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RESEARCH ARTICLE

Inhibition of DNA Topoisomerases by a Series of Benzoxazoles and their Possible Metabolites

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Abstract: ***Background*:** A series of 2-substituted benzoxazoles and their possible metabolites, N-(2-hydroxy-4(or 5)-nitrophenyl)benzamides and phenylacetamides, which were previously synthesized by our group, were investigated for their inhibitory activities on both eukaryotic DNA topoisomerase I (Topo I) and II (Topo II).

***Methods*:** DNA topoisomerase I and II inhibitory activity of compounds were determined by relaxation assay which could measure the conversion of supercoiled pBR322 plasmid DNA to its relaxed form.

***Results*:** According to DNA topoisomerase relaxation assay results, four derivatives (**1c, 1f, 2b, 2e)** among tested 21 compounds inhibited human Topo I, whereas three compounds (**1c, 1e, 1f**) inhibited human Topo IIα. 2-(4'-bromophenyl)-6-nitrobenzoxazole (**1f**) was observed to be the most effective Topo II inhibitor with the lowest IC_{50} value of 71 μM and 2-(4'-tert-butyl-phenyl)-6-nitrobenzoxazole (**1c**) with the IC_{50} value of 104 μM was considered the most effective Topo I inhibitor. However, it is noteworthy that these two compounds affected both Topo I and II enzymes. When the relationship between chemical structures and biological activities of the compounds were examined, the following two results were obtained: (i) CH_2 bridge seems to decrease the Topo I and II inhibitions and (ii) bulky groups at R_1 position increased both Topo I and II inhibitions for benzoxazole derivatives while small groups at R1 position increased the Topo I inhibition for benzamide derivatives.

***Conclusion*:** Biological activity and SAR results of tested derivatives may provide some predictions in order to design novel topoisomerase inhibitors.

Keywords: Benzoxazole, benzamide, phenylacetamide, topoisomerase I, topoisomerase II, topoisomerase inhibitors.

ARTICLE HISTORY

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1. INTRODUCTION

DNA topoisomerases are essential enzymes for nuclear processes of DNA. They have role solving topological problems related to DNA replication, transcription and also recombination and repair by homeostatic control of supercoiling. Topoisomerases exist in all organisms from archaea to human [1-3]. Despite having differences in specificity, their catalytic mechanism is a common feature between various topoisomerases. In all cases, this mechanism consists of a nucleophilic attack of a DNA phosphodiester bond by a catalytic tyrosine residue from enzyme. There are two classes of topoisomerases that are distinguished by their catalytic mechanisms. While type I enzymes cleave only one strand of DNA for catalysis, type II cleave both strands to overcome the entanglements or to avoid supercoiling. Temporary intermediates between DNA and enzyme were formed in this process (cleavable complexes) [2, 4].

In the past several years, topoisomerases have become one of the most expedient and strategic molecular targets for anticancer drugs and numerous patents have been filled and published on topoisomerase inhibitors [5]. They are the targets of antimicrobial and anticancer drugs, and hence, have deserved investigation to understand the biochemical and pharmacological basis of drug action [6, 7]. Topoisomerase inhibitors can be divided into two main groups, which differ in the mechanism of inhibition of the enzyme catalytic activity. Topoisomerase poisons inhibit the enzyme catalytic activity by stabilizing cleavable complexes. Accumulation of DNA damage triggers cell cycle arrest and activates apoptotic cascades. The second group inhibitors which can be named as catalytic inhibitors or suppressors impair the functions of topoisomerases without forming a covalent complex between enzyme and DNA. Therefore, suppressors can block process progressing by topoisomerase [8].

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Recent patents indicated that heterocyclic compounds have immense potential to inhibit topoisomerase enzyme [5]. Therefore, the researchers lead to design new heterocyclic compounds which can inhibit topoisomerases [9-16]. Benzoxazole skeleton is a constituent of several natural products and often incorporated in drug design because of its structural similarity of purine bases and ability of engagement in a number of distinct energetically favourable interactions with its host protein. 2-phenyl substituted bisbenzoxazole UK-1, a natural compound isolated from *Streptomyces sp*. is a good example for Topo II inhibitor compounds derived 2-phenyl substituted benzoxazoles [17, 18]. In the last few years, it was reported that especially 2-substituted benzoxazole derivatives were investigated for various types of biological properties such as anti-inflammatory [19], analgesic [20], antiepileptic [21, 22], antimalarial [23], anti-HIV [24, 25], anticancer [25-30], topoisomerase inhibitors [12], kinase inhibitors [31, 32], protease inhibitors [33], GSH inhibitors [34] and cyclooxygenase inhibitors [35].

Information about biotransformation of benzoxazoles is inadequate in the literature. However, according to the several studies, it has been suggested that primary metabolites of benzoxazoles are amid derivatives, which are formed from the hydrolysis of the oxazole ring [36]. It was reported that benzamide and phenylacetamide derivatives like benzoxazoles exhibited various types of biological properties such as antifungal [37], antibacterial [38], antihelmintic [39], antiviral [40-42] and anticancer [43-45]. Especially, benzamides are known to be an important class of HDAC inhibitors [46] and Parp inhibitors [47].

Previously, we synthesized a series of 2-phenyl/benzylbenzoxazole derivatives and some of their possible primary metabolites such as benzamides and phenylacetamides in order to investigate their antimicrobial activities [48, 49]. In this study, we aimed to evaluate the inhibition effects of these compounds on human DNA topoisomerase I and II enzymes by relaxation assay for developing new antitumor compounds.

2. EXPERIMENTAL

2.1. Reagents and Test Compounds

Chemical structures of test compounds synthesized previously by our group are shown in Table **1**. They were solubilised in DMSO and kept frozen until use. In all experiments, the final concentration of DMSO was 200-fold less than the stock concentration and control samples contained an equivalent amount of vehicle. Recombinant purified human Topo I and IIα were purchased from TopoGEN (Port Orange, FL, USA). All other common laboratory chemicals were of the highest grade available.

2.2. Topoisomerase I Enzyme Inhibition Study

DNA Topo I activity of the compounds were tested by plasmid relaxation assay described in the literature [50]. Relaxation activity of DNA Topo I was determined by measuring the conversion of supercoiled pBR322 plasmid DNA to its relaxed form. Each reaction mixture had a total volume of 10 µL containing 1 unit of recombinant human DNA Topo I, 0.1 µg pBR322 supercoiled DNA, 50 mM Tris-HCl, pH 7.5, 60 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 50 mg/mL BSA, and varying amount of test compounds or CPT in DMSO. The reaction mixture was incubated at 37 ºC for 30 min and then the reaction was terminated by adding stop solution (1% SDS, brome phenol blue, Xylene cyanol and 15% (v/v) glycerol). The samples were electrophoresed in a horizontal 1% agarose gel in TAE buffer (40 mM Tris acetate, 2 mM EDTA, pH 8.0) at 45V for 3h at room temperature. The gels were stained with bromide (1µg/ml) and photographed under UV illumination. Band distributions were analysed by a GDS 8000 Complete Gel Documentation and Analysis System (Gel Works 1D Intermediate, version 2.5; Ultra Violet Products). All experiments were repeated for a minimum of two times.

2.3. Topoisomerase II Enzyme Inhibition Study

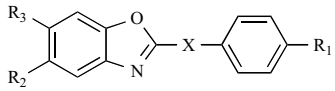
DNA topoisomerase II inhibitory activity of compounds were determined by relaxation assay which could measure the conversion of supercoiled pBR322 plasmid DNA to its relaxed form (Stewart and Champoux, 2001). The mixture of 100 ng of plasmid DNA and 1 unit of human DNA Topo IIα was incubated with or without tested compounds in the assay buffer (10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, and 15 mg/mL bovine serum albumin) for 30 min at 37ºC. Then, reactions were terminated by adding stop solution (1% SDS, brome phenol blue, Xylene cyanol and 15% (v/v) glycerol). The samples were electrophoresed in a horizontal 1% agarose gel in TAE buffer (40 mM Tris acetate, 2 mM EDTA, pH 8.0) at 45V for 3h at room temperature. The gels were stained with ethidium bromide (1µg/ml) and photographed under UV illumination. Band distributions were analysed by a GDS 8000 Complete Gel Documentation and Analysis System (Gel Works 1D Intermediate, version 2.5; Ultra Violet Products). All experiments were repeated for a minimum of two times.

3. RESULTS AND DISCUSSION

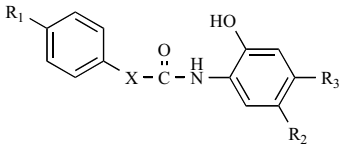
The development of innovative chemotherapeutic treatments for cancer has taken scientists into close research of DNA. Scientists have investigated the topoisomerase enzymes as important targets for the generation of new cancer treatments because they are essential molecules for all DNA related mechanisms of cells such as replication, recombination and repair. When these events are trapped in cancer cells, genomic instability occurred which leads to cell death.

In spite of the remarkable elucidation of topoisomerase structures, enzymatic mechanisms, biological functions, and mechanisms of action of inhibitors as antibacterial and anticancer agents over the past 40 years, only a small number of desirable agents have been used effectively in routine. Therefore, scientists continue their efforts to find agents with fewer side effects and more effective agents [51]. In the study, all of the compounds were tested for Topo I and II inhibitory activity. CPT and etoposide, well known Topo I and II inhibitors, respectively, were used as positive controls. Topo I and II inhibitory activities of tested compounds were measured by relaxation assay which based on detecting the conversion of supercoiled pBR322 plasmid DNA to its

Table 1. Topo I and II inhibitory activity of compounds.



Comp.	X	R1	R_2	R_3	Enzyme Inhibition (%)			
					Topo I		Topo II	
					400 µM	50 µM	800 µM	80 µM
1a	-	CH_3CH_2	NO_2	H	0.0	ND	**66**	**2.3**
1b	-	F	NO_2	H	0.0	ND	0.0	ND
1c	-	$C(CH_3)_3$	H	NO_2	**90.3**	**34.8**	**73**	**21**
1d	-	H	H	NO_2	**25**	ND	0.0	ND
1e	-	F	H	NO_2	0.0	ND	**100**	**13**
1f	-	Br	H	NO_2	**67.6**	**21.2**	**83**	**43**
1g	CH_2	Br	H	NO_2	**21**	ND	0.0	ND
1h	CH_2	Cl	H	NO_2	0.0	ND	**56**	**6.0**
1i	CH_2	F	NO_2	H	**25.4**	ND	0.0	ND



Comp.	X	R1	R_2	R_3	Enzyme Inhibition (%)			
					Topo I		Topo II	
					400 µM	50 µM	800 µM	80 µM
2a	-	$C(CH_3)_3$	H	NO_2	**47**	ND	**77**	**3.0**
2b	-	H	H	NO_2	**79**	**15.8**	**71**	**6.0**
2c	-	F	H	NO_2	0.0	ND	**63**	**6.0**
2d	-	CH_3CH_2	H	NO_2	**25**	ND	**32**	ND
2e	-	H	NO_2	H	**87**	**8.5**	**66**	**3.0**
2f	-	CH_3CH_2	NO_2	H	0.0	ND	0.0	ND
2g	-	F	NO_2	H	0.0	ND	0.0	ND
2h	CH_2	Cl	H	NO_2	**19**	ND	**83**	**8.0**
2i	CH_2	CH_3	H	NO_2	0.0	ND	**56**	**3.0**
2j	CH_2	F	H	NO_2	0.0	ND	0.0	ND
2k	CH_2	CH_3	NO_2	H	0.0	ND	**70**	2.0
2l	CH_2	F	NO_2	H	0.0	ND	0.0	ND
Camptothecin (100 µM)					100		-	
Etoposide (50 µM)					-		100	

ND: Not determined.

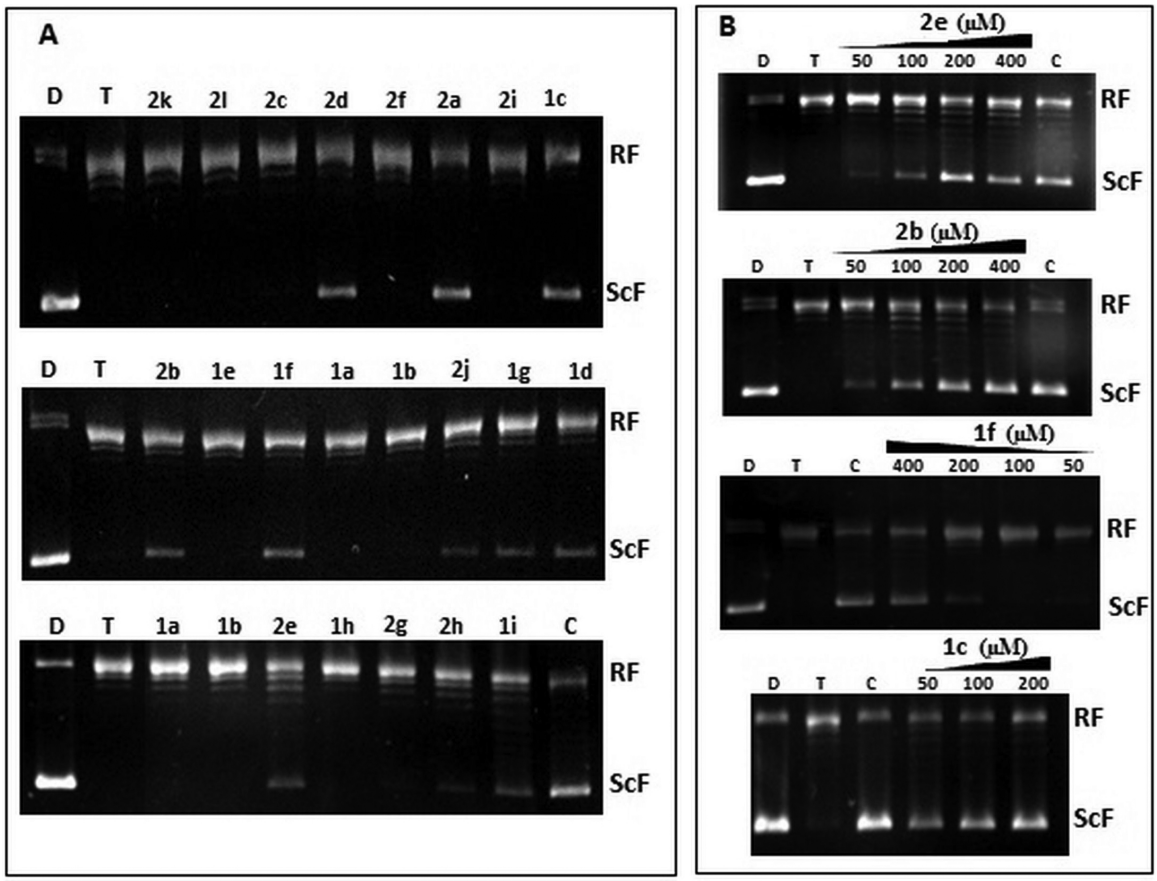


Fig. (1). Topo I inhibitory activities of tested compounds. (A) Effect of 1a-1l and **2a-2i** on DNA relaxation catalysed by Topo I at concentration of 400μM. (B) Effect of **1e, 1k, 2g** and **2a** on DNA relaxation catalysed by Topo I at various concentrations. Lane D: pBR322 DNA only; lane T: pBR322 DNA+Topo I; lane C: pBR322 DNA+Topo I+Camptothecin (100 μM); RF: relaxed form; ScF: supercoiled form.

relaxed form in the presence of the tested compounds. While Topo I and II inhibitory activities of the compounds were illustrated in Figs. (**1** and **2**), percentages of enzyme inhibition at the indicated concentrations were shown in Table **1**. These values were calculated by comparisons of the optical densities of the supercoiled DNA bands in the presence and absence of tested compounds.

If the tested compound had no inhibitory activity at 400 μM for TopoI and 800 μM for Topo II, then that compound was assumed as having no inhibitory activity and no lower concentrations were tested. Only compounds which inhibited TopoI and/or TopoII over 50% were evaluated in a concentration-dependent manner. After enzyme inhibition percentages of various concentrations of the compounds were calculated, IC_{50} values, shown in Table **2**, of effective compounds were obtained by using S-probit analysis.

It was shown that only four compounds (**1c, 1f, 2b** and **2e**) inhibited Topo I catalytic activity over 50% at 400 μM, when screening data of Topo I enzyme inhibition were analyzed. Compounds **1c** (2-(p-tert-butylphenyl)-6-nitrobenzoxazole) and **1f** (2-(p-bromophenyl)-6-nitrobenzoxazole) displayed 90.3% and 67.6% inhibition when **2b** and **2e** showed 79.0% and 87.7% inhibition, respectively. Among these compounds, **1c** was found as the most effective compound with the IC_{50} value of 104 μM among all of the tested structures. Besides, N-(2-hydroxy-4-nitro)-4-tert-butylbenzamide (**2a**) which was a possible metabolite of **1c** showed 47% Topo I catalytic inhibitory activity at 400 μM. These results showed that when the compound **1c** was hydrolyzed and returned to the amide form as the compound **2a**, **2a** remained its inhibitory activity well even if not as **1c**. In this context, the compound **1c** itself and the possible metabolite **2a** were thought to have potent inhibitory activity on Topo I.

Topo IIα increases 2- to 3-fold during G2/M and in order of magnitude higher in rapidly proliferating cells than in quiescent cells [52]. Therefore, Topo IIα inhibitors are especially attractive target for rapidly dividing cancer cells. According to screening data of DNA Topo II inhibition of the tested compounds, it was shown that most of the compounds had good Topo II inhibitory activities at 800 μM except compounds **1b**, **1d**, **1g**, **1i**, **2f**, **2g**, **2j** and **2l**. Further screening assays showed that compounds **1c**, **1e**, and **1f** displayed significant Topo II inhibitory activity at 80 μM (21%, 13% and 43% inhibition, respectively). According to these results, it was revealed that **1f** was the most effective compound among the tested compounds with the IC_{50} value of 71 μM on hTopo II.

According to inhibition percentages of Topo I and II, all of the tested compounds showed less inhibitory effects compared to positive controls, CPT and etoposide. However, it is

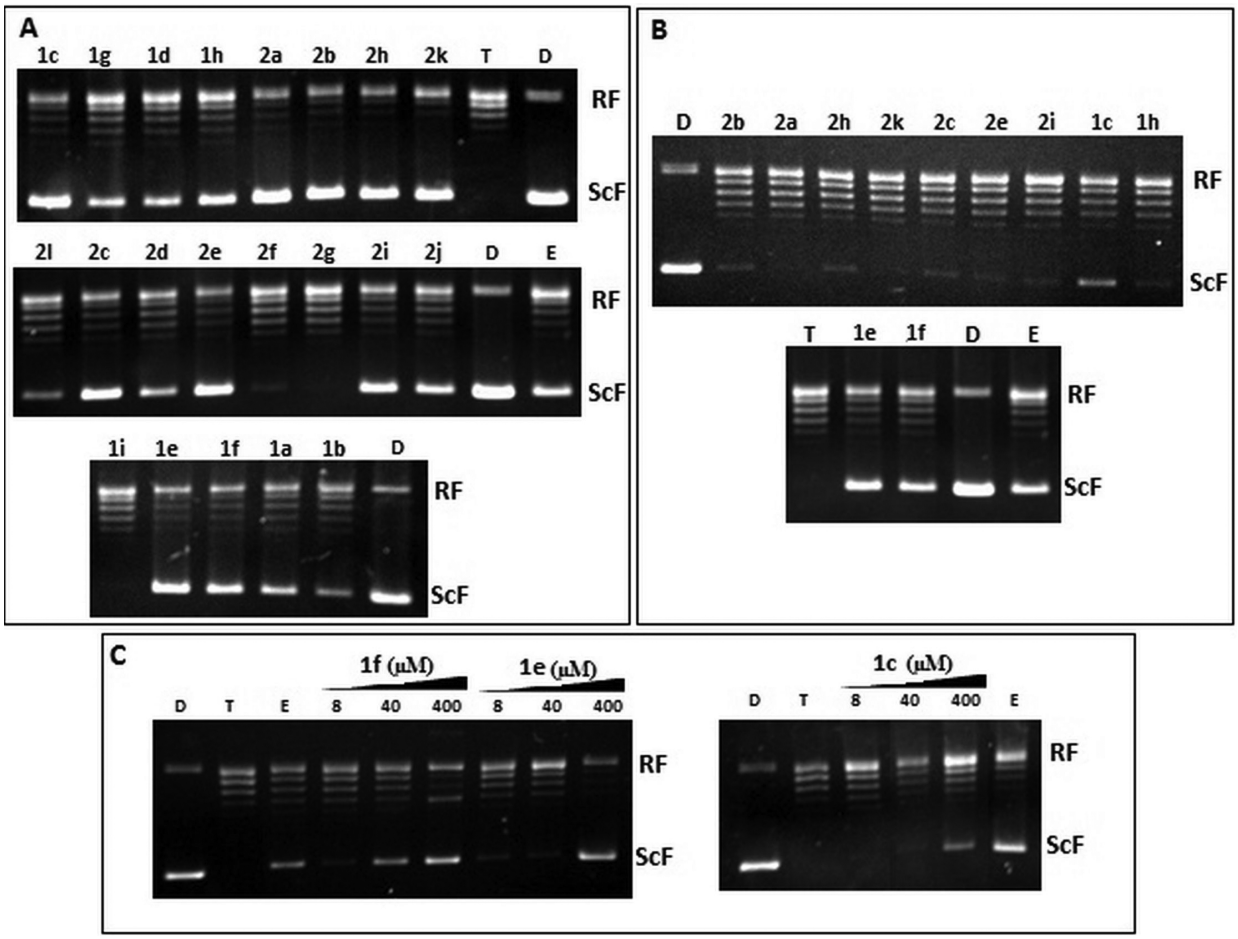


Fig. (2). Topo II inhibitory activities of tested compounds. (**A**) Effect of 1a-1l and **2a-2i** on DNA relaxation catalysed by Topo II at concentration of 800μM; (**B**) Effects of selected compounds on DNA relaxation catalysed by Topo II at concentration of 80μM; (**C**) Effect of **2a, 2f** and **2g** on DNA relaxation catalysed by Topo II at various concentrations. Lane D: pBR322 DNA only; Lane T: pBR322 DNA+Topo II; Lane E: pBR322 DNA+Topo I+Etoposide (50 μM); RF: relaxed form; ScF: supercoiled form.

well known that Topo I inhibitors such as Hoechst dyes, CPT and Topo II inhibitors such as doxorubicin exhibit poisoning activity at low concentrations, when they act as catalytic supressors at high concentrations [53-55]. In this context, the poisoning effects of **1c, 1e, 1f**, and **2b** compounds with the IC_{50} values around 100 μM should also be assessed.

Table 2. Topo I and Topo II inhibitory concentrations (IC_{50} values) of selected compounds.

Compounds	Topo I (μM)	Topo II (μM)
1c	104	120
1e	-	167
1f	217	71
2b	166	-
2e	243	-
CPT	35.34	-
Etoposide	-	10

Topoisomerase inhibitors can be classified primarily as inhibitors of Topo I, Topo II, or dual Topo I/II inhibitors. Some inhibitors, such as topotecan, affect only Topo I activity when doxorubicin is only a Topo II inhibitor. Similarly, BN-80927 is a homocamptothecin and a potent Topo I poison, but also a catalytic inhibitor of Topo II enzyme. It has excellent cytotoxicity against a number of human tumor cell lines in culture [56].

Compound **1e** had inhibitory activity on Topo II when **2b** and **2e** inhibited Topo I enzyme at the similar concentrations. Furthermore, **1c** and **1f** inhibited catalytic activity of both Topo I and Topo II at about 100 μM and they might be referred as dual inhibitors.

Topoisomerases are still good targets for cancer chemotherapy but toxicity of sequential or simultaneous combinations of Topo I and II inhibitors is the most important use-limiting factor. In this regard, a single compound able to inhibit both Topo I and II may present the advantage of improving topoisomerase inhibition, with reduced toxic side effects [57]. In this context, dual inhibitors **1c** and **1f** can be good alternatives among the tested compounds. However, cytotoxicity of these compounds against tumour cells should be evaluated to test this prediction. According to the activity

results, compound **1c** showed better Topo I inhibition and compound **1f** exhibited better Topo II inhibition than the other tested compounds. For benzoxazole derivatives, bulky groups at R1 position such as tert-butyl and Br increased both Topo I and Topo II inhibitions. For benzamide derivatives, it was found that small groups at R1 position such as H atom increased the Topo I inhibition and CH_2 bridge at the X position decreased both Topo I and Topo II inhibition.

It is well known fact that benzoxazole, benzimidazole and benzothiazole derivatives are desirable compounds as topoisomerase inhibitors for medicinal chemists as well as for our group. It is also known that a benzamide derivative, bisbenzoxazole UK-1, which is structurally very similar to the compounds tested in this study, is one of the strong inhibitors of Topo II [17, 53]. Oksuzoğlu reported some benzoxazole and benzimidazole derivatives as eukaryotic Topo I and II poisons and Kaplan-Ozen reported some benzothiazole derivatives as eukaryotic Topo II-α suppressors [12, 14].

Furthermore, studies on inhibitory effects of benzamide and /or benzenacetamide derivatives as possible metabolites of the benzoxazoles on the topoisomerase enzymes appear to be limited in the literature [58, 59]. Therefore, our data obtained from this present study may contribute to the literature about topoisomerase inhibitory activity of phenyl benzamides and benzenacetamides.

CONCLUSION

The most widely used approach for anticancer drug design is leading to cell death by the impairment of DNA repair and DNA damage accumulation in the cancer cells. In this respect, DNA topoisomerases are considered as important targets for cancer chemotherapy. Heterocyclic compounds included NO_2 are attractive derivatives for topoisomerase inhibitory activity. In this study, we tested topoisomerase inhibitory activities of some phenyl/benzyl benzoxazole derivatives and their possible metabolites. Our results showed that only four compounds had inhibitory activity on Topo I and three compounds on Topo II. Additionally, **1c** and **1f** inhibited both Topo I and Topo II activity. While CH_2 bridge on the X place seemed to decrease the Topo I and Topo II inhibition, bulky groups at R1 position such as tert-butyl and Br increased both Topo I and Topo II inhibition. These observations may provide some predictions in order to design novel topoisomerase inhibitors.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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