HSF2, HSPA1A, HSPA1B, HSPA2, HSPA5, HSPA8, HSPA9, HSPB1, HSPB6, HSPD1, HSPE1, HSPG2, HTR1B, HTR2B, HTR4, HTRA1, HTRA2, ICA1L, ICAM1, ID1, ID3, IDH2, IER3, IFIH1, IFIT3, IFNA2, IFNG, IFNGR1, IFT122, IGF1, IGF1R, IGF2, IGF2R, IGFBP3, IKBKB, IL10, IL10RB, IL11, IL12A, IL12RB1, IL17A, IL17RA, IL18, IL1A, |

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|---|---|
| **Common 1,438 genes between TCDD and cardiovascular diseases** | IL1B, IL1RN, IL2, IL22, IL4, IL6, IL6RA, IL6ST, ILK, INPP5D, INPPL1, INS, INS1, IRAK3, IRAK4, IRF1, IRF2BPL, IRF4, IRS1, IRX5, ITGA2, ITGA2B, ITGA5, ITGAL, ITGAM, ITGAV, ITGB1, ITGB2, ITGB3, ITM2B, ITPKC, ITPR2, JAG1, JAK2, JCAD, JPH2, JUN, JUNB, JUP, KANK2, KAT6A, KAT6B, KAT8, KCNA5, KCND3, KCNE1, KCNE2, KCNE3, KCNH2, KCNJ1, KCNJ2, KCNJ5, KCNJ8, KCNK3, KCNN2, KCNN3, KCNQ1, KDM1B, KDR, KIF3C, KIF6, KITLG, KL, KLF13, KLF15, KLF4, KLF9, KLK1, KLKB1, KNG1, KRAS, KRIT1, LAMA4, LAMC1, LAMP2, LBR, LCN2, LDB3, LDHA, LDHB, LDLR, LEFTY2, LEMD2, LEP, LEPR, LGALS2, LGALS3, LHB, LHX3, LIF, LIG4, LIPA, LIPC, LMCD1, LMNA, LMOD1, LOX, LPL, LRCH1, LRIG1, LRP6, LRP8, LSP1, LTA, LTA4H, LTBP3, LTF, LY86, LZTR1, MACO1, MAOA, MAOB, MAP2K1, MAP2K2, MAP2K7, MAP3K5, MAP3K7, MAP3K8, MAPK1, MAPK11, MAPK14, MAPK3, MAPK8, MAPK9, MAPRE1, MAPT, MAS1, MASP1, MAT2A, MBD5, MBP, MC2R, MCL1, MDH1, MED12, MED13L, MEF2A, MEIS2, MEOX2, MERTK, METTL3, MEX3C, MFAP5, MFF, MFN2, MGP, MIA3, MIB1, MIR106B, MIR126, MIR140, MIR144, MIR15A, MIR15B, MIR17, MIR188, MIR18A, MIR191, MIR193A, MIR193B, MIR19A, MIR20A, MIR20B, MIR23B, MIR27A, MIR27B, MIR301A, MIR301B, MIR330, MIR335, MIR337, MIR34A, MIR34C, MIR362, MIR369, MIR374A, MIR411, MIR542, MIR590, MIR98, MIRLET7G, MKKS, MLXIPL, MMP1, MMP12, MMP14, MMP2, MMP3, MMP9, MOCOS, MPO, MRAS, MRLC2, MRPS6, MS4A6A, MSN, MSTN, MT1, MT2, MT2A, MTHFD2, MTHFR, MTOR, MUC1, MX1, MYC, MYD88, MYH11, MYH6, MYH7, MYH8, MYH9, MYL2, MYL3, MYL4, MYL7, MYL9, MYLK, MYLK2, MYO18B, MYO1C, MYO6, MYOCD, MYOT, MYOZ1, MYOZ2, MYPN, NACA, NAT2, NAV2, NBEAL1, NCF1, NCF4, NCL, ND1, ND5, ND6, NDRG2, NDUFB11, NDUFS1, NDUFS2, NDUFV2, NEBL, NECTIN2, NEDD4, NEDD4L, NEURL1, NEXN, NF1, NFATC2, NFE2L2, NFKB1, NFKBIA, NGF, NHERF2, NIPBL, NISCH, NKX2-5, NKX2-6, NME1, NOS2, NOS3, NOTCH1, NOTCH2, NOTCH3, NOX4, NPC1, NPPA, NPPB, NPR1, NPTN, NPY, NQO1, NR1H4, NR2F2, NR3C1, NR3C2, NR4A1, NRAS, NRG1, NRIP1, NT5E, NTN1, NTS, NUAK1, NUCKS1, NUP155, NUPR1, OCLN, OGDHL, OGG1, OLR1, OPRK1, OPRL1, OPRM1, ORAI1, OXSR1, P2RY1, P2RY12, P4HB, PAK2, PAMR1, PAOX, PAPPA, PARP1, PCDHA13, PCDHA9, PCK1, PCNA, PCSK5, PCSK9, PDE3A, PDE4B, PDGFD, PDGFRA, PDHA1, PDIA3, PDK2, PDK4, PDLIM5, PDPK1, PDYN, PEBP1, PECAM1, PF4, PFKFB3, PFKM, PGM1, PHLDA1, PHLDA3, PHLDB2, PIGL, PIGM, PIK3CA, PIK3CG, PIK3IP1, PINK1, PITX2, PKD1L1, PKD2, PKP2, PLA2G2A, PLA2G7, PLAT, PLAU, PLD1, PLD2, PLG, PLN, PLOD1, PLPP3, PLXND1, PNOC, PNPLA2, PNPO, PODXL, POLE, POMC, PON1, PON2, POR, POU5F1, PPA2, PPARA, PPARD, PPARG, PPARGC1A, PPAT, PPBP, PPFIA4, PPM1B, PPM1K, PPP1R1A, PPP2CA, PPP2R3A, PPP3CA, PPP3R1, PQBP1, PRAME, PRDM16, PRDM6, PRDX6, PRG4, PRKAA2, PRKAG2, PRKAR1A, PRKAR2B, PRKCA, PRKCB, PRKCD, PRKCE, PRKCH, PRKCZ, PRKD1, PRKG1, PRL, PRMT5, PROC, PROCR, PROS1, PRPF8, PRRX1, PRTN3, PSEN1, PSEN2, PSMA2, PSMA6, PSMB1, PSMB5, PSMB7, PSORS1C2, PSRC1, PTEN, PTGER3, PTGIR, PTGIS, PTGS1, PTGS2, PTH, PTK2, PTP4A2, PTPN1, PTPN11, PTPN3, PTPN6, PTPRA, PTPRF, PUS1, PYCARD, PYGB, RAB12, RAB1B, RAB5A, RAB7A, RAB7B, RAC1, RAC2, RAF1, RALBP1, RARG, RASA1, RASSF1, RB1, RBCK1, RBM10, RBM20, RBP4, RCAN1, RECQL4, REEP1, REEP3, REG3G, RELA, REN, RENBP, RERE, RETN, RETNLA, RETSAT, RGCC, RGS2, RGS5, RGS7, RHOC, RHOJ, RIPK3, RIT1, RNF10, RNF213, ROBO4, ROCK2, RPL17, RPS2, RPS6KB1, RRAD, RSAD2, RT1-N3, RTN1, RXRG, RYR2, S100A1, S100A4, S100A5, S100A6, S100B, S1PR1, S1PR2, SALL4, SAP130, SARS1, SARS2, SCD1, SCMH1, SCN10A, SCN1B, SCN3B, SCN4B, SCN5A, SCN9A, SCNN1A, SCO1, SCO2, SCT, SELE, SELL, SELP, SEMA3C, SENP1, SERPINA1, SERPINA10, SERPINA3K, SERPINC1, SERPIND1, SERPINE1, SERPINF1, SERPING1, SERPINH1, SFRP1, SGCA, SGCB, SGCD, SGCG, SGO1, SH2B3, SH3PXD2A, SH3PXD2B, SHBG, SHC1, SHOC2, SIK1, SIPA1L1, SIRT1, SIRT4, SIRT6, SIRT7, SKI, SLC12A2, SLC12A3, SLC20A1, SLC22A3, SLC22A4, SLC22A5, SLC23A1, SLC25A4, SLC27A6, SLC29A3, SLC2A10, SLC2A4, SLC31A1, SLC35F1, SLC39A13, SLC4A3, SLC5A3, SLC6A2, SLC6A4, SLC6A6, SLC8A1, SLC9A1, SLC9A3, SLC9B1, SLIT3, SLPI, SMAD3, SMAD4, SMAD6, SMAD7, SMARCAL1, SMG9, SMOX, SNAP23, SNCA, SNRNP27, SNTA1, SOCS1, SOCS3, SOD1, SOD2, SOD3, SORD, SORL1, SORT1, SOS1, SOS2, SOX15, SOX18, SOX4, SOX5, SP110, SPARC, SPATS2L, SPHK2, SPN, SPP1, SRC, SRCAP, SSPN, STAMBP, STAT3, STAT4, STAT5A, STIM1, STING1, STK11, STK39, STK4, STRA6, STXBP2, SULT1A1, SULT1E1, SUN1, SYNE2, SYNPO2L, TAB2, TAC1, TACR1, TAFAZZIN, TAP1, TBC1D7, TBCA, TBX1, TBX2, TBX20, TBX3, TBX5, TBXA2R, TCF21, TCF4, TCF7L2, TECRL, TEK, TERT, TFAP2B, TFPI, TFRC, TGFA, TGFB1, TGFB2, TGFB3, TGFBI, TGFBR1, TGFBR2, TGM2, TGOLN1, TH, THBD, THBS1, THBS2, THOC6, THRB, THY1, TICAM1, TIMP1, TIMP2, TJP1, TLE4, |

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| **Common 1,438 genes between TCDD and cardiovascular diseases** | TLE5, TLL1, TLR2, TLR4, TM6SF2, TMED2, TMEM43, TMEM70, TMEM71, TMPO, TNC, TNF, TNFAIP3, TNFAIP6, TNFRSF11B, TNFRSF12A, TNFRSF13B, TNFRSF1A, TNFRSF1B, TNFRSF4, TNFRSF8, TNFRSF9, TNFSF12, TNFSF4, TNFSF8, TNFSF9, TNNC1, TNNI3, TNNT2, TNXB, TP53, TPH1, TPM1, TPM4, TRAF1, TRAF6, TRDN, TREX1, TRH, TRIB1, TRIM28, TRIM63, TRP53, TRPC1, TRPC3, TRPM4, TRPV1, TSPAN2, TTN, TTPA, TUBA1A, TUBA1C, TUBA8, TUBB1, TUBB5, TWIST1, TXN1, TXNL4A, TXNRD1, TXNRD3, TYRO3, UBE4B, UBIAD1, UCN, UCP1, UCP2, UFD1, UGCG, ULK4, UNC45B, UNC5B, UQCRFS1, USP3, USP34, UST, UTS2, VAMP5, VAMP8, VAV3, VCAM1, VCL, VDAC1, VDR, VEGFA, VEGFD, VHL, VIM, VIP, VKORC1, VWF, WDPCP, WDR1, WDR12, WDR45, WIPF1, WNK1, WNK4, WNT2B, WNT3, WNT3A, XDH, XIAP, XPNPEP2, XPO1, XPO7, XYLT1, XYLT2, YARS2, YES1, YTHDF1, YWHAH, YY1, ZC3H12A, ZC3HC1, ZCCHC14, ZEB1, ZEB2, ZFHX3, ZFP36, ZFP532, ZFPM2, ZIC3, ZPBP2 |

**Table S2.** Top 10 transcription factor (TF) related to the hub genes

| Rank | TF | Mean Rank | Overlapping Genes |
|---|---|---|---|
| 1 | NFKB1 | 13.0 | ACTB, AKT1, EGFR, GAPDH, IL1B, IL6, TNF, TP53 |
| 2 | ETV3L | 28.0 | GAPDH, IL1B, IL6, TNF |
| 3 | MSC | 67.33 | ACTB, IL1B, IL6, TNF |
| 4 | SNAI1 | 70.33 | IL1B, IL6, TNF |
| 5 | NFKB2 | 73.0 | ACTB, EGFR, IL1B, IL6, TNF |
| 6 | MTF2 | 81.0 | AKT1, EGFR, TNF |
| 7 | ZNF267 | 85.67 | IL1B, IL6, TNF |
| 8 | NR4A3 | 97.0 | IL1B, IL6, TNF |
| 9 | NFE2L2 | 105.0 | ACTB, EGFR, GAPDH, IL1B, TNF |
| 10 | ZNF813 | 108.5 | AKT1, TP53 |

**Table S3.** Top 10 miRNA related to the hub genes

| Rank | TF | p-value | FDR | Odd ratio | Overlapping Genes |
|---|---|---|---|---|---|
| 1 | hsa-miR-19a-3p | 0.000357 | 0.0331 | 0.0948 | TNF, AKT1, ACTB, TP53 |
| 2 | hsa-miR-125b-5p | 0.000122 | 0.0312 | 0.0717 | TP53, AKT1, EGFR, TNF |
| 3 | hsa-miR-125a-5p | 0.000622 | 0.0426 | 0.0597 | TP53, EGFR, AKT1 |
| 4 | hsa-miR-937-5p | 0.00131 | 0.0510 | 0.0275 | TP53, ACTB |
| 5 | hsa-miR-644a | 0.00164 | 0.0538 | 0.0309 | GAPDH, ACTB |
| 6 | hsa-miR-5702 | 0.00107 | 0.0510 | 0.0249 | TP53, ACTB |
| 7 | hsa-miR-451a | 0.000183 | 0.0312 | 0.0103 | AKT1, IL6 |
| 8 | hsa-miR-382-3p | 0.00125 | 0.0510 | 0.0269 | ACTB, AKT1 |
| 9 | hsa-miR-27a-5p | 0.000387 | 0.0331 | 0.0149 | AKT1, EGFR |
| 10 | hsa-miR-133b | 0.00134 | 0.0510 | 0.0279 | EGFR, AKT1 |