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FULL PAPER

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Biological evaluation and pharmacophore modeling of some benzoxazoles and their possible metabolites

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Abstract

A series of benzoxazole derivatives and some possible primary metabolites were evaluated as anticancer agents. *In vitro* anti-proliferative activities of the compounds were tested using the SRB assay on cancerous (HeLa) and non-cancerous (L929) cell lines. It was found that 17 of 21 tested compounds had cytotoxic activity on HeLa cells and the cytotoxic activities of the compounds were 15–700 times higher than on L929 cells. We generated two distinct pharmacophore models for the cytotoxic activities of the compounds on HeLa and L929 cells. While active compounds such as camptothecin and **X8** fitted the two models generated for both cell lines, selective cytotoxic compounds such as **XT3B** fitted only the model generated for HeLa cells. Evaluation of the genotoxic activities of the cytotoxic compounds with the alkaline comet assay revealed that compounds **X17** and **XT3** showed strong genotoxic effects against HeLa cells at low concentrations whereas they had no genotoxic effect on L929 cells. Due to the selective ability for inducing DNA strand breaks only on cancerous cells, the compounds were identified as effective derivatives for anticancer candidates.

KEYWORDS
anticancer activity, benzoxazole, comet assay, pharmacophore, SRB assay

1 | INTRODUCTION

Cancer is a disease caused by the uncontrolled proliferation of cells. Due to its complexity and the leading high rates of mortality worldwide, it is one of the most studied diseases investigated from different perspectives. While some researchers seek to discover its cellular mechanisms, others study to develop new treatment regimens to slow the progress of the disease and to prolong life of the patient. New anticancer agents are consistently being developed in many ways to target disease-related various cellular mechanisms and different biological molecules such as enzymes, hormones, and receptors are being investigated. However, the discovery and development of new treatments are still urgently required due to the problems with current treatments, such as toxicity and drug-resistance.[1,2] It has been reported that the anti-tumor efficacy of chemotherapeutic agents correlates with their growth inhibiting, differentiation-inducing or apoptosis-inducing abilities.[3]

Heterocycle compounds are defined as an important class of chemical entities in life science research. A benzoxazole skeleton is a constituent of several natural products and is often incorporated in drug design because of its structural similarity to purine bases. A benzoxazole scaffold may engage in a number of distinct energetically favorable interactions with its host protein. Both the 1-oxygen as well as the 3-nitrogen atoms can act as HBAs, and due to its aromatic planar nature both π-π stacking and π-cation interactions are possible. Due to its lipophilic character, hydrophobic interactions with its host protein are possible.[4] Moreover, they have, in fact, high versatility in chemical modifications, allowing changes to the characteristics of side-chains on a rigid platform.[5] Especially 2-substituted benzoxazoles have been well

characterized pharmacologically up to today. It has been reported that 2-substituted benzoxazole derivatives investigated as ligands for one or more biological targets have exhibited various types of biological properties such as anti-inflammatory,[6] analgesics,[7] antiepileptic,[8,9] antimalarial,[10] anti-HIV,[11,12] anticancer,[13–17] topoisomerase inhibitors,[18] kinase inhibitors,[19,20] protease inhibitors,[15] GSH inhibitors,[21] and cyclooxygenase inhibitors.[22] Anticancer activities of 2-substituted benzoxazole derivatives were of a great interest after the discovery that a natural compound 2-substituted bis(benzoxazole), UK-1, had anti-cancer activity against lymphoma and leukemia.[23]

Information in the literature about biotransformation of benzoxazoles is inadequate. However, several studies suggest that primary metabolites of benzoxazoles are amid derivatives, which form from the hydrolysis of the oxazole ring.[24] As with benzoxazoles, it has been reported that benzamide and phenylacetamide derivatives exhibit various antimicrobial,[25,26] antiviral,[27] analgesic,[28] anticonvulsant,[29] antihelmintic,[30] and especially anticancer[31–36] biological properties. Imatinib, structurally a phenyl benzamide derivative, is a well-known first targeted anticancer agent and a small molecule kinase inhibitor used to treat certain types of cancer. According to cell culture studies, IC_{50} values of imatinib against different cell lines varied from 0.073–100 μM.[37]

We previously synthetized a series of 2-phenyl/benzyl benzoxazole derivatives and some possible primary metabolites (benzamide and phenylacetamides) and investigated their antimicrobial activities.[38,39] In this study, we focused on their *in vitro* anticancer activities based on investigations in the literature about anticancer activities of both benzoxazole, benzamide, and phenylacetamide derivatives. So the main goal of this research was to develop new potential anticancer agents. It is important that an anticancer agent has cytotoxic activity on cancer cells but is not cytotoxic against normal cells. Thus, we evaluated cytotoxic activities of test compounds on cancer and non-cancerous cell lines. It is known that some cytotoxic agents cause DNA damage in the cells which results in cell death and rapidly divided cancer cells were affected by the damage more than normal cells. Many cytotoxic agents commonly used to treat cancer patients cause high levels of DNA damage, that lead to cell cycle arrest and/or cell death.[40] As a consequence, we assessed DNA damaging effects of more cytotoxic compounds (**X8, X23, XT3, X17,** and **XT9B**) for HeLa cancer cells with comet assay on both HeLa and L929 cells.

In this study, we also used generating pharmacophore hypothesis methods to analyze the structure–activity relationships between biological activity and molecular structures of the tested compounds. Three-dimensional approaches such as HipHop and HypoGen are useful in building 3D pharmacophore models from the activity data and conformational structure. However, for HypoGen ranging activity values of a collection of conformational models of compounds there should be at least four orders of magnitude. In the other algorithm in 3D pharmacophore generation, HipHop, the scalar affinity values of the molecules are not regarded and this model is based on alignment of common features present in highly potent compounds.[41]

2 | RESULTS

2.1 | *In vitro* cytotoxicity study

In vitro anticancer activities of nine 2-substitute phenyl/benzyl benzoxazoles, 12 benzamide and phenylacetamide derivatives were screened on HeLa (human cervix adenocarcinoma) and L929 (rat fibroblast) cell lines by SRB cytotoxicity assay. Both cell lines were incubated with different concentrations of compounds at 48 h and the % survival for each was calculated. Then IC_{50} values for each compound were determined by S-probit analyses. To evaluate cell line specific cytotoxic activities specificity index (SI) calculated by IC_{50}L929/ IC_{50}HeLa formula were used. All cytotoxic activity data of the compounds were summarized in Table 1. We defined the compounds, which had IC_{50} value less than 100 μM as cytotoxic.

2.2 | Analysis of DNA damage with the comet assay

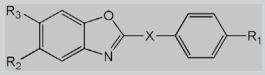
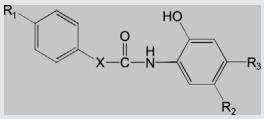
Single cell gel electrophoresis (Comet assay) is an available and common method for measuring DNA damage in various eukaryotic cells. The assay depends on the relaxation of supercoiled DNA on agarose embedded nucleoids and detects DNA strand breaks in cells treated with tested compounds. After electrophoresis, the relative amount of DNA in the comet tail indicates DNA break frequency.[42] We evaluated DNA damaging potentials of the most cytotoxic 5 compounds (**XT3**, **X8**, **X17**, **X23,** and **XT9B**) for Hela cells according to SRB assay on both L929 and HeLa cells. For 48-h treatment, cells were incubated with IC_{50} concentrations of the compounds.

DNA tail intensity data obtained from comet analysis is shown in Table 2. Etoposide (ETOP), camptothecin (CPT), and H_2O_2 were used as positive controls. According to the tail intensity data of the compounds, only **X23** and **XT9B** have any DNA damaging activity on both cell lines. It was shown that treatment with **XT3** was caused increasing of tail intensity for HeLa cells ($p < 0.001$), although L929 cells with treated higher doses compound was not increased tail intensity. **X8** and **X17** have genotoxic activity for both cells. However, **X17** was genotoxic at 10 μM for HeLa cells ($p < 0.001$), while it increased tail intensity at 75 μM for L929 cells ($p < 0.0001$). We assessed genotoxic potentials of **X17** in dose dependent-manner to observe its effects at 10 μM and related data are shown in Figure 1.

2.3 | Analysis of morphological changes

Morphological changes of the HeLa cells treated with the compounds (**XT3, X8, X23, X17,** and **XT9B**), which showed to have the most cytotoxic effect on HeLa cells were evaluated under an inverted microscope. As seen in Figure 2, each compound at IC_{50} doses decreased cell proliferation markedly. Likewise, exposure of HeLa cells to the compounds resulted in rounding and retraction in some cells, as well as divergence from normal morphology at 48-h treatment, while most of the cells maintained normal cell shape and morphology. Therefore, it was thought that IC_{50} dose of each compound significantly reduced cell adhesion to the surface compared with the

TABLE 1 Cytotoxic activity data

A 							
Compound	**X**	**R_1**	**R_2**	**R_3**	**L929 IC_{50} (μM)**	**HeLa IC_{50} (μM)**	**SI***
XT8B	–	CH_3CH_2–	NO_2	H	3188.9	15.89	201
XT9B	–	F	NO_2	H	1312	10.88	121
X8B	–	$C(CH_3)_3$	H	NO_2	249.7	605.86	0
X17B	–	H	H	NO_2	859.8	46.06	19
XT3B	–	F	H	NO_2	21494.5	31.02	693
XT4B	–	Br	H	NO_2	1301.7	43.54	30
X10B	CH_2	Br	H	NO_2	836.8	144	6
X23B	CH_2	Cl	H	NO_2	5938.9	388.6	15
XT2B	CH_2	F	NO_2	H	439.92	285.67	2
B 							
X8	–	$C(CH_3)_3$	H	NO_2	4.46	8.9	1
X17	–	H	H	NO_2	77.71	12.65	6
XT3	–	F	H	NO_2	33.08	10.02	3
XT5	–	CH_3CH_2–	H	NO_2	34.44	22.66	2
XT6	–	H	NO_2	H	98.59	40.94	2
XT8	–	CH_3CH_2–	NO_2	H	66.25	18.14	4
XT9	–	F	NO_2	H	43.11	29.45	1
X23	CH_2	Cl	H	NO_2	11.6	10.17	1
XT11	CH_2	CH_3	H	NO_2	95.86	33.62	3
XT12	CH_2	F	H	NO_2	86.5	46.61	2
XT1	CH_2	CH_3	NO_2	H	103.8	32.68	3
XT2	CH_2	F	NO_2	H	120.58	46.13	3
CPT					0.93	0.5	2
ETOP					22.87	3.21	7
GSP					31.34	9.52	3

SI, IC_{50} L929/ IC_{50} HeLa; A, benzoxazole derivatives; B, benzamide (X8–XT9) and phenylacetamide (X23–XT2) derivatives; CPT, camptothecin; ETOP, etoposide; GSP, gossypol.

control. On the other hand, almost all cells exposed to the most cytotoxic compounds displayed cellular alterations including nuclear condensation and cell degradation.

2.4 | Pharmacophore modeling study

2.4.1 | HipHop method

A set of known drugs (CPT, ETOP, and gossypol [GSP]) and most active compounds on HeLa cell line (**X8, XT3, X17, X23, XT9B**) shown in Table 1 were selected as the training set for use in the HipHop pharmacophore generation method. The selected pharmacophore model contained two HBAs and one Hp, and possessed a high ranking score. Figure 3 represents the mapping of most active compounds. It was revealed that at the given positions two HBA atoms or groups were necessary for the activity. Almost all of the tested compounds fitted the pharmacophore model (Figure 3). Fit values of the tested compounds are shown in Table 3.

2.4.2 | HypoGen method

According to the cytotoxic activity results for L929 cell line, the HypoGen method was applied to build the pharmacophore model. The best pharmacophore model was selected based on the highest correlation coefficient out of 10 hypotheses with a value of 0.91. The selected model strongly predicts the activity of the tested compounds. The correlation between the experimental activity and predicted activity of tested compounds is shown in Table 4. The

TABLE 2 Comet assay results of selected compounds

	HeLa		L929	
Compounds	**μM**	**Tail intensity (%)**	**μM**	**Tail intensity (%)**
Control	0	14.09 ± 10.68	0	12.56 ± 10.23
XT3	10	19.55 ± 12.52**	30	14.80 ± 13.20
X8	10	19.86 ± 11.43**	5	16.98 ± 12.26*
X17	10	20.85 ± 15.31**	75	26.15 ± 15.03***
X23	10	16.48 ± 13.69	10	15.30 ± 13.04
XT9B	10	12.33 ± 11.34	400	12.29 ± 7.94
H_2O_2	25	75.75 ± 17.24***	25	71.04 ± 12.18***
CPT	0.5	59.53 ± 19.99***	1	23.32 ± 11.40***
ETOP	3	69.97 ± 18.90***	20	64.43 ± 15.34***

*$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$.

selected pharmacophore model contained two HBAs, one HBD and one Hp feature. The selected pharmacophore model and mapping of the compounds are shown in Figure 3.

3 | DISCUSSION

Through *in vitro* pre-screening studies, newly synthesized compounds can be tested for their cytotoxic and genotoxic properties, and their activities on the molecules to which they are targeted can be investigated.[40,43] In this research, the cytotoxic activities of the compounds were assessed on a non-cancerous (L929) and a cervix cancer (HeLa) cell line by the SRB assay that American National Cancer Institute (NCI) used and recommend for drug researches.[44] To the best of our knowledge, while there is no definite conclusion for the dose range of the compounds tested by the investigators that will be effective, organizations such as NCI appear to regard compounds with an IC_{50} value below 100 μM as effective compounds.[45] So, only derivatives with IC_{50} values below 100 μM were defined as cytotoxic and those with IC_{50} values below 10 μM were evaluated as strong cytotoxic compounds in the study.

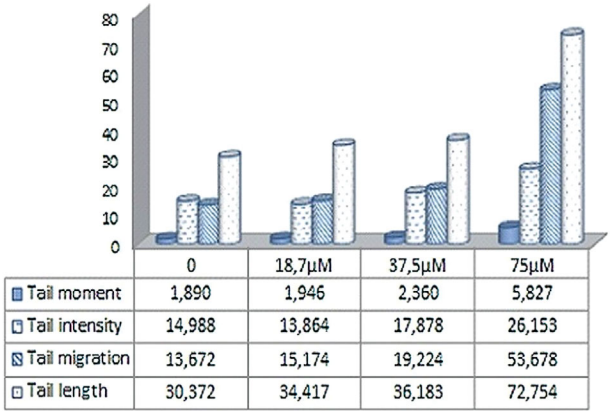


FIGURE 1 Comet DNA damage parameters for L929 cells treated with **X17** in a dose-dependent manner

According to the SRB assay data for L929 cell line, 11 of 21 test compounds did not show a remarkable cytotoxic activity. **X8**, **X23**, **XT3**, **XT5,** and **XT9** which had less than 50 μM IC_{50} values were determined as the most cytotoxic compounds. It was shown that any of benzoxazole derivatives are not cytotoxic, whereas IC_{50} values of benzamide and phenylacetamide derivatives have between 5 and 120 μM. According to these results, it could strictly be expressed that tested benzoxazoles were less cytotoxic against L929 cells than their possible metabolites.

In terms of the cytotoxicity data for HeLa cell line, 17 compounds with IC_{50} values below 50 μM had good cytotoxic potential. Among this series, **X8**, **X23**, **XT3,** and **XT9B** (IC_{50} < 10 μM) were most cytotoxic compounds. Whereas IC_{50} values of benzoxazole derivatives were in range of 10–600 μM, IC_{50} values of all benzamide and phenylacetamide derivatives were less than 50 μM.

According to this data, it was remarkable that benzamide and phenylacetamide derivatives exhibited more cytotoxic activity than benzoxazole derivatives on HeLa cells and L929 cells. However, when the chemical structures of the compounds were considered, it was not surprising. It was expected that metabolite derivatives, benzamide and phenylacetamides, were more cytotoxic against both cell lines through active groups occurring from the hydrolysis of the oxazole ring of benzoxazole scaffold and increasing polarity such as OH groups.[46] In fact, it was suggested clearly that cytotoxic activities increased with metabolic activation when IC_{50} values of seven benzoxazole derivatives and their metabolites (**X8-X8B**, **X17-X17B**, **XT3-XT3B**, **XT8-XT8B, XT9-XT9B**, **X23-X23B**, **XT2-XT2B**) among these series were evaluated. It was remarkable that all of the tested compounds except for **X8** and **X8B** exhibited against HeLa cells more cytotoxicity than against L929 cells, when cytotoxic activities of the compounds against both cell lines were investigated.

The therapeutic index is defined as the ratio between the toxic dose and the therapeutic dose of a drug, used as a measure of the relative safety of the drug for a particular treatment.[47] In *in vitro* anticancer drug screening, the selectivity index (SI) compares cytotoxic activities of a tested compound on both cancer and non-cancerous cells, and can be used as a measure of the relative safety of the compounds. Because, specific cytotoxicity against cancer cells is a desirable condition for anticancer agents to avoid side effects. Studies indicate that the agent selectively killed cancer cells when the SI was at least 2.[48] In this study, SI demonstrated a comparison of cytotoxic activities of a tested compound on L929 and HeLa cells. Based on this, the SI data shown in Table 1 indicated that most of the tested compounds exhibited cytotoxic selectivity for HeLa cells. Especially **XT3B**, **XT4B**, **XT8B**, **XT9B**, **X23B,** and **X17B** among the benzoxazoles which their SI values were ranging in 15–700 and **X17** and **XT8** (SI = 6 and 4, respectively) among the benzamides were the most remarkable compounds as *in vitro* anticancer agents. According to this data, it can be generally said that tested benzoxazoles are more specific against cancer cells than their possible metabolites. However only **XT8B**, **XT9B,** and **X17** had promising cytotoxicity on cancer cells as an anticancer agent.

Additionally, it was shown that **X8** (IC_{50}(L929): 4.46 μM, IC_{50}(HeLa): 8.9 μM) and **X23** (IC_{50}(L929): 11.6 μM, IC_{50}(HeLa):

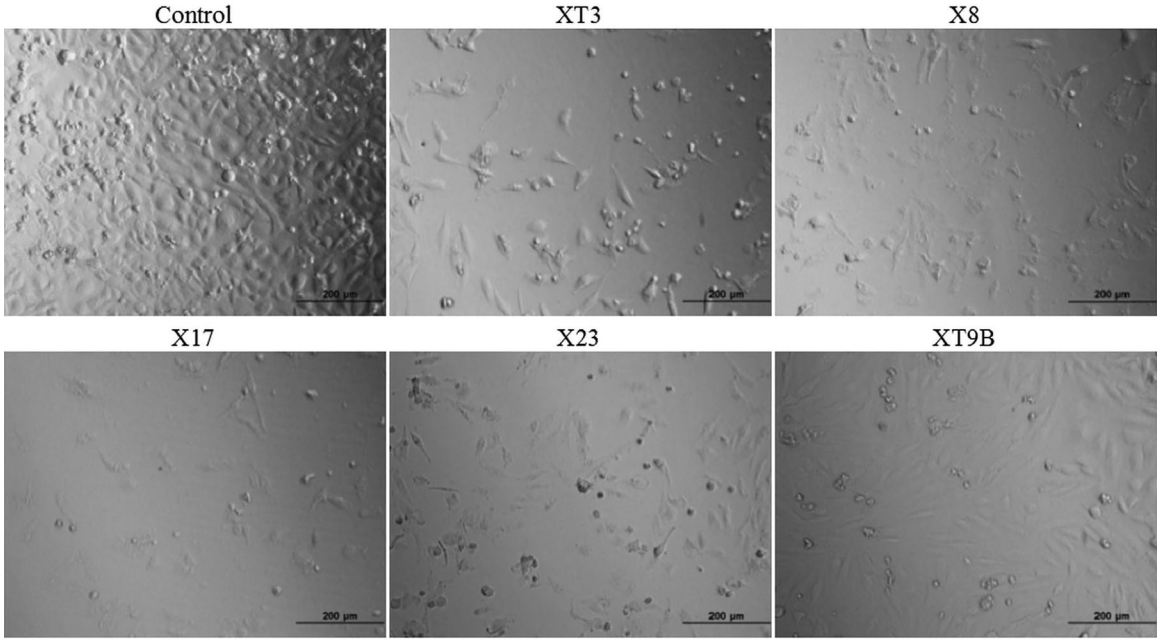


FIGURE 2 The morphological changes of the HeLa cells treated with most cytotoxic compounds (cells were incubated with the compounds at IC_{50} concentrations for 48 h)

10.17 µM) were the most cytotoxic compounds against both cell lines and also exhibited similar activity compared with the reference compounds (IC_{50}(L929): 0.93–31.4 µM, IC_{50}(HeLa): 0.5–9.5 µM). Although these compounds were not specific for HeLa cancer cells, it should be reviewed whether they would be specific for other various cancer cell lines. Furthermore, **XT3** (IC_{50}(L929): 33.08 µM, IC_{50}(HeLa): 10.02 µM), **XT5** (IC_{50}(L929): 34.44 µM, IC_{50}(HeLa): 22.26 µM), and **XT9** (IC_{50}(L929): 43.11 µM, IC_{50}(HeLa): 29.45 µM) exhibited moderate activity thanks to their IC_{50} below 50 µM. Among them, **XT3** is most promising compound due to its threefold greater cytotoxic activity against HeLa cancer cells than L929 cells. In our previous study about the antimicrobial activity of these compounds, it was reported that **X8**, **XT3**, **XT8,** and **XT9** exhibited broad spectrum antibacterial and antifungal activities.[38] This suggested that they might have the same target sites in prokaryotic and eukaryotic cells.

UK-1, a product isolated from a strain of *Streptomyces* is a well-known 2-phenyl substituted compound as is our tested benzoxazole derivatives. It was reported that it has IC_{50} values for some cancer cell lines were in range between 0.02–65 µM and for HeLa cells 1.22 µg/mL.[23,49] Similarly, **XT9B**, the most cytotoxic compound of this series exhibited cytotoxic activity at 2.81 µg/mL.

Since DNA replication is an essential phase of the cell cycle, DNA is one of the main targets of cancer therapies. Many of the drugs commonly used in cancer therapy are genotoxic agents—that cause high levels of DNA damage. They initiate cell cycle checkpoints and lead to cell cycle arrest and/or cell death.[39] However, genotoxicity of anticancer drugs to normal cells is one of the most serious problems of chemotherapy due to the possibility of inducing secondary malignancies. Therefore, it is a necessity for chemotherapy to determine the DNA-damaging effect of these drugs on normal cells.[50] Comet assay is used in different research areas including bio-monitoring, routine genotoxicity assessment, and studies of DNA repair. On the other hand, it is used to predict cell response to drugs, such as anticancer agents that affect DNA structure.[51] We therefore assessed potential DNA damage by the compounds tested in this present study to see whether they were associated with their cytotoxic effect on cancer cells. **XT3, X8, X23, X17,** and **XT9B** were determined as the most cytotoxic compounds against Hela cells with IC_{50} values of approximately 10 µM. We tested DNA damaging effects of them with alkaline comet assay. In this way, we assessed whether the cytotoxic activity of compounds related to their DNA damaging activities. According to tail DNA intensity for HeLa and L929 cells treated with the compounds, it was revealed that **X23** and **XT9B** did not cause DNA strand breaks while **X8** and **X17** had increased tail DNA intensity of both cells. A remarkable cytotoxic activity on cancer cells at low concentrations is desirable for a potent anticancer agent. However, **X8** might kill the normal cells at lower concentration than that which killed the cancer cell. It was also found that it had DNA damaging activity on normal cells at these concentrations. For the possibility that it might cause secondary tumors due to genotoxic activity restricted its use as an anticancer agent. On the contrary, it was found that **X17** had a genotoxic effect at 10 µM concentration, which was genotoxic for HeLa cells while it caused DNA strand breaks on L929 cells at 75 µM. Similarly, **XT3** did not increase DNA tail intensity on L929 cells at IC_{50} concentration (30 µM), but it caused DNA strand breaks on HeLa cells at 10 µM. These results show that **X17** and **XT3** had selective cytotoxic and genotoxic activities against HeLa cancer cells.

It is well known that DNA damage caused by DNA single and double strand breaks can be detected with alkaline comet assay.[43,52] Increased DNA double strand breaks in cells result in genomic instability and cell death. Therefore, DNA damage or inhibition of repair pathways have become targets in cancer therapy.[53] According

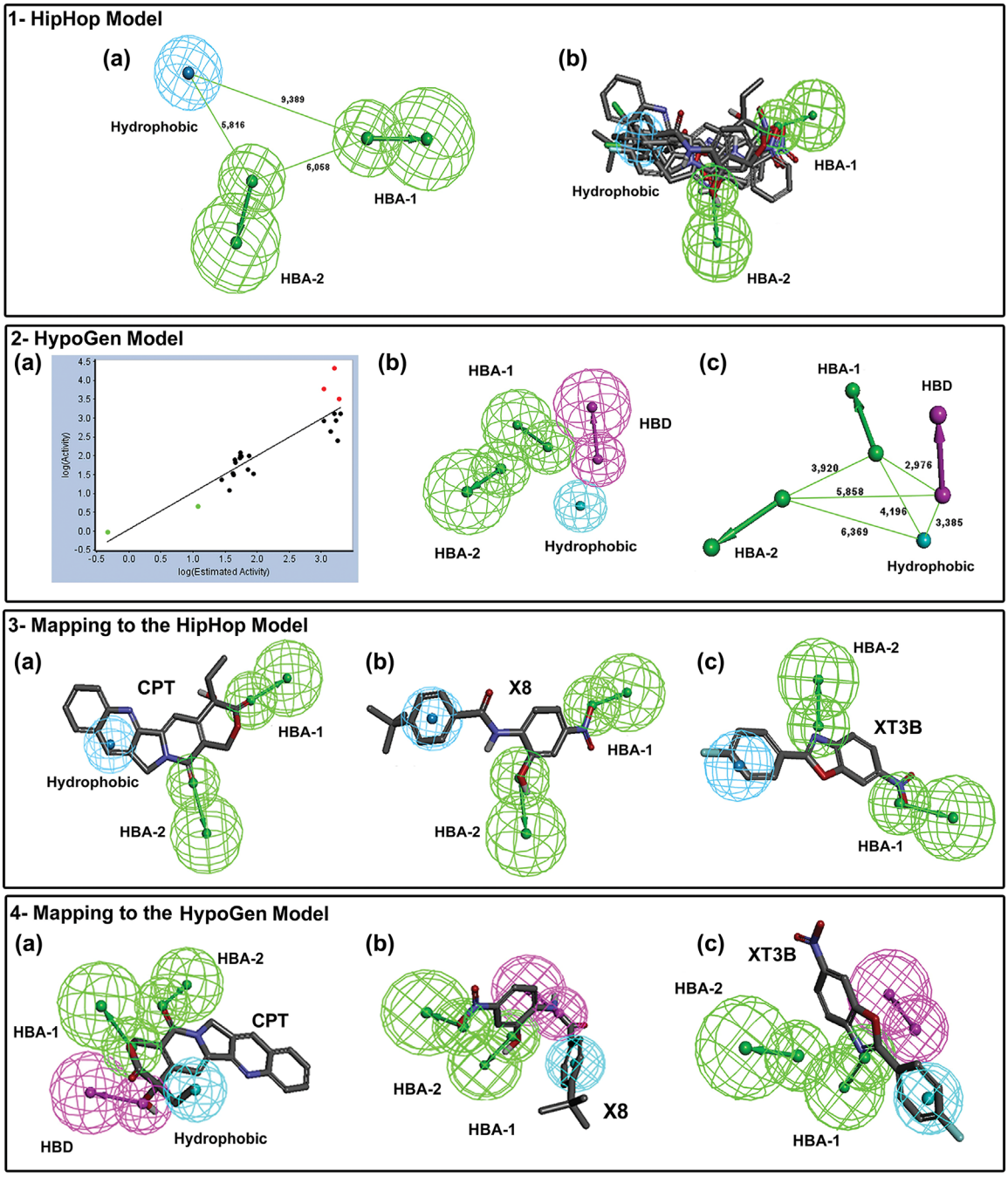


FIGURE 3 The selected pharmacophore models and mapping of the compounds. (1a) Distances between the generated common features calculated in the participated HipHop pharmacophore model on the HeLa cell line. (1b) Mapping of all tested compounds to the HipHop model. (2a) Correlation diagram of the selected HypoGen pharmacophore model on the L929 cell line. (2b) Features of the HypoGen model. (2c) Distances between the generated common features calculated in the participated HypoGen model. (3a) Mapping of CPT to the HipHop model. (3b) Mapping of **X8** to the HipHop model. (3c) Mapping of **XT3B** to the HipHop model. (4a) Mapping of CPT to the HypoGen model. (4b) Mapping of **X8** to the HypoGen model. (4c) Mapping of **XT3B** to the HypoGen model

to the results, it might be said that **X17** and **XT3** could kill HeLa cells by creating DNA strand breaks.

According to bioactivity results for HeLa cell line, the amide derivatives (Group A in Table 1) and benzoxazole derivatives (Group B in Table 1) had similar activities and almost all of the tested compounds fitted the selected HipHop pharmacophore model (Figure 3). It was found that while the methylene bridge at the 2. position of benzoxazole ring was decreasing the activity, the nitro groups on the R2 position increased this activity. It can be seen that there is a good correlation between the activities and the Fit Values of the compounds (Table 3). According to L929 cell line activity results, the possible metabolites of benzoxazoles, amide derivatives had higher activity than the benzoxazole derivatives and they also fitted the HypoGen pharmacophore model better. According to the activity results, the

TABLE 3 Fit values of the tested compounds

Compounds	FitValue
CPT	2.99969
ETOP	2.82632
X8	2.72546
GSP	2.66767
XT3	2.41268
X17	2.28308
XT3B	2.11522
X23	2.05482
XT9B	2.02757

reference drug CPT had potent activity both on HeLa and L929 cell lines. As shown in Figure 3, CPT fitted well all of the features of both the HipHop and HypoGen pharmacophore models. This can be explained with its potent activity and lower selectivity against HeLa cell line. When we looked at the other tested compounds, the most active compound **X8** fitted all of the features of the two models like CPT, so it had lower selectivity too. The most selective compound **XT3B**, as the SI value of 693 (Table 1), fitted to the HipHop model and showed a significant activity on Hela cell line. Besides, **XT3B** fitted HBA-1 and hydrophobic features of the HypoGen model but did not fit the HBA-2 and HBD features (Figure 3). This can be explained with the higher selectivity. According to all these results, it could be concluded that the compounds fitted the HipHop model well but did not fit the HypoGen model at the same time, could show potent and selective anticancer activity on HeLa cell line.

TABLE 4 Correlation between the experimental and predicted activities

Compound	Fit Value	Predicted activity	Observed activity	Error cost
CPT	6.93	0.47	0.93	−2
X8	5.50	12	4.5	+2.8
X23	5.03	37	12	+3.1
ETOP	5.16	28	23	+1.2
GSP	4.96	43	31	+1.4
XT3	4.65	88	33	+2.7
XT5	4.97	42	34	+1.2
XT9	4.74	72	43	+1.7
XT8	4.93	46	66	−1.4
X17	4.94	45	78	−1.7
XT12	4.87	54	87	−1.6
XT11	4.84	57	96	−1.7
XT6	4.72	75	99	−1.3
XT1	4.85	55	100	−1.9
XT2	4.86	55	120	−2.2
X8B	3.33	1800	250	+7.4
XT2B	3.46	1400	440	+3.1
X10B	3.56	1100	840	+1.3
X17B	3.36	1700	860	+2
XT4B	3.30	2000	1300	+1.5
XT9B	3.39	1600	1300	+1.2
XT8B	3.32	1900	3200	−1.7
X23B	3.54	1100	5900	−5.3
XT3B	3.39	1600	21,000	−13

4 | CONCLUSION

In this present study, we aimed to evaluate *in vitro* anticancer activity of some 2-phenyl substituted benzoxazoles and their potent metabolites (*N*-phenyl benzamide and phenyl acetamides) by commentating their structure activity relationships.

Results obtained from SRB assay indicated that the 17 of 21 tested compounds possessed good-to-moderate cytotoxic activity against HeLa cells and 14 compounds among them were particularly promising, since they could kill cancer cells 2–700 times more effectively than the noncancerous cells. Therefore, the most active compounds, **XT8B**, **XT9B**, **X8**, **X23**, **X17**, **XT3,** and **XT8**, may be further evaluated in other cancer and normal cell lines and *in vivo* animal models in the line of further development. Among them **X17** and **XT3** caused DNA strand breaks on HeLa but not L929.

It is known that almost all effective anticancer drugs have the potential to produce toxicity or side-effects because of the less selectivity to the cancer cells. Selectivity of the drugs is important for using them efficiently. According to our pharmacophore studies, the compounds that fitted the HipHop model well but did not fit the HypoGen model at the same time, could show potent and selective anticancer activity. Therefore, these models could be useful for further studies in order to design new potent and selective anticancer agents.

5 | EXPERIMENTAL

5.1 | Test compounds

Test compounds synthesized previously by our group were shown in Table 1.[38,39] They were dissolved in dimethyl sulphoxide (DMSO) and stored at −20 °C prior to use.

The InChI codes of the investigated compounds together with some biological activity data are provided as Supporting Information.

5.2 | Cell lines and culture conditions

Non-cancerous mouse fibroblast (L929) cell lines and human cervix epithelial adenocarcinoma (HeLa) cell lines (kindly provided from Hacettepe University, Department of Physiology) were employed in this study. Both cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (Lonza, Belgium), 45 IU/mL penicillin and 45 IU/mL

streptomycin (HyClone, USA) at 37°C in a humidified atmosphere of 5% CO_2.

5.3 | *In vitro* cytotoxicity screening

The cytotoxicity of test compounds was assessed using a cell death assay based on detection of cells by sulforhodamine-B (SRB) dye (Sigma, USA) according to procedure of Vichai and Kitikira.[54] The cells were inoculated into 96-well microtiter plates at a density of 1×10^4 cells per well. After cell inoculation, the microtiter plates were incubated for 24 h to attach cells onto the well surface. Test compounds prepared in DMSO were dissolved in cell culture medium at appropriate final concentrations and added to each well. Plates were incubated for 48 h at 37°C and 5% CO_2 after the addition of compounds. Cells were fixed with cold 10% (w/v) trichloroacetic acid (TCA) for 60 min at 4°C. After incubation, plates were washed with water and the plates added to an SRB solution at 0.4% (w/v) in 1% acetic acid and were incubated for 30 min at room temperature. The plates were then rinsed with 1% acetic acid to remove unbound excess dye. Bound stain was subsequently eluted with 10 mM Tris base solution (pH 10.5) and the absorbance was read on an Elisa plate reader (BioTek-μQuant) at 510 nm. Percentage growth for each concentrations of the compound was calculated on a plate by plate basis for test wells relative to control wells. S-probit analysis was used to calculate the 50% cell growth inhibition (IC_{50}) value of each test compound. All experiments were repeated a minimum of two times with each experiment done in three replicates. CPT (Sigma, USA), ETOP (Sigma, USA), and GSP (Sigma, USA) were used as positive controls in the assay.

5.4 | Morphological analysis

Morphological changes in L929 and HeLa cells exposed to IC_{50} concentrations of selected compounds were taken using an inverted microscope (Olympus IX71, Japan) at 200× magnification.

5.5 | *In vitro* genotoxicity screening

The alkaline comet assay was performed to assess genotoxic potentials of the compounds by following the Singh et al. (1988)[41] protocol with a few modifications. HeLa cells were inoculated into 24-well microtiter plates at a density of 6×10^4 cells per well. A stock solution of test compounds in DMSO was dissolved in cell culture medium at appropriate final concentrations and the final concentration of DMSO in the culture medium was less than 0.5%. Cells were exposed to different concentrations of compounds for 48 h at 37°C and 5% CO_2. After cells were trypsinized, cell suspensions were centrifuged at 1200 rpm for 5 min. Cell suspension in low melting point agarose was pipetted onto slides pre-coated with 1% high melting-point agarose. Slides with embedded cells in agarose were allowed to solidify at 4°C. The slides were then incubated overnight in a freshly prepared lysis buffer (100 mM EDTA, 2.5 M NaCl, 10 mM Tris, 1% Triton X–100 pH 10, 4 °C). After lysis, the slides were placed in a horizontal electrophoresis chamber and incubated for 40 min, at 4°C in an alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA pH 13) for alkali DNA unwinding. Electrophoresis was performed in the same buffer for 40 min at 25 V and 300 mA (0.75 V/cm) at 4°C. After the electrophoresis, the slides were rinsed three times with a neutralizing buffer (0.4 M Tris pH 7.5) for 5 min. The slides were stained ethidium bromide solution (20 μg/mL).

Two identical slides per samples were prepared and at least 50 cells were randomly analyzed per slide. Tail intensities of the selected 200 cells per sample at two repeated experiments were measured by Comet assay IV image analysis software (Perceptive Instruments Ltd., USA). All statistical analysis was performed with Prism GraphPad Software (Version 5.01).

The suitability of groups for normal distributions was assessed by Kolmogorov–Smirnov analysis. The Kruskal–Wallis test was used to compare the results of dose groups and followed by Dunn's multiple comparison post-hoc tests, $p \leq 0.05$ were considered as statistically significant. To compare the results of two groups a Mann–Whitney *U*-test was used.

5.6 | Pharmacophore modeling studies

In this research, the HipHop and HypoGen methods were used to generate a pharmacophore hypothesis to explain the specification of the structure–activity relationships of pharmacophoric sites of the tested compounds. Molecules were built using the Discovery Studio 3.5 software, and standard 3D structures were generated and the geometry of all molecules was optimized with ABNR Minimization Method and conformational models for each compound were automatically generated. The "best conformer generation" procedure was applied to provide the best conformational coverage for a maximum number of conformers generated, defaulted to 255 in a 0–20 kcal/mol range from the global minimum. The generated conformations were used to align common molecular features and generate a pharmacophore hypothesis.

5.6.1 | HipHop method

According to the activity results on HeLa cell line, the HipHop method was applied to build the pharmacophore model. Then the hypothesis was generated from these aligned structures.[41,55,56] A set of known drugs (CPT, ETOP, and GSP) and most active compounds against HeLa cell line (**X8**, **XT3**, **X17**, **X23, XT9B**) shown in Table 1 were selected as the training set for use in the HipHop pharmacophore generation method. CPT was considered as the "reference compound," specifying a principal value of 2 and a maximum omitting features (MaxOmitFeat) value of 0. Then 10 pharmacophoric hypotheses were generated from these aligned structures using the Common Feature Pharmacophore Generation protocol. A preparative test was performed with a hydrogen bond acceptor (HBA), a hydrogen bond donor (HBD), a hydrophobic aromatic (HpAr), a hydrophobic aliphatic (HpAl), a hydrophobic (Hp), and a ring aromatic (R). The hypothesis shown in Figure 3 was chosen for the further evaluation within the generated 10 hypotheses.

5.6.2 | HypoGen method

According to the activity results on the L929 cell line, the HypoGen method was applied to build the pharmacophore model. HBA, HBD, HpAr, HpAl, Hp, and R features were selected to generate the pharmacophore hypotheses.[57] For the HypoGen method we used, differently from HipHop method, the activity values to generate the hypothesis. For this, the ranging activity values of a collection of conformational models of compounds should be at least four orders of magnitude. The HypoGen generated pharmacophore model is based on chemical features of active compounds in the training set. The best pharmacophore model was selected based on the highest correlation coefficient, value of 0.91 out of 10 hypotheses.

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