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DNA Interactions, Mutagenic, Anti-Mutagenic And Antimicrobial Activities of (E)-2-((3,5-Bis(Trifluoromethyl)Phenylimino)Methyl)-4,6-Dimethoxyphenol

Nuray YILDIRIM^{*1}, Neslihan DEMİR²

Abstract

Small molecules that interact with DNA are known to be effective as anticancer and antimicrobial agents. Therefore it is significant to search for new molecules interacting with DNA as potential new therapeutic agents. In this study, we aimed to investigate interactions of novel fluorine substituted imine compound with DNA, (E)-2-((3,5-bis(trifluoromethyl)phenylimino)methyl)-4,6-dimethoxyphenol, and investigate its biological activities. DNA interactions of the compound were investigated by UV-Vis absorption spectroscopy and gel electrophoresis. The results demonstrated that the compound binds to DNA via intercalation. Agarose gel electrophoresis experiments showed that the compound does not cleave pBR322 plasmid DNA hydrolytically or oxidatively. Furthermore, mutagenic, anti-mutagenic, and antimicrobial activities of the compound were studied by Ames and broth microdilution test, respectively. The compound showed mutagenic activity on both TA98 and TA100 strains. Also, the antimutagenic activity was observed in TA100 strain of *S. typhimurium*. It demonstrated antimicrobial activity against the microorganisms tested in the concentration range of 16-64 µg/µL. The results show that the compound intercalates with DNA and has promising biological activities.

Keywords: DNA binding, DNA cleavage, mutagenicity, antimicrobial activity

1. INTRODUCTION

DNA is the main target for many drugs including anticancer drugs and antibiotics since DNA codes the genetic information to carry out replication and transcription in the cells. Small molecules interacting with DNA were reported as effective anticancer, antiviral, and antibiotic agents [1-2]. Cisplatin is the first inorganic antineoplastic drug,

which was used in modern medicine to treat several cancers [3]. However, due to the adverse side effects and high toxicity, its effectiveness is limited so, there is a necessity for developing more effective, less toxic anticancer drugs [4]–[6]. Today, many antitumors, antiviral, and antibiotics that are used clinically show their effects by binding to DNA, such as netropsin, mithramycin, etc. [7]. Therefore, it is significant

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to search novel molecules for developing more effective anticancer drugs [2].

During the last decade, there has been an increasing focus on the small molecule-DNA binding studies, because many drugs perform their antitumor effects via binding to DNA consequently inhibiting the replication process and resulting in growth inhibition and death of the cells [8]. Developing new drugs that target the DNA requires an understanding of how these molecules interact with DNA [9]. Small molecules non-covalently bind to DNA via three binding modes: groove binding, intercalation, and electrostatic interactions. Intercalation, and groove-binding are the most effective binding modes leading to cell degradation [10-11].

Imine compounds are an important class of compounds and have many applications in medicine and pharmaceutical fields as antibacterial, antifungal, and chemotherapeutic agents [12-14]. Fluorine substitution is a widely used strategy for developing drugs that can change the conformation, membrane permeability, and electrical charge of a ligand [15]. Keeping the view the facts mentioned, we decided to study a novel fluorine substituted imine compound, (E)-2-((3,5-bis(trifluoromethyl)phenylimino)methyl)-4,6-dimethoxyphenol, which we predicted to have useful biological activities. For this purpose, we studied DNA binding properties of the compound by UV-Vis spectroscopy and DNA cleavage activity with gel electrophoresis techniques. Furthermore, mutagenic, antimutagenic, and antimicrobial activities were investigated by the Ames and broth microdilution tests, respectively.

2. MATERIALS AND METHODS

2.1. Materials

All chemicals and reagents were obtained commercially and prepared as instructed by the manufacturer. The ^1H and ^{13}C NMR spectra were recorded on a Bruker AVANCE- 500 spectrometer operating at 400 and 101,6 MHz. Infrared absorption spectra were obtained from a Perkin Elmer BX II spectrometer in KBr discs and

were reported in cm^{-1} units. The UV-VIS spectra were measured using a SHIMADZU 1800 series spectrometer. Elementary analyses were performed on a Vario EL III CHNS elemental analyzer. Melting points were measured with an Electro Thermal IA 9100 apparatus using a capillary tube. 3,5-Dimethoxysalicylaldehyde, 3,5-bis(trifluoromethyl)aniline, calf thymus DNA (CT-DNA), ethidium bromide, Mueller Hinton Broth, RPMI 1640 were purchased from Sigma-Aldrich. pBR322 supercoiled plasmid DNA and EcoRI enzyme were purchased from Thermo Fisher Scientific.

DNA stock solution was prepared by the manufacturer's instructions and stored at 4°C for up to ten days. To determine the purity and concentration of the CT-DNA, UV absorbance was measured at 260 nm and 280 nm. A_{260}/A_{280} gave a ratio of 1.8-1.9 demonstrating that DNA was free of protein. The molar concentration of the DNA was calculated from its absorption intensity at 260 nm with a molar extinction coefficient of $6600\text{ M}^{-1}\text{cm}^{-1}$ [16].

2.2. Synthesis of 2-((3,5-bis(trifluoromethyl)phenylimino)methyl)-4,6-dimethoxyphenol

3,5-Dimethoxysalicylaldehyde (0.396 g, 2.18×10^{-3} mol) was added to EtOH (100 mL) solution of 3,5-bis(trifluoromethyl)aniline (0.499 g, 2.18×10^{-3} mol). The mixture was stirred and refluxed for 1 h. Compound was obtained from the evaporation of EtOH (Fig. 1). It was crystallized from CHCl_3 :n-hexane (3:2) as a yellow crystal, mp 125°C , 0.73 g (85%) yield. Found: C, 51.85; H, 3.31; N, 3.56. Calc. For $\text{C}_{17}\text{H}_{13}\text{F}_6\text{NO}_3$; C, 51.90; H, 3.30; N, 3.56 %. IR(KBr, cm^{-1}); $\nu_{\text{O-H}}$; 3435 m, $\nu_{\text{Ar-H}}$; 3051 w, $\nu_{\text{C-H}}$; 2980-2940-2848 m, $\nu_{\text{C=N}}$; 1631 s, $\nu_{\text{C=C}}$; 1601-1571-1517 s, $\nu_{\text{C-N}}$; 1474 s, $\nu_{\text{C-O}}$; 1371 s, $\nu_{\text{C-O-C}}$; 1180-1170-1100 s. $^1\text{H-NMR}$ (DMSO); δ ppm, 10.03 (s, 1H, Ar-OH); 9.03 (s, 1H, Ar-CH=N-); 8.10-6.09 (m, 7H, Ar-H); 3.81 and 3.79 (s, 3H and 3H, OCH_3). $^{13}\text{C-NMR}$ (DMSO); δ ppm, 119.79 (s, 1C, C1); 162.13 (s, 1C, C2); 165.25 (s, 1C, C3); 103.33 (s, 1C, C4); 166.60 (s, 1C, C5); 106.38 (s, 1C, C6); 168.03 (s, 1C, C7); 161.38 (s, 1C, C8); 125.32 (s, 2C, C9); 150.50 (s, 2C, C10); 122.57 (s, 1C,

C₁₁); 130.63 (s, 2C, C₁₄); 94.29 (s, 1C, C₁₅); 91.38 (s, 1C, C₁₆).

2.3. DNA Binding

The UV-Vis absorption spectra titrations were investigated to study the binding affinity between DNA and ligand in 5 mM Tris-HCl, 50 mM NaCl buffer (pH 7.2) at room temperature. 1.6 mL solutions of the ligand (4×10^{-5} M) and the blank were added into quartz cuvettes. One aliquot of the CT-DNA solution (5.25 mM) was added to each cuvette. To eliminate the absorbance of DNA itself, it was also added to the blank. In each step, the ligand-DNA solution was incubated for 5 min at room temperature.

2.4. Cleavage Activity

DNA cleavage experiments were studied by agarose gel electrophoresis [17]. pBR322 supercoiled plasmid DNA ($0.1 \mu\text{g } \mu\text{L}^{-1}$) treated with the various concentrations of the ligand (25-400 μM) in the presence or absence of H_2O_2 in Tris-HCl buffer (10 μM , pH 7.2) and incubated for 3 h at 37 °C. H_2O_2 was used as an oxidizing agent to investigate oxidative cleavage activity. Then, loading buffer was added to reaction mixture and run on 1% agarose gel in TBE (Tris-Boric acid- EDTA, pH 8.0) buffer at 60 Volt for an hour. Bands were visualized by UV light.

2.5. Mutagenic Activity and Antimutagenic Activity

Potential mutagenic and antimutagenic activities of the compound were studied with the Ames/*Salmonella* test system using the standard plate incorporation method [18]. The experiments were carried out with *Salmonella typhimurium* TA98 (for frame-shift mutagens) and TA100 (for base-substitution mutagens) strains in the absence of S9 metabolic activation [18-19]. In mutagenicity assay, 0.1 mL of each bacterial culture and 0.5 mL of sodium phosphate buffer (0.2 M, pH 7.4) were mixed with 2 mL of top agar which contained different concentrations of the compound. This mixture was then poured onto minimal agar plates (MGA). After the incubation of the plates for 48 h at 37 °C, the revertant

bacterial colonies (his^+) were counted on each plate. In the antimutagenic activity assay, the standard mutagens were also added to the mixture before pouring onto MGA.

During the mutagenicity and antimutagenicity studies, as parallel to the study, the positive, negative, and spontaneous controls were used. The standard mutagens 4-nitro-*o*-phenylenediamine (NPD) for TA98 strain and sodium azide (SA) for TA100 were used as positive controls, Dimethyl sulphoxide (DMSO) was used for both strains as negative control.

A compound was considered mutagenic when the two-fold increase was detected in the revertant colonies as compared to negative control [19]. The antimutagenic activity was calculated according to the rate of inhibition using the given formula:

$$\text{Inhibition rate (\%)} = (A-B/A-C) \times 100 \quad (1)$$

Where A is the number of revertant colonies in the plate containing mutagen, B is the number of revertant colonies in the presence of mutagen and compound/plate, C is the number of spontaneous colonies/plate.

The antimutagenic effect was considered moderate when the inhibition rate was 25–40% and strong when it was more than 40%. Inhibitory rate less than 25% was considered as not antimutagenic [20-22].

2.6. Antimicrobial Activity

To determine antimicrobial activities of the compound against bacterial and fungal strains; *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 254992, *Escherichia coli* ATCC 35218, *Bacillus cereus* NRRL B-3711, *Proteus vulgaris* ATCC 13315, *Candida albicans* ATCC 60193, and *Candida tropicalis* ATCC 13803 reference strains were used. Minimal inhibitory concentration (MIC) of the compound was determined using the broth microdilution method according to procedures

and principles of the Clinical Laboratory Standards Institute [23].

Gentamicin and Fluconazole were used as positive controls. The compound stock solution was prepared with DMSO which was tested against the microorganisms and showed no effect. 100 μ L of Mueller Hinton Broth for bacteria and RPMI 1640 medium for fungi were added in 96-well microplates. Two-fold dilutions of compounds and reference drugs were distributed into the wells (256-0.5 μ g/mL). Inoculum suspensions of the bacteria and fungi strains were prepared and adjusted to 0.5 McFarland turbidity and 100 μ L of inoculum were added to the wells. After addition of inoculum, each well contained approximately 5×10^5 CFU/mL bacterial concentration in the final test. Then plates were incubated at 37 °C for 16-20 h for bacteria and 20-24 h for fungi. The lowest concentration of compound that prevented visible growth was evaluated as MIC.

3. RESULTS AND DISCUSSION

3.1. FT-IR, ^1H NMR and ^{13}C NMR Spectroscopic studies

The FT-IR, ^1H -NMR and ^{13}C -NMR data are given in synthesis for the compound. The OH, C=N, C=O and C-O-C vibration band are observed at 3435, 1631, 1371 and 1180-1170-1100 cm^{-1} of compound, respectively (Fig. 1). The ^1H -NMR data for the compound show that the tautomeric equilibrium favours the enol-imine in DMSO. The $-\text{OH}$ and $-\text{CH}=\text{N}-$ protons are observed at 10.03 and 9.03 ppm singlets for the compound. The phenyl protons of the compound gave multiplets at 8.10-6.09 ppm (Fig. 2). According to the ^{13}C -NMR spectra, the compound has 14 signals, showing that the structures in solution are symmetrical (Fig. 3).

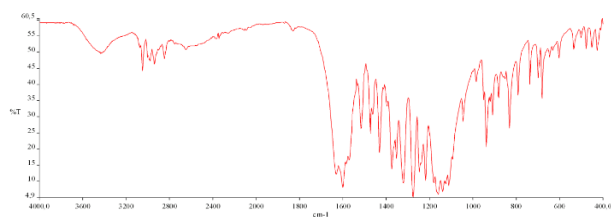


Figure 1 FT-IR spectrum of the compound

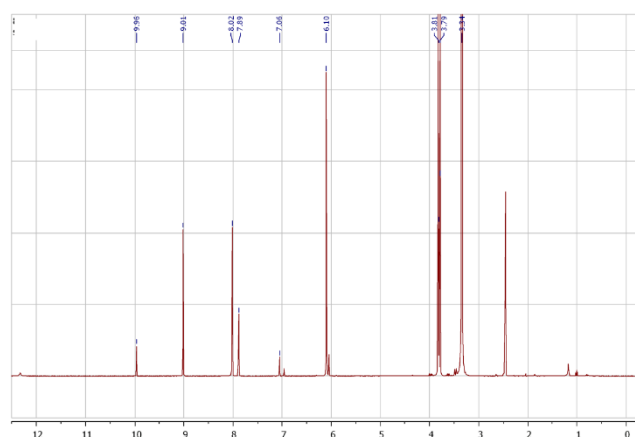


Figure 2 ^1H -NMR spectrum of the compound

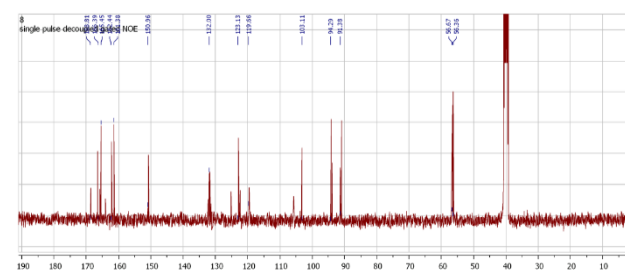
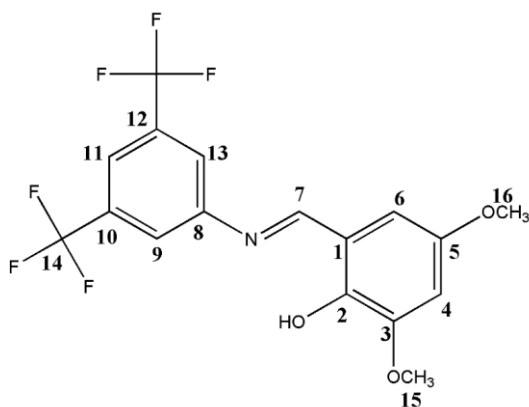


Figure 3 ^{13}C -NMR spectrum of the compound

3.2. DNA Binding

It is widely known that for many antitumor drugs DNA is a primary pharmacological target. Therefore, studying interactions between DNA and new compounds are important to discover new potential drugs and understand the mechanism of binding. Spectrophotometric titration is a widely used technique to investigate small molecule-DNA interactions and binding affinity of a molecule [24]. Thus, whether the compound (Fig. 4) has a binding ability to the CT-DNA was studied by UV spectra titrations. The absorption spectrum of the compound at 20 μM concentration, in the absence and presence of the CT-DNA, is shown in Fig. 5.

Binding of a small molecule to DNA usually causes changes in the absorbance and shift in the wavelength [24]. The compound as seen in Fig. 5 shows maximum absorbance in 353 nm and after



2-((3,5-Bis(trifluoromethyl)phenylimino)methyl)-4,6-dimethoxyphenol

Figure 4 Chemical formula of the compound

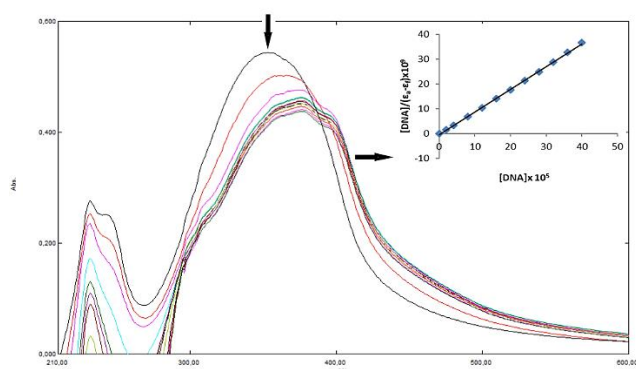


Figure 5. Absorption spectra of the compound in the absence and presence of increasing amounts of CT-DNA. Conditions: $[DNA]/[Compound]=0-10 \mu M$. Inset: Plot of $[DNA]/(\epsilon_a-\epsilon_b)$ versus $[DNA]$ for the titration of DNA with the compound.

the addition of increasing amounts of DNA, while compound concentration was fixed, its absorption spectra showed %8-19 hypochromic effect and 24 nm red-shift. Hypochromism and red-shift are associated with intercalation binding mode suggesting that there is a strong stacking interaction where an aromatic chromophore inserted between DNA base pairs [5]. After a ligand intercalates to the DNA, the π^* orbital of the intercalated ligands could pair up with π orbitals of the base pairs on the DNA, hence decreases the $\pi-\pi^*$ transition energies [10], [25]. Therefore, the extend of the hypochromic effect is usually associated with the strength of intercalation [26]. This result shows that the compound binds to DNA via intercalation.

To determine DNA binding affinity, the intrinsic binding constant K_b was calculated by using the equation:

$$[DNA]/(\epsilon_a-\epsilon_b) = [DNA]/(\epsilon_b-\epsilon_f) + 1/K_b(\epsilon_a-\epsilon_b) \quad (2)$$

Where, K_b is the binding constant, $[DNA]$ is the concentration of the DNA in base pairs, ϵ_a is apparent coefficient, ϵ_b is the extinction coefficient of the fully bound drug, ϵ_f extinction coefficient of the free drug [27]. By using this equation, the binding constant K_b was found $2.5 \times 10^5 M^{-1}$. In the intercalation process, planar molecules insert between the base pairs of the DNA and change the helical structure, resulting in lengthening of the DNA. It is reported that even though this process needs a serious amount of free energy, with the favorable help of hydrogen bonding, hydrophobic, ionic, and van der Waals forces give rise to 10^5 to $10^{11} M^{-1}$ association constants [28-29]. Results of the experiment show that the binding constant of the compound indicates intercalation binding mode.

3.3. DNA Cleavage Activities

DNA cleavage activities of the compound were determined by agarose gel electrophoresis. When agarose gel electrophoresis conducted, supercoiled plasmid DNA migrates faster due to its size while nicked plasmid DNA migrates relatively slow. Linear form migrates between these two.

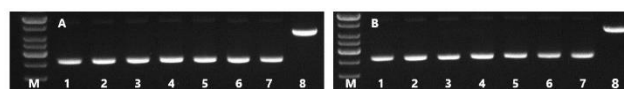


Figure 6. DNA cleavage activity of the compound. A. Hydrolytic cleavage B. Oxydative cleavage. M. Marker (1 Kb), 1. Supercoiled pBR322, 2-7. Supercoiled pBR322+ 5, 25, 50, 100, 200, 400 μM compound), 8. Positive control (linearized pBR322 using EcoRI enzyme).

The cleavage activity of the compound was studied by using supercoiled pBR322 plasmid DNA as a substrate in Tris-HCl buffer under physiological conditions. The compound was tested for hydrolytic and oxidative cleavage activities with concentrations in the range of 5-

400 μM and as shown in Fig. 6 no cleavage activity was observed.

3.4. Mutagenic and Antimutagenic Activities

As shown in the Table 1, there is almost a 2-fold increase in the number of revertants at the concentration of 50 and 500 ppm in compare to negative control (12 ± 1.09) for TA98, and there is even more than 3-fold increase in the number of revertants at the concentrations of 5, 50, and 500 ppm in compare to negative control (148 ± 2.65) for TA100. Based on these findings, the compound shows mutagenic activity on both TA98 and TA100 strains.

Although DNA intercalators have a capacity to alter the genetic material of the cell and can result in a toxic effect, not all intercalators have a genotoxic effect. For toxicity, basic electrophilic or cationic functional groups are often necessary [29-31].

The compound did not have antimutagenic activity on strain TA98 (< 25% inhibition) but had antimutagenic activity on strain TA100. The moderate antimutagenic activity was observed at 0.5 and 5 ppm/plate concentrations (25-40% inhibition) The strongest antimutagenic activity was observed at 50 and 500 ppm/plate concentration (>40% inhibition) against *S. typhimurium* TA100 strain (Table 2).

Table 1

Mutagenicity assay results of the compound for *S. typhimurium* TA98 and TA100 strains

Sample/ Standard	Concentration (ppm)	His ⁺ Revertant Number of Colony /Plate	
		TA98	TA100
		Mean \pm SE	Mean \pm SE
Positive Control	NPD	10^{-2}	770 ± 5.06
	SA	10^{-3}	1043 ± 5.41
Compound		0.5	13 ± 0.05
		5	15 ± 0.06
		50	21 ± 0.14
		500	23 ± 0.08
	Negative Control	DMSO	12 ± 1.09
Spontaneous Control		33 ± 2.16	124 ± 5.89

* NPD: 4-nitro-*o*-phenylenediamine, SA: Sodium azide, DMSO: Dimethyl sulphoxide

Table 2

Antimutagenicity assay results of the compound for *S. typhimurium* TA98 and TA100 strains

Treatment	Concentration (ppm)	His ⁺ Revertant Number of Colony /Plate			
		TA98	% Inhibition	TA100	% Inhibition
		Mean \pm SE		Mean \pm SE	
Positive Control	NPD	10^{-2}		1006 ± 18.48	
	SA	10^{-3}			
Compound		0.5	8.29	706 ± 10.74	33.71
		5	12.83	638 ± 9.67	36.58
		50	16.31	647 ± 8.55	40.33
		500	6.68	572 ± 6.23	48.76
	Negative Control	DMSO	12 ± 1.05		140 ± 6.53
Spontaneous Control		26 ± 3.01		116 ± 5.89	

* NPD: 4-nitro-*o*-phenylenediamine, SA: Sodium azide, DMSO: Dimethyl sulphoxide

Table 3

MIC ($\mu\text{g}/\mu\text{L}$) of the compound

Microorganisms	Compound ($\mu\text{g}/\mu\text{L}$)	Gentamicin ($\mu\text{g}/\mu\text{L}$)	Fluconazole ($\mu\text{g}/\mu\text{L}$)
<i>Staphylococcus aureus</i> ATCC 25923	64	8	-
<i>Enterococcus faecalis</i> ATCC 29212	64	8	-
<i>Bacillus cereus</i> NRRL B-3711	64	1	-

<i>Bacillus subtilis</i> ATCC 6633	32	1	-
<i>Escherichia coli</i> ATCC 25922	32	0.125	-
<i>Escherichia coli</i> ATCC 35218	64	0.125	-
<i>Pseudomonas aeruginosa</i> ATCC 254992	32	0.125	-
<i>Proteus vulgaris</i> ATCC 13315	32	1	-
<i>Candida albicans</i> ATCC 60198	16	-	0.125
<i>Candida tropicalis</i> ATCC 13803	32	-	1

3.5. Antimicrobial Activities

Antibacterial and antifungal activities of the compound were screened using the broth microdilution method according to the procedures of CLSI [23]. The lowest concentration of the compound that inhibits the visible growth of the microorganisms was recorded as MICs. Average data from the three replicated experiments are presented in Table 3. The compound inhibited the growth of the tested microorganisms and showed antibacterial and antifungal activity with the MIC values in the range of 32-64 $\mu\text{g}/\mu\text{L}$ and 16-32 $\mu\text{g}/\mu\text{L}$, respectively. However, the compound was less effective compared to reference drugs gentamicin and fluconazole.

The compound demonstrated similar antibacterial and antifungal effect against the tested bacteria and fungi. Among the tested microorganisms the compound was the most effective against *C. albicans* with the MIC value of 16 $\mu\text{g}/\mu\text{L}$.

4. CONCLUSION

The most encouraging ways to develop more effective drugs is to research new molecular structures that present more effective antitumor activities. Therefore, many scientists have been driven to look for new molecules with better biological activities.

In this paper, we studied the DNA binding properties of novel fluorine substituted imine compound, (E)-2-((3,5-bis(trifluoromethyl)phenylimino)methyl)-4,6-dimethoxyphenol. In addition, cleavage activity, mutagenicity, antimutagenicity, and antimicrobial activity of the compound were also investigated. The compound displayed intercalative binding to CT-DNA with the $K_b = 2.5 \times 10^5 \text{ M}^{-1}$. The observed value of binding constant K_b shows that the compound is

strongly bound with CT-DNA. Although, it is lower than the classical intercalators like ethidium bromide ($7 \times 10^7 \text{ M}^{-1}$), it is comparable to other intercalators such as propidium ($3.1 \times 10^5 \text{ M}^{-1}$), DAPI ($1.2 \times 10^5 \text{ M}^{-1}$) [32-33]. Cleavage experiments using pBR322 supercoiled plasmid DNA showed that the compound does not have cleavage activity. Although DNA intercalators have the ability to alter the DNA, not all intercalators have cleavage activity. Many anticancer drugs initiate their extended damage to the DNA by their cleavage activity which eventually leads cells to apoptosis and cell death. However, even without the cleavage activity, intercalation alters the DNA structure and this prevents DNA replication and transcription by interfering with the action of topoisomerases [34]. The compound showed mutagenic activity on both TA98 and TA100 strains. The compound demonstrated antimutagenic activity only on TA100 strain. Intercalation in DNA can alter the helical structure of the DNA in the cell and this may lead to triggering the activity of the DNA repairing mechanisms which can explain antimutagenic activity of the compound [35]. The compound showed antimicrobial activity against tested microorganisms in the concentration range of 16-64 $\mu\text{g}/\mu\text{L}$.

The results show that the compound has promising biological activities that may have potential practical applications. Further studies are needed to investigate its potential pharmacological properties such as topoisomerase inhibition, DNA footprinting for sequence specificity and cytotoxicity assay to determine its activity on human cells. The results obtained from this study will be useful in understanding the molecular intercalation mechanism and in designing its potential biological and pharmacological effects in the future.

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Conflict of Interest

There is no conflict of interest in this study.

Authors Contributon

N.Y: Writing the article, general literature review, experimental section of DNA binding and antimicrobial activities, general data analysis, discussion of all experimental results, and conclusions.

N.D: Experimental section of mutagenic and antimutagenic activities, data analysis, discussion of results and conclusions.

Ethics Committee Approval

This paper does not require ethics committee permission or any special permission.

Research And Publication Ethics

In the writing process of this study, international scientific, ethical and citation rules were followed, and no falsification was made on the collected data. Sakarya University Journal of Science and its editorial board have no responsibility for all ethical violations. All responsibility belongs to the responsible author and this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

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