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

Fatma Zilifdar*a, Egemen Fotob, Tugba Ertan-Bolellib, İlkay Yildizb, Esin Aki-Yalccinb and Nuran Dirila

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, 2c) 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 IC50 value of 71 µM and 2-(4'-tert-butyl-phenyl)-6-nitrobenzoxazole (1e) with the IC50 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) CH2 bridge seems to decrease the Topo I and II inhibitions and (ii) bulky groups at R1 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.

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].
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 drug-receptor 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/benzyl-benzoxazole 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 solubilized 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 MgCl2, 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 °C 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 MgCl2, 1 mM EDTA, 1 mM ATP, and 15 mg/mL bovine serum albumin) for 30 min at 37°C. 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...
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Table 1. Topo I and II inhibitory activity of compounds.

<table>
<thead>
<tr>
<th>Comp.</th>
<th>X</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>Enzyme Inhibition (%)</th>
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<td></td>
<td></td>
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<td></td>
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<td>800 µM</td>
</tr>
<tr>
<td>1a</td>
<td>-</td>
<td>CH₃CH₂</td>
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<tr>
<td>1b</td>
<td>-</td>
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<td>NO₂</td>
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<td>H</td>
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<td>NO₂</td>
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<tr>
<td>1e</td>
<td>-</td>
<td>F</td>
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<th>Enzyme Inhibition (%)</th>
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<td>Topo I</td>
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<td></td>
<td></td>
<td>400 µM</td>
<td>50 µM</td>
<td>800 µM</td>
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<td>2a</td>
<td>-</td>
<td>C(CH₃)₃</td>
<td>H</td>
<td>NO₂</td>
<td>47</td>
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<td>H</td>
<td>H</td>
<td>NO₂</td>
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<tr>
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<tr>
<td>2g</td>
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<td>NO₂</td>
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<td>CH₃</td>
<td>H</td>
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<td>NO₂</td>
<td>H</td>
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</table>

Camptothecin (100 µM)  100  -
Etoposide (50 µM)  -  100

ND: Not determined.
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 TopoII, 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, IC50 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 TopoI catalytic activity over 50% at 400 µM, when screening data of TopoI 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 IC50 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% TopoI 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 TopoI.

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 IC50 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
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 suppressors at high concentrations [53-55]. In this context, the poisoning effects of 1c, 1e, 1f, and 2b compounds with the IC50 values around 100 μM should also be assessed.

Topoisomerases 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].

Compounds 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.

Table 2. Topo I and Topo II inhibitory concentrations (IC50 values) of selected compounds.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Topo I (µM)</th>
<th>Topo II (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1c</td>
<td>104</td>
<td>120</td>
</tr>
<tr>
<td>1e</td>
<td>-</td>
<td>167</td>
</tr>
<tr>
<td>1f</td>
<td>217</td>
<td>71</td>
</tr>
<tr>
<td>2b</td>
<td>166</td>
<td>-</td>
</tr>
<tr>
<td>2e</td>
<td>243</td>
<td>-</td>
</tr>
<tr>
<td>CPT</td>
<td>35.34</td>
<td>-</td>
</tr>
<tr>
<td>Etoposide</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

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.
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 CH₂ 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 found 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-Özen reported some benzothiazole derivatives as eukaryotic Topo II-α suppressors [12, 14].

Furthermore, studies on inhibitory effects of benzamide and/or benzenacatamides 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 benzenacatemides.

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 NO₂ 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 CH₂ 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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