Clinical and Experimental Health Sciences

Novel Thiazole-Hydrazide Derivatives and Their Anticancer Properties

Asaf Evrim Evren[1,2,3], Demokrat Nuha[1,4], Sam Dawbaa[1,5,6], Abdullah Burak Karaduman[7], Leyla Yurttas[1]

[1] Anadolu University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Eskişehir, Türkiye.
[2] Anadolu University, Faculty of Pharmacy, Department of Analytical Chemistry, Eskişehir, Türkiye.
[3] Bilecik Şeyh Edebali University, Vocational School of Health Services, Department of Pharmacy Services, Bilecik, Türkiye.
[4] University for Business and Technology, Faculty of Pharmacy, Prishtina, Kosovo.
[5] Thamar University, Faculty of Medical Sciences, Department of Pharmacy, Dhamar, Yemen.
[6] Al-Hikma University, Faculty of Medical Sciences, Department of Pharmacy, Dhamar, Yemen.
[7] Anadolu University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Eskişehir, Türkiye.

Correspondence Author: Asaf Evrim Evren
E-mail: asafevrim.evren@bilecik.edu.tr

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ABSTRACT

Objective: Cancer is described as uncontrolled cell division, and it is a major problem in Türkiye, as well as around the world. Current treatment options are insufficient in some cases, particularly the treatment rate for lung cancer cases, which is very low. Meanwhile, current pharmaceuticals have several side effects, such as drug-drug interactions, and cognitive disorders. Additionally, developing drug resistance is a major problem for current and future management of the disease. Accordingly, the search for new molecules or alternative treatment options is actively achieved.

Methods: In this study, eight novel thiazole-hydrazide analogs were designed and synthesized, and their structural elucidation was performed via HRMS, 1H-NMR, and 13C-NMR. Their biological activity profile was investigated on A549 lung carcinoma and MCF7 breast adenocarcinoma cells. To determine the selective cytotoxicity on cancer cells, they were also tested against NIH/3T3 healthy cell line. Besides that, an *in silico* study was performed to understand the binding modes of the compounds.

Results: The results showed that in the serial 4f and 4g, the most bulky analogues, showed no inhibition against any cell type, even at the highest concentration tested. On the other hand, 4a, 4b, 4d, 4e, and 4h showed less cytotoxicity on healthy cells than A549 cells, so they exhibited significant cytotoxicity and a selective profile against A549 cancer cells. While they also inhibited MCF7 cells. The major point is that para-chlorophenyl analogs at the fourth position on thiazole (4a and 4d) displayed a better anticancer profile than ortho-chlorophenyl analogs. These two compounds were also investigated for their apoptotic effects using in silico studies. Both experimental and *in silico* studies revealed that the combination of thiazole and hydrazinoacetyl has a significant impact against cancer cells, and *in silico* study also suggested that tri-substitute thiazole ring has anticancer potential that induced cancer cell death via apoptosis.

Conclusion: Results of this study was presented that compound 4a was the most potent compound against lung cancer cells (A549) and 4d was the most potent compound against breast cancer cells (MCF-7). Furthermore, analyzing the molecular docking study for promising compounds (4a and 4d) suggested that interactions with the loop region residues have a pivotal role in inducing caspase-3 enzyme activity. It was concluded that hybridization of thiazole and hydrazinoacetyl moieties is responsible for the anticancer activity.

Keywords: Thiazole; hydrazide; anticancer; A549 lung cancer; MCF7 breast cancer

1. INTRODUCTION

Cancer is a public health problem with high incidence and mortality worldwide, including Türkiye (1). According to the WHO 2020 report (2), there were approximately 37070 lung and 7161 breast cancer deaths in Türkiye with an annual incidence of close to 41264 and 24175 cases, respectively. Chemotherapy is one of the treatment options, however, there are a lot of challenges, such as drug resistance (3, 4), and side effects (5, 6). Therefore, it is obvious that humanity needs the development of new therapeutic agents. For this purpose, pharmaceutical chemists have a great responsibility in designing and finding new molecules. The cytotoxic effects of molecules containing the thiazole ring system in their structure on various cells, from cancer (7, 8) to invasive microbial organisms (9, 10), have been investigated to date. Some studies specifically reported that the effect of thiazole-containing compounds on A549 and MCF7 cell lines is significant because of its lower cytotoxicity against healthy cell lines (11-14). Moreover, this ring system has also been

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revealed to overcome developing drug resistance issues (15, 16). Molecular mechanistic studies have indicated that the effectiveness of thiazole derivatives in cancer treatment may be achieved through many mechanisms (15, 17, 18) such as aromatase inhibition (19, 20), EGFR inhibition (21), and MMP-9 (22) inhibition. This ring system has also reported as apoptosis inducer (23-25). All this information points to the anticancer potential of thiazole-derived compounds. On the other hand, previous published papers have revealed that the parts substituted to the thiazole ring system are decisive for the inhibitory strength of the molecule, its mechanism of action or some side effect profiles. Although the substituted parts augment the cytotoxic activity of the molecule mainly by ensuring and/or increasing the stability of the ligand-protein complex, they can also be responsible for serious problems such as drug-drug interactions (26, 27), high cytotoxicity on healthy cells (28, 29), and cognitive dysfunctions (30, 31). For example, substitution of the thiazole ring with hydroxamate has been shown to reduce oral bioavailability, metabolism and selectivity and cause in vivo instability problems in many drugs. For this reason, hydroxamate has been reported as an undesirable group in designing drug molecules. Therefore, it is of great importance to realize substitutions intelligently and evaluate the designed molecules in terms of possible negative effects during the drug design process (32-34). It is known that some groups such as amides, esters and hydrazides are frequently used in drug design studies. Studies have shown that acetamide moiety substitution increases the anticancer effect of thiazoles (35, 36). Moreover, some other studies have reported that hydrazide has a significant effect on the anticancer activity of the molecule because it can form H-bonds (37, 38). In this study, we aimed to design and synthesize effective, safe, stabile, and economic (39-41) anticancer molecules by bringing these parts together on the same molecule. Moreover, *in silico* studies have attempted to elucidate whether the caspase-3 enzyme is a possible molecular target of the tested thiazole-hydrazide analogues.

2. METHODS

2.1. Chemistry

All chemicals used in the syntheses were purchased either from Merck Chemicals (Merck KGaA, Darmstadt, Germany) or Sigma-Aldrich Chemicals (Sigma-Aldrich Corp., St. Louis, MO, USA). The reactions and the purities of the compounds were observed by thin-layer chromatography (TLC) on silica gel 60 F_{254} aluminum sheets obtained from Merck (Darmstadt, Germany). The melting points of the synthesized compounds were recorded by the MP90 digital melting point apparatus (Mettler Toledo, Ohio, USA) and presented as uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded by a Bruker 300 MHz NMR spectrometer (Bruker Bioscience, Billerica, MA, USA) using DMSO-d_6 as a solvent for the samples. In the NMR spectra, splitting patterns were designated as follows: s: singlet; d: doublet; t: triplet; m: multiplet. Coupling constants (*J*) were reported as Hertz. High-resolution mass spectrometric (HRMS) studies were performed using an LC/MS-IT-TOF system (Shimadzu, Kyoto, Japan).

2.1.1. General Synthesis of Ethyl 2-(2-chlorophenoxy) acetate (1)

2-chlorophenol (0.05 mol, 6.4 g), and ethyl 2-chloroacetate (0.06 mol, 6.42 mL) were added into a flask and then potassium carbonate (0.075 mol, 10.35 g) was put in this mixture. This mixture was refluxed in 150 mL of acetone for 20 h. The completion of the reaction was controlled with TLC analysis. The solvent was evaporated, and the raw material was filtered and washed with water before recrystallization with ethanol.

2.1.2. General Synthesis of 2-(2-chlorophenoxy) acetohydrazide (2)

Ethyl 2-(2-chlorophenoxy)acetate (**1**) (0.025 mol, 5.36 g), and hydrazine hydrate (0.1 mol of 85 %) were stirred at room temperature in ethanol (100 mL). The completion of the reaction was controlled using TLC. Stirring was stopped and the mixture was awaited till a precipitate was formed. The precipitated material was filtered. After drying, the product was recrystallized with ethanol.

2.1.3. General Synthesis of 2-(2-(2-chlorophenoxy)acetyl)-N-phenylhydrazinecarbothioamide Derivatives (3)

2-(2-Chlorophenoxy)acetohydrazide (**2**) (0.017 mol, 3.4g) and phenylisothiocyanate derivatives (0.02 mol) were refluxed in 100 mL of ethanol for 3h. TLC was used to observe the reaction. The solvent was evaporated, and the scraped material was removed. The raw product was recrystallized from ethanol.

2.1.4. General Synthesis of 2-(2-chlorophenoxy)-N'-(3,4-diphenylthiazol-2(3H)-ylidene) acetohydrazide Derivatives (4a–4h)

The synthesized intermediates 2-[2-(2-chlorophenoxy)acetyl]-*N*-phenyl hydrazinecarbo-thioamide derivatives (**3**) (0.001 mol) were refluxed with phenacyl bromide derivatives (0.001 mol) for 2h in ethanol. After overnight standing in a cool place, crystals of the final thiazole compounds were filtered off.

2.2. Antiproliferative Activity

The cytotoxic activities of the compounds were performed against NIH/3T3, MCF-7, and A549 cell lines according to the previously reported method (39), using doxorubicin as the standard drug. The inhibitory concentrations were determined for each compound against the tested cell lines, and the results are presented in µg/mL.

2.3. ADME Parameters

Some physicochemical properties of the compounds were predicted with Swiss ADME software (40, 41).

2.4. In silico study on caspase-3 enyzme

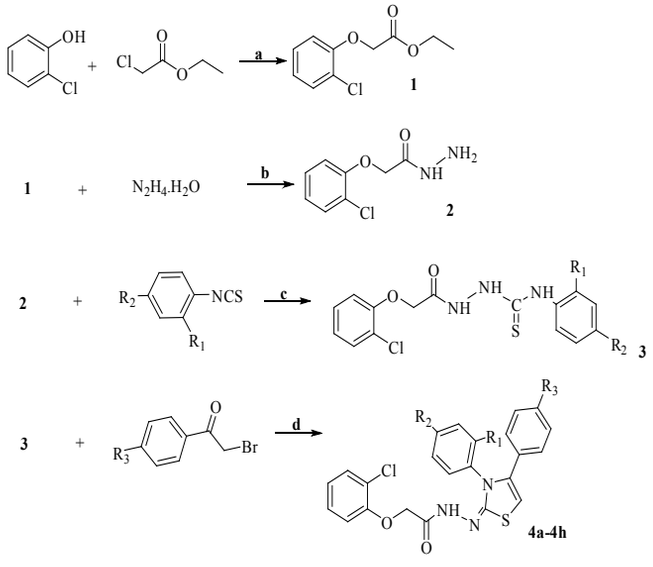
The *in silico* study was run using the Schrodinger Maestro Drug Discovery program (42). The most active compounds (4a and 4d) were prepared using the LigPrep module for the docking study. The X-RAY crystal structure of the caspase-3 enzyme was retrieved from the Protein Data Bank web server (PDBID: 4QTX), this structure was prepared using the Protein Wizard module in the default setting. The area of residues in the allosteric binding cleft was mapped by the Grid Generation module, then the docking procedure was applied similarly to our previous studies (43).

3. RESULTS

3.1. Chemistry

The synthesis diagram of the final molecules 4a-4h was displayed in Figure 1. At first, the starting material, ethyl 2-(2-chlorophenoxy)acetate (1), was obtained from the reaction of 2-chlorophenol and ethyl 2-chloroacetate. Then, compound 1 was treated with hydrazine monohydrate to gain 2-(2-chlorophenoxy)acetohydrazide (2) (44). The resulting molecule (2) was reacted with phenylisothiocyanate derivatives to synthesize 2-[2-(2-chlorophenoxy)acetyl]-N-phenylhydrazinecarbothioamide derivatives (3) (44), and those products were also refluxed with phenacyl bromide derivatives to gain 2-(2-chlorophenoxy)-N'-(3,4-diphenylthiazol-2(3H)-ylidene)acetohydrazide derivatives (4a-4h). The structures of all synthesized compounds (4a-4h) were confirmed by high-resolution mass spectroscopy (HRMS), 1H-NMR, and 13C-NMR. All analysis results were shared with the spectra in the supplementary file.

In both NMR (1H-NMR and 13C-NMR) spectra, the peaks of the aromatic and aliphatic regions were observed at estimated areas. The mass spectra of the compounds' [M+1] peaks were observed in agreement with their predicted molecular formula (4a-4h).



Compound	R^1	R^2	R^3	Compound	R^1	R^2	R^3
4a	-H	-Cl	-H	4e	-Cl	-H	-H
4b	-H	-Cl	-Cl	4f	-Cl	-H	-Cl
4c	-H	-Cl	$-NO_2$	4g	-Cl	-H	$-NO_2$
4d	-H	-Cl	-OMe	4h	-Cl	-H	-OMe

Figure 1. *The synthesis scheme of the compounds* ***4a-4h.*** *Reagents and conditions:* ***(a)*** *K_2CO_3, Acetone, ref., 20 h;* ***(b)*** *EtOH, 0-5 °C, then r.t 3 h;* ***(c)*** *Acetone, Potassium carbonate, r.t.,* ***(d)*** *EtOH, reflux, 2h.*

3.2. ADME Parameters

The predictable physicochemical properties which are number of H-bond acceptor (HBA), H-bond donor (HBD), topologic polar surface area (TPSA, Å²), partition coefficient (Log Po/w), water solubility (Log S), gastrointestinal absorption level (GIA), skin permeation (Log Kp, cm/s), violation number of drug-likeness rules and synthetic accessibility (SA) of the final compounds and standard drug, was determined and they are shown in Table 1. Accordingly, it was determined that the compounds had an HBA number of 3-5, an HBD number of 1 and a TPSA of 83.86-129.68 Å². Log P values of the compounds were determined in between 4.56-5.89 whereas they have Log S values of – 8.72 – -7.93. Except for compounds 4c and 4g, the synthesized compounds were predicted to have high gastrointestinal absorption. The synthetic accessibility indicates that the compounds can be synthesized easily., that SA values were calculated at a range of 3.55-3.73 (1 is for very easy and 10 is for very difficult.)

Table 1. *Physicochemical, pharmacokinetic, and medicinal chemistry properties of the final compounds (by SwissAdme) (4a-4h).*

	HBA	HBD	TPSA	Log $P_{o/w}$	Log S	GIA	RoF (V)	SA
4a	3	1	83.86	5.36	-7.93	High	Yes (1)	3.55
4b	3	1	83.86	5.89	-8.59	High	No (2)	3.56
4c	5	1	129.68	4.56	-8.72	Low	Yes (1)	3.66
4d	4	1	93.09	5.29	-8.10	High	No (2)	3.70
4e	3	1	83.86	5.35	-7.93	High	Yes (1)	3.59
4f	3	1	83.86	5.86	-8.59	High	No (2)	3.60
4g	5	1	129.68	4.57	-8.72	Low	Yes (1)	3.69
4h	4	1	93.09	5.26	-8.10	High	No (2)	3.73
Ref. D.	12	6	206.07	0.52	-3.91	Low	Yes (1)	5.81

HBA: *H-bond acceptor,* ***HBD:*** *H-bond donor,* ***TPSA:*** *Topologic polar surface area (Å²)* ***Log $P_{o/w}$:*** *Consensus Log $P_{o/w}$ (Average of all five predictions),* ***Log S:*** *Water Solubility,* ***GIA:*** *Gastrointestinal absorption,* ***Log K_p:*** *skin permeation (cm/s)* ***RoF (V):*** *Rule of Five (violation number),* ***SA****: Synthetic accessibility from 1 (very easy) to 10 (very difficult).* ***Reference drug:*** *Doxorubicin*

3.3. Cytotoxic Activity

The antiproliferative activities of the final synthesized compounds (4a-4h) were tested on healthy mouse embryoblast cells NIH/3T3, lung cancer cell line A549, and breast cancer cell line MCF-7 cells. The results in micromolar are presented in Table 2. The reference compound Doxorubicin found to be non-toxic against healthy cells, and its IC50 values against A549 and MCF-7 were calculated as 10.76 µM and 5.51 µM, respectively.

Table 2. *IC_{50} values of the compounds 4a-4h against NIH/3T3, A549, and MCF-7 cell lines (μM)*

Compound	NIH/3T3	A549	MCF-7	Selectivity index A547/ MCF7
4a	>500	26.53±0.08	64.41±0.02	>18.85/>32.21
4b	319.31±0.95	31.59±0.12	246.81±0.09	10.11/1.29
4c	280.94±0.69	429.14±0.57	195.67±0.61	0.66/1.44
4d	>500	37.82±0.06	40.18±0.05	>13.22/>12.44
4e	>500	45.86±0.17	108.62±0.52	>10.90/>4.60
4f	>500	>500	>500	n.c./n.c.
4g	>500	>500	>500	n.c./n.c.
4h	461.46±0.41	69.46±0.33	127.33±0.16	6.64/3.62
Doxorubicin	>500	10.76±0.04	5.51±0.03	n.c.

n.c.: *not calculated*

Compounds 4f and 4g did not show inhibition against all three cells even at the highest concentration tested. The lowest limit of cytotoxicity of all compounds against healthy cells was observed to be an IC50 value of 280 μM. Compound 4a was determined to be the most potent compound among the compounds, with an IC50 value of 26.53 μM. This is approximately half the potency of the standard drug against A549 cells. Significant cytotoxicity potency against A549 was observed in the following order 4a, 4b, 4d, 4e, and 4h, where 4a is the most potent and 4h is the least potent compound. However, none of the compounds reached the potency of doxorubicin. The IC50 value of doxorubicin against MCF-7 was 5.51 μM, while that of compound 4d was 40.18 μM which is considered the most potent of the synthesized compounds followed by compound 4a with an IC50 value of 64.41 μM. The potencies of compounds 4e and 4h followed these compounds. On the other hand, the selectivity index calculations for the compounds' selectivity against A549 cancer cells were determined in the following order 4a> 4d>4e>4b>4h>4c while against MCF7 cancer cells were as 4a>4d>4e>4h>4c>4b.

3.4. In silico results

Possible molecular interactions of the test compounds with caspase-3, a cysteine-protease group enzyme that plays an important role during apoptosis (45, 46), were investigated using in silico methods. The best poses collected from molecular docking studies performed for both compounds are shown in Figure 2. Obtained findings indicated that both compounds docked to enzyme cavity and interacted commonly with Glu123 (aromatic H-bond), Cys163 (H-bond/ Halogen bond), Tyr204 (π-π stacking and π-cation contact/ Ar H-bond), Ser205 (H-bond), Trp206 (π-π stacking), Arg207 (Ar H-bonds), and Phe256 (π-π stacking and π-cation contact) residues. On the other hand, compound 4a interacted specifically with Gly122 (Halogen bond) while 4d formed halogen bond with Gly165 at the same cavity, in addition to that, oxygen of 4-methoxyphenyl group of 4d also formed H-bond with Ser251 residue. The interactions were summarized in Table 3. As seen in the table, hydrazide moiety was localized between loop regions (Cys163 and Arg207 residues) which are identified for allosteric stimulation.

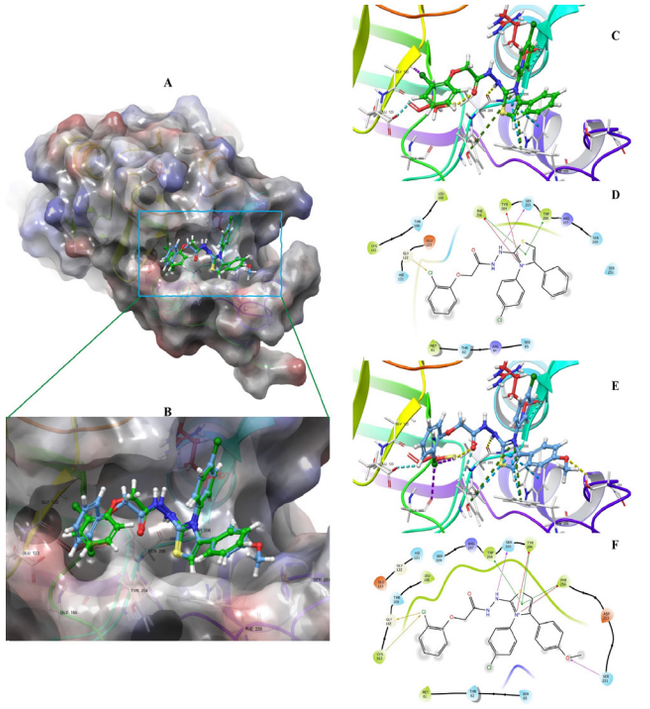


Figure 2. *Poses obtained from Molecular docking study.* ***A and B:*** *Superimposed of* ***4a*** *(green carbons) and* ***4d*** *(blue carbons) at the allosteric cavity of caspase-3 (PDBID: 4QTX);* ***C*** *and* ***D****: 3D and 2D interaction diagrams between* ***4a*** *and enzyme binding cavity;* ***E*** *and* ***F****: 3D and 2D interaction diagrams between* ***4d*** *and enzyme binding cavity.*

Table 3. *Interaction sites of the caspase-3 with the active compounds*

Compound	Moiety	Interaction type
4a	Chlorine of 2-chlorophenoxy	Gly122 (Halogen bond)
	$Hydrogen_3$ of 2-chlorophenoxy	Glu123 (Aromatic H-bond)
	Oxygen of hydrazide	Cys163 (H-bond)
	$Nitrogen_2$ of hydrazide	Ser205 (H-bond)
	Nitrogen of thiazole	Trp206 (π-cation)
		Phe256 (π-cation)
	$Hydrogen_3$ of 4-chlorophenyl	Arg207 (Ar H-bond)
	$Hydrogen_2$ of 4-chlorophenyl	Arg207 (Ar H-bond)
	Thiazole ring	Trp206 (π-π stacking)
		Phe256 (π-π stacking)
4d	$Hydrogen_3$ of 2-chlorophenoxy	Glu123 (Aromatic H-bond)
	Chlorine of 2-chlorophenoxy	Cys163 (Halogen bond)
		Gly165 (Halogen bond)
	Oxygen of hydrazide	Tyr204 (Ar H-bond)
	Nitrogen of thiazole	Tyr204 (π-cation)
		Phe256 (π-cation)
	$Nitrogen_2$ of hydrazide	Ser205 (H-bond)
	$Hydrogen_3$ of 4-chlorophenyl	Arg207 (Ar H-bond)
	$Hydrogen_2$ of 4-chlorophenyl	Arg207 (Ar H-bond)
	Thiazole ring	Tyr204 (π-π stacking)
		Trp206 (π-π stacking
		Phe256 (π-π stacking)
	Methoxy of 4-methoxyphenyl	Ser251 (H-bond)

4. DISCUSSION

Eight new thiazole-hydrazide analogs (4a-4h) were designed and synthesized in this work. The identity and purity of the final molecules were confirmed by using analytical and spectral methods. The 1H-NMR spectra of the compounds indicated that the signals at δ 3.82-4.80 ppm were observed as singlet peaks for Ph-OCH3 protons. O-CH2 protons peaked as a singlet at δ 4.68-4.86 ppm. The propanehydrazide N–H proton was observed as a broad singlet peak at δ 11.42-11.88 ppm. The appearance of a pair of singlet, doublets, triplets and/or multiplets at δ 6.57–8.25 ppm indicated that the aromatic protons. Meanwhile, the 13C-NMR spectra of final molecules peaked at δ 55.77–55.80 ppm for Ph-OCH3 carbon, at δ 66.68-66.94 ppm for O-CH2 carbon, at δ 96.67-160.79 ppm for aromatic carbon and at δ 167.48-167.99 for the carbonyl carbon (C=O). M±1 peaks in HRMS spectra agreed with the calculated molecular weight of the final compounds (4a-4h).

To determine their antitumor potential, they were evaluated on A549 lung carcinoma and MCF7 breast adenocarcinoma cells. They were also tested against the NIH/3T3 healthy cell line to ascertain the selective cytotoxicity on cancer cells. Results showed that even at the highest dose tested, 4f and 4g, were unable to demonstrate inhibition against all three cells. On the other hand, 4a, 4b, 4d, 4e, and 4h exhibited significant cytotoxicity and a selective profile against A549 while they also inhibited MCF7 cells, except 4b because of its low selectivity on cancer cells.

When molecular structures are examined, the serial compounds were divided into two parts according to whether they contained N-(2-chlorophenyl)thiazole or N-(4-chlorophenyl)thiazole substitution. Both series were derived by substituting the phenyl ring at the 4th position of the thiazole ring with the same groups (H, 4-Cl, 4-NO2, 4-OCH3). At the end, compounds 4c and 4g were the lowest cytotoxicity profile against both cancer cells in the series. These molecules are containing nitro group and chlorine atom together.

It was determined that derivative containing non-substituted phenyl ring (4a) were more active than the derivatives containing other substituents (Cl, NO2 and OCH3) against A549 cancer cells. Similar to previous results (10, 27), the results indicated that the small groups more attractive in contributing to the anti-non-small cell lung carcinoma (NSCLC) activity than bulky groups. In another previous study (39), it was declared that the non-substituted thiazole ring is more effective than the 4-substituted thiazole analogs because of its ability to form interactions via nitrogen and aromatic ring system.

The major point is that para-chlorophenyl analogs at the fourth position on thiazole displayed a better anticancer profile than ortho-chrlorophenyl analogs. Among them, 4a against for A549 cells and 4d against for MCF7 were reported as more promising compounds than others.

The results of in vitro studies revealed that compounds 4a and 4d may be effective agents for both NSCLC and breast cancer. Within the scope of this study, the effects of these two compounds, which were comparable to the standard drug, and their possible relationship with apoptosis were also investigated. Based on the knowledge that the caspase-3 enzyme plays a role in intrinsic and extrinsic pathways in both types of cancer (48, 49), possible allosteric activation of the caspase-3 enzyme was investigated by molecular docking studies. As seen clearly in Figure 2-B, the superimposed pose indicated that the variable groups have an impact directly on the localization of the 2-chlorophenoxy as an invariable moiety of the final molecules while the 4-chlorophenyl group was stabilized and was not affected by the variable groups. These findings revealed that hydrazinoacetyl moiety enabled the rotation of the ligands, thus, its flexibility was found important for the activity since the ligands can take a position against loop amino acids, that's why these interactions between ligand and loop residues stabilized the ligand-protein complex. The results of in silico studies suggest that molecules carrying thiazole and hydrazinoacetyl pharmacophores in their structures may lead to the death of cancer cells through caspase-3 activation, and the in vitro anticancer activities of these compounds revealed in this study may be mediated by the activation of apoptotic mechanisms.

In the ADME estimation studies, obtained results showed that there was no more than one violation of Ro5 (Lipinski's rule of five) (47). These results of basic computational chemistry suggested that the final compounds might have good pharmacokinetic profiles. Therefore, it can be suggested that the hit molecules (4a, 4c, 4e, and 4g) might be good candidate anticancer agent(s).

5. CONCLUSION

In the scope of this study, new thiazole-hydrazide structured compounds that may play a role in the treatment of non-small cell lung cancer and breast cancer were designed and structural analyses were performed after the synthesis with the high yield. Then, the activity profiles were evaluated on A549 lung carcinoma and MCF7 breast adenocarcinoma cell lines. They were also tested against the NIH/3T3 healthy cell line to ascertain the selective cytotoxicity on cancer cells. Results showed that 4a, 4c, 4e, and 4g appear to have good druggable profiles. However, even at the highest dose tested, 4f and 4g, the bulkiest analogs in the series, were unable to demonstrate inhibition against all three cells. On the other hand, 4a, 4b, 4d, 4e, and 4h exhibited significant cytotoxicity and a selective profile against A549 while they also inhibited MCF7 cells, except 4b because of its low selectivity on cancer cells. The major point is that para-chlorophenyl analogs at the fourth position on thiazole displayed a better anticancer profile than ortho-chlorophenyl analogs. Among them, 4d and then 4a were reported as more promising compounds than others. Furthermore, analyzing the molecular docking study for promising compounds (4a and 4d) suggested that

interactions with the loop region residues have a pivotal role in inducing caspase-3 enzyme activity. Briefly, the 3-substituted thiazole-hydrazide combination is marked as a promising pharmacophore structure against non-small cancer cells.

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Design of the study: *AEE, LY*
Acquisition of data for the study: *SD, ABK, DN, AEE*
Analysis of data for the study: *SD, ABK, DN, AEE*
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Drafting the manuscript: *AEE, LY, SD, DN*
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