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Antiproliferative and Genotoxic Activities in L929 and HeLa Cell Lines, Mutagenic Effects in *Salmonella* Strains of Novel Benzoxazole Derivatives

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***SUMMARY*. Some novel fused heterocyclic compounds of 2,5-disubstituted-benzoxazole derivatives, which were previously synthesized by our group, were investigated for their mutagenic properties on *Salmonella typhimurium* TA 98 and TA 100 strains, cytotoxic activity in L929 and HeLa cell lines by Sulforhodamine B (SRB) cytotoxicity test, and genotoxic potentials in the comet assay. By using Ames/*Salmonella* assay in the presence of S9 fraction, B22 (5-nitro-2-(*p*-nitrobenzyl)benzoxazole) was found to be mutagenic in both *S. typhimurium* TA98 and TA100 strains at all tested doses. IC_{50} values which were evaluated by SRB cytotoxicity assay revealed that B11 (2-(*p*-nitrobenzyl)benzoxazole) (IC_{50} = 99.16 μM) was the most anti-proliferative compound on HeLa cancer cells. Compounds were also tested for their genotoxicity by using comet assay, and it was found that all the compounds had DNA-damaging genotoxic activity on HeLa cells. The comet assay results showed that B11 produced DNA damage at lower concentrations than the other compounds tested on HeLa cancer cells. The results obtained from all the tests suggest that B11 could be a good candidate as a new anticancer agent.**

RESUMEN. Algunos nuevos compuestos heterocíclicos fusionados derivados de 2,5-benzoxazol-disustituidos previamente sintetizados por nuestro grupo fueron investigados por sus propiedades mutágenas sobre cepas de *Salmonella typhimurium* TA98 y TA100, por la actividad citotóxica en líneas celulares L929 y HeLa mediante el ensayo de citotoxicidad con sulforodamina B (SRB) y el potencial genotóxico por el ensayo cometa. Mediante el uso del ensayo Ames/*Salmonella* en presencia de la fracción S9, B22 (5-nitro-2- (*p*-nitrobencil)benzoxazol) resultó ser mutagénico en ambas cepas de *S. typhimurium* TA98 y TA100 en todas las dosis probadas. Los valores de IC_{50} que fueron evaluados por el ensayo de citotoxicidad con SRB revelaron que B11 (2-(*p*-nitrobencil)benzoxazol) (IC_{50} = 99,16 mM) fue el compuesto con mayor acción anti-proliferativa en las células cancerosas HeLa. Los compuestos también se ensayaron por su genotoxicidad mediante el ensayo cometa, encontrándose que todos los compuestos tenían actividad genotóxica dañante del ADN en células HeLa. Los resultados del ensayo cometa mostraron que B11 produce daños en el ADN a concentraciones más bajas que los otros compuestos ensayados sobre las células cancerosas HeLa. Los resultados obtenidos a partir de todas las pruebas sugieren que B11 podría ser un buen candidato como un nuevo agente contra el cáncer.

INTRODUCTION

Cancer is a mortal disease worldwide. The discovery and development of new treatments are urgently needed because of problems with current treatments such as toxicity and drug-resistance [1]. Thus, research is directed towards novel drug designs with lower side effects and increased chemotherapeutic efficacy. Benzazoles, which are the substituted benzoxazole and benzimidazole derivatives, have been targeted by much research for many years because they constitute an important class of heterocyclic compounds that exhibit substantial chemotherapeutic activity [2-5]. Benzoxazole and its ring derivatives structurally resemble adenine

KEY WORDS: anticancer drugs, benzoxazole derivatives, comet assay, DNA damage-genotoxicity, Ames/*Salmonella* test, SRB cytotoxicity assay.

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and guanine heterocyclic bases present in the nucleic acid structure. Therefore, assuming that the chemotherapeutic activities of the benzoaxazole derivatives occur by inhibiting the nucleic acid synthesis, research interest in biological activities of these compounds has increased.

Most cancer cells divide more often than normal cells, and the process of cell division can be targeted to treat cancer patients. The aim of targeting cell proliferation is to arrest the cell cycle and/or cause cancer cell death using cytotoxic compounds (chemotherapy) or ionising radiation (radiation therapy) [6] . DNA is one of the main targets of these therapies because DNA replication is an essential phase of the cell cycle. Many of DNA damaging (genotoxic) drugs commonly used to treat cancer patients cause high levels of DNA damage that initiate cell cycle checkpoints leading to cell cycle arrest and/or cell death [7,8]. Genotoxic drugs affect nucleic acids and alter their function. These drugs may directly bind to DNA, or they may indirectly lead to DNA damage by affecting enzymes involved in DNA replication [9]. Rapidly dividing cancer cells are particularly sensitive to genotoxic agents because cancer cells have problems with the DNA repair system, and they actively synthesize new DNA [10,11]. The goal of treatment with any of these agents is the induction of DNA damage in the cancer cells. DNA damage, if severe enough, will induce cells to undergo apoptosis: the equivalent of cellular suicide. It has been reported that the antitumor efficacy of chemotherapeutic agents correlated with their growth inhibiting, differentiation-inducing, or apoptosis-inducing abilities [12]. However, the genotoxic chemotherapy drugs affect both normal and cancer cells, therefore the genotoxicity of these drugs is one of their most serious side effects owing to the possibility of inducing secondary malignancies.

Genotoxicity tests can be defined as *in vitro* or *in vivo* tests designed to detect drugs which can induce genetic damage directly or indirectly by various mechanisms of action [13]. The Ames *Salmonella*/microsome assay is a short-term bacterial mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage which leads to gene mutations [14]. The Ames assay is used worldwide as an initial screen to determine the mutagenic potential of new drugs [13]. It is perhaps the most rapid, simple, sensitive, and economical screening test for mutagenicity [15], and has an extensive database and good correlation with carcinogenicity [16].

The comet assay is a quick, sensitive, and reliable genotoxicity test [17-19] which is widely used to evaluate the genotoxic potentials of chemical and drugs [13,20]. Ostling & Johanson [21] first demonstrated "comets" and described their tails in terms of DNA with relaxed supercoiling through a process of electrophoresis (pH 9.5) of cells embedded and lysed in agarose on a microscope slide [22]. Since then, the worldwide acceptance of comet assay makes it a good assay to detect DNA damage (single and double-strand breaks) in eukaryotic cells [20].

Many researchers found that some benzoxazoles and related fused-heterocyclic compounds such as benzimidazoles, benzothiazoles, and benzoxazines exhibited antimicrobial [23,24], antihistaminic [25-27], antiviral [28], topoisomerase inhibiting [29-33], and antitumor activities [3-5,34,35]. Consequently, these derivatives were subjected to application of anticancer drug development [36].

A series of novel 2,5-disubstituted-benzoxazole derivatives (as seen in Fig. 1 and Table 1) was previously synthesized by our group [25-27,31].

In our previous study, we reported that these compounds exhibited strong inhibitory activity on eukaryotic DNA topoisomerase II [31]. In this research, these compounds were investigated with the perspective of developing new anticancer drugs. For this purpose, we used the Ames/*Salmonella* microsome assay to examine the compounds for mutagenic activity. SRB cytotoxicity assay was used for cytotoxicity screening of the compounds in L929 and HeLa cell lines. DNA-damaging genotoxic potentials of these compounds were assessed by using the comet assay.

The goal of this study was to determine whether the synthesized compounds have any antiproliferative and DNA-damaging genotoxic activity or not, which could be the mode of their chemotherapeutic action, to discover new anticancer drug candidates.

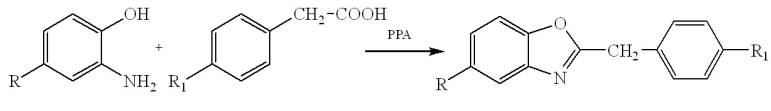
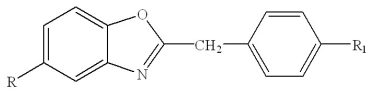


Figure 1. The chemical synthesis of the tested 2,5-disubstituted-benzoxazole derivatives. **R**: H, Cl, NO_2, NH_2, $\mathbf{R_1}$: NO_2, OCH_3, CH_3.



Compounds	R	R_1
B11	H	NO_2
B20	Cl	OCH_3
B22	NO_2	NO_2
B26	NH_2	CH_3

Table 1. The chemical structures of the tested 2,5-disubstituted-benzoxazole derivatives.

MATERIALS AND METHODS

Chemicals and reagents

The tested benzoxazole derivatives (compounds B11, B20, B22, and B26) were previously synthesized by our group respectively [25-27,31]. The chemical synthesis and structures of the compounds could be seen in Fig. 1 and Table 1, respectively. All benzoxazole derivatives were dissolved in dimethylsulfoxide (DMSO) and freshly prepared just before the experiments.

DMEM medium was obtained from HyClone (Utah, USA), fetal bovine serum was purchased from Lonza (Belgium), reagents for cell culture and the other assay chemicals were obtained from Sigma–Aldrich (Germany). All the chemicals were of analytical grade.

Mutagenicity assay

Mutagenicity of the benzoxazole derivatives was assessed by Ames/*Salmonella* microsome assay. The test strains were kindly provided by Dr. Bruce Ames (University of California, Berkeley, CA., USA). For this assay, two strains of *S. typhimurium* bacteria, namely TA98 and TA100, were used. The tester strains were checked for their genetic integrity for histidine dependence, biotin dependence, histidine/biotin dependence, rfa marker (crystal violet), and presence of the plasmid pKM101 (ampicillin resistance) before the experiments were undertaken [14]. The doses of the compounds to be tested in the *Salmonella* mutation assays were selected in cytotoxicity assay. Briefly, 0.1 mL of overnight grown bacterial culture (1-2 × 10^8 cfu/mL) was added to 2 mL top agar along with different concentrations of the tested compounds. The top agar was poured onto nutrient agar plates, and cytotoxic assessment was performed after 24 h incubation at 37 °C [37] .

Preparation of the liver S9 metabolic fraction was based on the procedure of Garner *et al.* [38]. Sprague-Dawley male rats were used in the preparation of liver S9 fraction. 3-methylcholanthrene and phenobarbital were used for the induction of rat liver enzymes. The protein content of S9 fraction was found to be 12 mg/mL.

Ames/*Salmonella* mutagenicity assay was carried out according to the method described by Maron & Ames [37]: 0.1 mL compound, 0.1 mL bacterial culture, and 0.5 mL of S9 fraction were added to 2 mL molten top agar for plate incorporation assays. The contents were mixed and poured on minimal agar plates. After 48-72 h of incubation, revertant colonies were counted. The tested compounds were assessed in independent experiments, each conducted in the absence and presence of the S9 metabolic activation system from rat livers. At least three plates were used for each dose, and each experiment was repeated two or three times. Daunomycin (6 µg/plate) for TA 98 and sodium azide (NaN_3, 1.5 µg/plate) for TA 100 were selected as positive controls without the S9 fraction. In presence of S9, 2-aminofluorene (2-AF, 50 µg/plate) for both TA98 and TA 100 strains was used as the positive control.

Cell cultures, conditions, and treatment

HeLa (human cervical cancer) and L929 (mouse fibroblast) cell lines were used in this study. The cell lines were provided by Dr. Aylin Gurpınar (Dept. of Biology, Faculty of Science, Hacettepe University, Turkey). Cells were routinely cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were grown in 25 cm^2 flasks at 37 °C in a humidified atmosphere containing 5% CO^2.

The cell lines were stored in liquid nitrogen, and the 5th to 8th passages from the stock cultures were used for the experiments after recultivation. The media were changed every 2-3 days; when the cultures reached confluency, the cells were washed with Dulbecco's Phosphate Buffered Saline, detached with Trypsin/EDTA, centrifuged, and subcultured.

Subsequently, the cells were treated with different concentrations of the tested compounds dissolved in DMSO for 1h in a dark incubator. Finally, the cells were collected by centrifugation at 250 × *g* for 5 min at 4 °C. A single-cell suspension of 3 × 10 cells/mL was prepared in

DMEM medium and then was mixed with low melting point agar for the comet assay as described below.

Sulforhodamine B (SRB) cytotoxicity assay

The antiproliferative SRB assay was performed to assess growth inhibition by a colorimetric assay which estimates cell number indirectly by staining total cellular protein with the dye SRB. The assay was carried out according to the method described by Vichai & Kirtikara [39]. Their method was optimized for the cytotoxic screening of the compounds to adherent cells in a 96-well format. Briefly, L929 and HeLa cells were seeded in 96-well microplates with 1×10^4 cells in 0.1 mL of DMEM medium supplemented with 10% FBS and routinely cultured in a humidified incubator (at 37 °C in 5% CO_2) for 24 h. After the compounds were added in serial concentrations (12.5, 25, 50, 100, 200, and 400 µM), the cells were incubated further for 48 h. After incubation, the cells were fixed by layering 100 µL of ice-cold 10% trichloroacetic acid on top of the growth medium. The cells were incubated at 4 °C for 1 h. Afterwards, the plates were washed 5 times with cold water, the excess water was drained off, and the plates were left to dry in air. SRB stain (100 µL; 0.4% in 1% acetic acid) was added to each well and left in contact with the cells for 30 min. Later, they were washed with 1% acetic acid, rinsed four times until only dye adhering to the cells was left. The plates were dried, and 200 µL of 10 mM Tris base (pH 10.5) was added to each well to solubilise the dye. The plates were shaken gently for 20 min on a gyratory shaker, and the absorbance (OD) of each well was read on a plate reader at 510 nm. Cell survival was measured as the absorbance percentage compared with the control (non-treated cells). The IC_{50} (50% inhibition concentrations) values were calculated with S-probit program and obtained by plotting the percentage of survival versus the concentrations.

Genotoxicity study. Comet assay

The comet assay was performed under alkaline conditions following some modifications as described by Chen *et al.* [40] and Who *et al.* [41]. Conventional microscope slides were dipped with a solution of 85 µL 0.5% of normal melting point agarose (NMPA) and 0.5% low melting point agarose (LMPA) in phosphate buffered saline (PBS) (pH 7.4), and they were left to dry on a flat surface at room temperature. 10 µL of the cell suspension (3×10^5 cells/mL) was gently mixed with 75 µL of 0.5% (w/v) of LMPA in PBS (pH 7.4); 75 µL of this suspension was rapidly layered onto the slides pre-coated with the mixtures of 0.5% NMPA and 0.5% LMPA, and they were covered with a cover glass. The slides were maintained at 4 °C for 5 min, and the cover glass was removed. Then, the cells were immersed in a fresh lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, and 1% (v/v) Triton X-100 at pH 10) at 4 °C for 2 h in a dark chamber. The slides were then placed in a wide horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM Na_2EDTA for 30 min. Later, the electrophoresis (1 V/cm, 300 mA) was conducted for 40 min at 4 °C. After the electrophoresis, the slides were soaked in a cold neutralizing buffer (400 mM Tris buffer, pH 7.5) at 4 °C for 10 min. Slides were dried in 100% methanol for 5 min and stored in a low humidity environment. After drying at room temperature, the slides were stained with 50 µL of ethidium bromide (EtBr) solution (60 µg/mL) and covered with a coverslip. Etoposide, a potent anticancer drug, was used as the positive control for both the SRB cytotoxicity assay and the comet assay.

For the visualisation of DNA damage, slides were examined at 400 × magnification using a fluorescence microscope connected to a CCD camera and an image analysis system (Comet assay IV version 4.11, Perceptive instruments). Randomly selected 100 cells (50 cells from each of the two replicate slides) were analyzed per sample. One hundred comets on each slide were scored according to the relative tail intensity and tail moment.

Statistical analysis

Statistical analysis was performed using Graphpad Prism5 package program. Ames mutation assay data were evaluated with One-Way ANOVA analysis. The results were reported as means ± standard error. Statistically significant differences were calculated compared with the control. In the assessment of the comet assay data, conformity to the normal distribution of the groups was evaluated by Kolmogorov-Smirnov analysis. The significance of difference between the groups was determined by Kruskal Wallis analysis. Nonparametric Mann–Whitney U-test was applied to compare each dose groups. Differences were considered to be statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Many clinically important chemotherapeutic drugs induce DNA damage such as base substitutions, intercalation, intra-strand or inter-strand crosslinks, and DNA breaks [8,42]. Rapidly dividing cancer cells are actively synthesizing new DNA, and they have problems with the DNA repair system or cell cycle control. Therefore, they are more sensitive to DNA damage compared with other body cells [10,11].

In this study, a series of previously synthesized [25-27,31] fused heterocyclic compounds of 2,5-disubstituted benzoxazole derivatives (Fig. 1 and Table 1) were investigated for their mutagenic potentials by Ames/*Salmonella* assay. Ames test is used worldwide as the key assay to confirm the mutagenic potentials of new chemicals and drugs. The test is also used for submission of data to regulatory agencies for registration or acceptance of many chemicals including drugs and biocides [13] .

The results of the mutagenicity obtained in the absence and presence of S9 fraction in *Salmonella* TA 98 and 100 strains are presented in Table 2. The maximum tested doses for each compound were chosen based on its solubility and sub-toxic effect of this dose on bacterial growth. The Ames test employs several histidine dependent *Salmonella* strains, each carrying different mutations in various genes. *S. typhimurium* TA 98 strain detects frame-shift mutagens. On the other hand, TA 100 strain detects mutagens that cause base-pair substitutions.

In Ames/*Salmonella* microsome test system, data are interpreted on the basis of a consistent doubling of the spontaneous reversion frequency confirmed by a dose-response relationship; however, when the number of induced revertants is less than twice the spontaneous rate, but a reproducible dose-related increase in revertants is detected, this is also interpreted as a positive response [14]. In our experiments, data were interpreted as described above.

Revertant Colony Numbers						
Compounds		**Doses (µg/plate)**	**S9 (-)**		**S9 (+)**	
			TA98 mean ± SD	**TA100 mean ± SD**	**TA98 mean ± SD**	**TA100 mean ± SD**
B11		0	20 ± 1	126 ± 20	44 ± 6	134 ± 6
		25	27 ± 5	136 ± 6	40 ± 7	104 ± 6
		50	25 ± 3	117 ± 9	42 ± 11	122 ± 13
		100	32 ± 4	110 ± 14	61 ± 5*	132 ± 14
B20		0	33 ± 3	126 ± 20	44 ± 6	134 ± 6
		100	32 ± 1	103 ± 3	40 ± 11	119 ± 12
		200	30 ± 4	112 ± 26	33 ± 4	111 ± 9
		400	31 ± 4	125 ± 23	33 ± 9	113 ± 13
B22		0	20 ± 1	126 ± 20	44 ± 6	134 ± 6
		50	41 ± 6*	125 ± 16	70 ± 15*	222 ± 53*
		100	43 ± 8*	112 ± 6	91 ± 15*	361 ± 33*
		200	64 ± 7*	109 ± 10	171 ± 17*	296 ± 57*
B26		0	33 ± 3	126 ± 20	44 ± 6	134 ± 6
		100	31 ± 5	110 ± 15	35 ± 6	136 ± 29
		200	32 ± 4	121 ± 15	33 ± 3	130 ± 13
		400	32 ± 4	130 ± 14	29 ± 5	143 ± 25
Positive controls	Daunomycin	6	91 ± 6	-	-	-
	Sodium azide	1.5	-	560 ± 28	-	-
	2-Aminofluorene	50	-	-	150,7 ± 36,1	>1000

Table 2. Mutagenic potentials of tested benzoxazole derivatives by Ames/*Salmonella* assay. Data represent the mean ± SD of data from three independent experiments. * Differences were considered to be statistically significant when * $p < 0.05$ compared with control.

In our study, compound B22 (5-nitro-2-(*p*-nitrobenzyl)benzoxazole) showed mutagenic activity at doses ranging 50-200 µg/plate on *S. typhimurium* TA 98 strain without S9. B22 was not mutagenic in the TA100 strain in the absence of S9. All of the other compounds were found to be non-mutagenic to the TA98 and TA100 strains in the absence of S9 (Table 2).

In the presence of S9 fraction, B22 was found to be exhibiting mutagenic activity in both *S. typhimurium* TA98 and TA 100 strains at doses ranging 50-200 µg/plate, whereas the B11 (2-(p-nitrobenzyl)benzoxazole) was very weakly mutagenic in the TA98 strain with S9 only at dose 100 µg/plate (Table 2).

Mutagenicity is likely to be a major factor in the carcinogenicity of the benzoxazole derivatives and may represent a serious limitation to the therapeutic use of potential drugs. This adverse property should be eliminated by rational drug design if possible [13]. Therefore B22, which was found to be mutagenic in both *Salmonella* tester strains in the presence of S9 fraction at all tested doses, should not be preferred as a candidate for potential drugs.

In this study, the viability of L929 and HeLa cells which were treated with different concentrations of the tested benzoxazole derivatives was measured using the SRB cytotoxicity assay. Growth inhibition of the cells was observed after incubation with the compounds for 48 h. The IC_{50} values of the compounds were calculated from the growth curves of cells (Table 3).

Among the benzoxazole derivatives tested, B11 was the most cytotoxic compound against HeLa cancer cells because the IC_{50} value (99.16 µM) of B11 was lower than the other compounds. The growth inhibition curve for B11 was obtained as survival % versus the concentrations (Fig. 2). The IC_{50} values of other tested compounds were high at both cell lines (Table 3). The applicability of such high doses of drugs for cancer therapy is not possible [13]. According to the expectation that anticancer agents have to be the most effective in low concentrations, it was found that B11 was the most effective compound on the death of cancer cells.

The cytotoxicity assay results were also used to determine the concentration ranges applied in comet assays. Thus, the compounds at the maximum sub-lethal doses were used separately for the determination of genotoxicity in L929 or HeLa cells using the alkaline comet assay. The comet assay is a versatile and sensitive method used by many researchers to measure DNA damage (single and double-strand breaks) in eukaryotic cells, and it is widely preferred to evaluate the genotoxic potentials of new chemicals and drugs [13,20].

The results obtained from the comet assay were given in Tables 4 and 5. As a tail parameter, we used tail moment and % tail DNA. Table 4 summarizes the results of DNA damage in L929 cells treated with varying concentrations of tested benzoxazoles, as measured by the comet assay. Results indicate that the positive control (etoposide) showed significant levels of DNA damage, while the negative control (DMSO as solvent for the tested compounds) revealed very low DNA damage. Among the tested benzoxazoles, only B22 produced DNA damage at concentration 120 µM in L929 cells. The other compounds were not genotoxic in L929 cells (Table 4).

Compounds	IC_{50} values (µM)	
	L929 cell line	HeLa cell line
B11	172.49	99.16 *
B20	582.03	86452
B22	135.76	338.5
B26	532.1	420.7
Etoposide (positive control)	3.21	22.87

Table 3. IC_{50} values of the benzoxazole derivatives exposed with L929 and HeLa cells by using the SRB cytotoxicity assay. * The most effective compound.

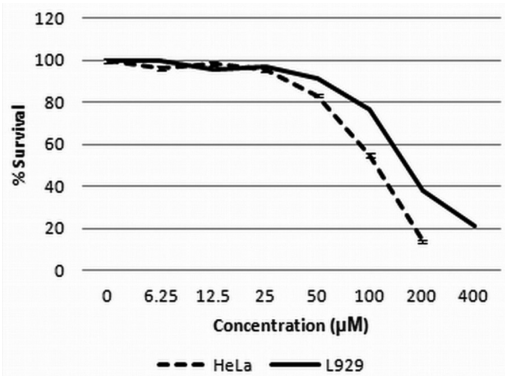


Figure 2. The cytotoxicity of B11 was determined using SRB assay in L929 and HeLa cells. B11 showed a dose dependent growth inhibition (survival % *versus* the concentrations) on the cells after 48 h incubation. in HeLa cells IC_{50} value of B11 = 99.16 µM, while in L929 cells IC_{50} value of B11 = 172.49 µM.

Compounds	Concentrations (µM)	L929 cell line	
		% Tail DNA	Tail moment
B11	150	13,50 (0,0051-68,11)	1,615 (0,001-15,06)
	75	10,98 (0,0-89,08)	1,430 (0,0-18,25)
	37,5	9,316 (0,0-50,47)	1,340 (0,0-9,268)
B20	500	14,94 (0,0-60,38)	1,945 (0,0-14,66)
	250	14,92 (0,0-53,30)	1,908 (0,0-10,51)
	125	11,71 (0,0-52,98)	1,611 (0,0-7,031)
B22	120	27,77 (0,214-72,10)*	3,260 (0,045-24,68)*
	60	17,05 (0,0726-46,89)	2,095 (0,0159-8,381)
	30	13,33 (0,0219-44,91)	1,789 (0,0054-8,50)
B26	400	12,13 (0,045-66,22)	1,791 (0,008-13,16)
	200	13,96 (0,0-56,96)	2,226 (0,023-9,420)
	100	8,353 (0,0232-41,19)	1,365 (0,0057-7,654)
Etoposide (positive control)	3,0	22,80 (0,692-60,93)*	26,02 (13,02-50,31)*
Control (negative control)	0	14,36 (0,0144-37,59)	1,804 (0,0-4,463)

Table 4. Assessment of DNA damage by the comet assays after exposure of L929 cells to the benzoxazole derivatives. *Differences were considered to be statistically significant when * $p < 0.05$ compared with control. The levels of DNA damage were calculated from the respective values of at least three treatments.

Compounds	Concentrations (µM)	HeLa cell line	
		% Tail DNA	Tail moment
B11	100	23,27 (0,05347-86,99) *	3,183 (0,361-7,484) *
	50	22,48 (1,539-51,25) *	2,862 (0,2699-10,67) *
	25	17,94 (1,396-87,79)	2,131 (0,0083-7,898)
B20	500	24,41 (0,0-64,51) *	3,823 (0,0-28,88) *
	250	21,00(0,0138- 63,17) *	2,643 (0,0026-13,41) *
	125	18,15 (0,0082-65,30)	2,228 (0,0002-16,01)
B22	300	24,69 (0,0-73,45) *	3,287 (0,0-35,36) *
	150	23,05 (0,124-70,73) *	2,798 (0,024-26,379) *
	75	25,13 (1,513-53,64) *	3,243 (0,3488-7,7569)*
B26	400	24,73 (0,0-74,83)*	3,135 (0,0-25,99) *
	200	30,15 (0,0787-61,56) *	3,813 (0,0214-10,01) *
	100	23,83 (0,6568-63,64) *	3,074 (0,124-23,36) *
Etoposide (positive control	20	70,24 (17,16-99,52) *	38,22 (3,431-102,4) *
Control (negative control)	0	18,96 (0,0309-54,23)	2,304 (0,007-6,747)

Table 5. Assessment of DNA damage by the Comet assays after exposure of HeLa cells to the benzoxazole derivatives. * Differences were considered to be statistically significant when * $p < 0.05$ compared with control. The levels of DNA damage were calculated from the respective values of at least three treatments.

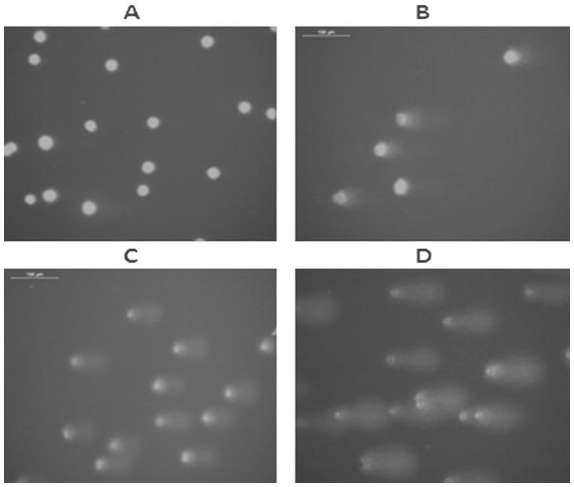


Figure 3. Representation of comet images, obtained after treatment with different concentrations of B11 in HeLa cells (**A**) Control (non-treated), (**B**) Cells treated with 25 µM of B11 (there is not DNA damage), (**C**) Cells treated with 50 µM of B11 (DNA damage was observed), and (**D**) Cells treated with 100 µM of B11 (more DNA damage was detected).

It was observed that B22 formed DNA damage in both normal (L929) and cancer (HeLa) cells as seen in Tables 4 and 5. This event is not preferred for the development of anticancer drugs because creating DNA damage on normal cells is one of their most serious side effects owing to the possibility of inducing secondary malignancies [11].

All of the tested compounds produced DNA damage on HeLa cancer cells as measured by the comet assay (Table 5). This result obtained from the comet assay indicates that all the tested compounds were genotoxic in HeLa cell line. Among these compounds, B11 was found to produce DNA damage at lower concentration (50 µM) on HeLa cancer cells when compared with the other compounds (Fig. 3 C). At the same time, it was observed that B11 formed DNA damage only in cancer cells (HeLa) while it did not do so in normal cells (L929 fibroblast) (Tables 4 and 5). Hence B11, which is assumed to have potential advantages in terms of reduced side-effects, could be preferred be as a candidate for the development and design of new anticancer drugs.

The structure-activity relationships for these tested compounds indicate that having a hydrogen (H) atom at position R is essential to achieve a hydrogen-bond interaction with the active side of the target. In addition, substitution with a nitro (NO_2) at position R_1 enhanced their genotoxic activity.

In our previous study, we also reported that B11 was found to be a strong topoisomerase II inhibitor having IC_{50} values of 17.4 µM, showing more potency than the reference drug etoposide [31]. Since the activity of topoisomerases enzymes is essential for cellular processes such as DNA replication, transcription, and chromosome condensation, inhibition of eukaryotic topoisomerases is widely used in the development and design of anticancer drugs [43].

Thus, we found that the data obtained from all assays for the benzoxazole derivatives showed correlation with each other. When the results obtained from all the experiments are compared, B11 seems to be the most effective compound.

CONCLUSION

In this study, previously synthesized 2,5-disubstituted-benzoxazole derivatives, which were originally designed to be chemotherapeutic agents, were evaluated from anticancer perspective by using various assays. Ames/*Salmonella* assay was used to examine mutagenic potentials of the compounds. SRB cytotoxicity test was performed to assess growth inhibition of L929 and HeLa cells treated with the compounds. DNA-damaging genotoxic potantials of the compounds were evaluated by using the comet assay. Among the tested compounds, B11 was found to be a remarkable compound. In fact, B11 showed the preferable outcome in all assays used. Obtained data suggest that B11 was the most cytotoxic compound in HeLa cancer cells, and it might cause DNA damage such as single and double-strand breaks in cancer cells.

In conclusion, B11 could be a potential candidate as a new anticancer agent. The present findings may provide future opportunities to design and develop more effective new chemotherapeutic agents.

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