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Critical structural motif for the catalytic inhibition of human topoisomerase II by UK-1 and analogs

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Abstract—Three new analogs of UK-1 have been synthesized and their efficacies as topoisomerase II inhibitors have been determined. Results show that UK-1 and two of these analogs are catalytic inhibitors of topo II and identifies a critical structural motif necessary for enzyme inhibition.

1. Introduction

DNA topoisomerases are nuclear enzymes responsible for controlling the topological state of DNA, carrying out a series of DNA cleavage and religation reactions.[1] Two major forms of topoisomerases are found in the eukaryotic cells, topoisomerase I (topo I) and topoisomerase II (topo II). Topo I creates single-stranded DNA breaks, allowing the opposing intact strand to pass through the nick, whereas topo II creates double stranded breaks, allowing intact duplex DNA to pass through the gap.[2,3] These actions are essential during DNA replication and transcription, where accumulated topological stress must be alleviated. Topo II is particularly important during cell replication, where the intertwined strands of DNA in the chromosomes must be separated before cell divisions can occur.

Topo II is a homodimer with each subunit containing two domains, the ATPase domain and DNA binding domain. The crystal structure of the DNA binding domain of yeast topo II reveals a conserved tyrosine residue located in the active site (tyrosine-783).[4] Although the details concerning the mechanism of topo II are not completely clear, a working model has been proposed based on the currently available biochemical and structural information.[5] The enzyme first associates with a section of duplex DNA, termed the G-segment. This signals the binding of two ATP molecules and causes the N-terminal domains to dimerize. This conformational change traps the second DNA duplex (the T-segment) and also induces cleavage of the G-segment. Hydrolysis of the ATP molecules results in the passing of the T-segment through the nicked G-segment and the religation of the G-segment, after which the enzyme dissociates from the DNA.[6]

It is well known that compounds which interfere with normal topoisomerase activity are highly cytotoxic; a number of these such as topotecan[7] (topo I) and etoposide[8] (topo II) are currently in clinical use as anticancer agents. Due to the complexity of the enzymatic process, drug inhibition mechanisms can also be highly complicated. Several compounds are known to interfere with the enzyme in different ways (Fig. 1). For example, etoposide inhibits topoisomerase by interfering with the religation of the G-segment, causing prolonged covalent attachment of the enzyme to the DNA (the 'cleavable complex').[9] Compounds that act by this mechanism are known as topo II 'poisons', and cause the accumulation of double-stranded DNA breaks, ultimately leading to apoptotic cell death. (Interestingly, recent studies show that etoposide can bind to the N-terminal ATPase domain as well as the enzyme active site, adding complexity to the mechanism of drug action.[10]) DNA binding compounds such as netropsin interfere with topo II activity not by stabilizing the cleavable complex, but by preventing the association of the enzyme with DNA.[11] This type of inhibition is a totally nonspecific

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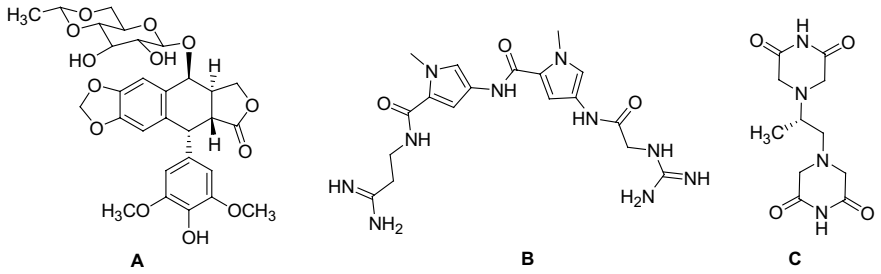


Figure 1. Examples of some topo II inhibitors: (A) etopside, (B) netropsin, and (C) ICRF-187.

effect and not limited to topo II inhibition. Additionally, drugs such as bisdioxopiperazine ICRF-187 interact with the ATPase domains, preventing ATP hydrolysis and enzyme turn-over.[12] Clearly, it is important to fully understand the mechanism of action for each class of compounds in order to design more effective topoisomerase II inhibitors.

A more recently discovered topoisomerase II inhibitor is the natural product UK-1 (Fig. 2).[13] First identified in 1993, UK-1 is a structurally unique bis(benzoxazole) metabolite of *Streptomyces* sp. 517-02.[14] This compound inhibits the growth of murine cancer cell line P388 with an IC_{50} in the 0.3–1.6 μM range.[15] UK-1 binds divalent metal ions[13] and binds DNA in a divalent metal ion-dependent manner.[16] Among Mg^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+}, Mg^{2+} was found to be the most effective to promote association with DNA. However, no hypothesis or data regarding the DNA binding mode of UK-1 has been offered. Recently, Kumar et al. showed that some truncated analogs of UK-1 share similar anticancer activities.[17] However, the exact mechanism of UK-1 is still unclear. To further the understanding of the anti-topo II activity of UK-1, three new analogs have been synthesized with more modest structural changes. These lack the methyl ester (**1**, Fig. 2), the *ortho*-hydroxyl (**2**), or both (**3**), allowing us to systematically evaluate the importance of these groups for activity. The syntheses of these compounds and results from topo II inhibition studies are presented below.

Analogs of UK-1 were prepared in which either the methyl-ester or the *ortho*-hydroxyl functionality was deleted as shown in Figure 1. The syntheses used parallel that previously reported for UK-1 with some modifications (Scheme 1).[18] 2-(Benzyloxy)benzoic acid (**4**) was coupled with methyl anthranilic acid (**5**) using 1-hydroxybenzotriazole (HOBT), 2-(1-*H*-benzotriazol-

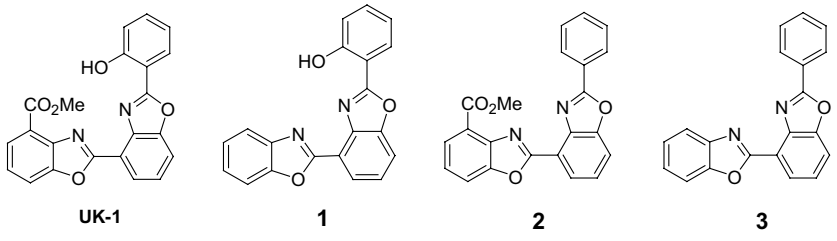
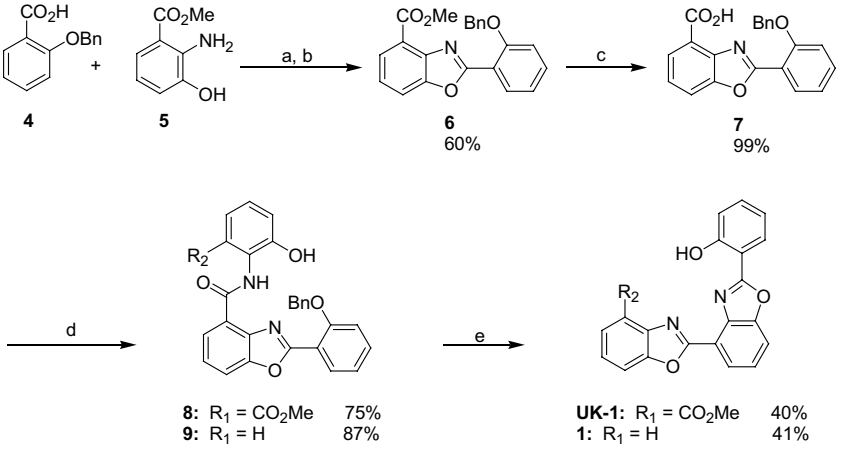


Figure 2. UK-1 and minimal analogs.



Scheme 1. Reagents and conditions: (a) HOBT, HBTU, DIPEA, DMF, rt; (b) 230 °C; (c) 5 M NaOH, THF, reflux; (d) **5** or 2-aminophenol, HOBT, HBTU, DIPEA, DMF, rt; (e) TsOH, toluene, reflux.

lyl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and diisopropylethylamine (DIPEA).[19] The amide product was isolated in 68% yield and subjected to thermal cyclodehydration to give the benzoxazole **6** in 89% yield.[20] The methylester was then hydrolyzed to the carboxylic acid in refluxing 5 M NaOH/THF to give **7** in essentially quantitative yield.

Using the previous coupling conditions (HOBT, HBTU), acid **7** was reacted with either **5** or 2-aminophenol to give **8** and **9**, respectively. Subsequent ring closure and benzyl deprotection were achieved in a single step by refluxing the amides with *p*-toluenesulfonic acid in toluene. The products, UK-1 and **1**, were isolated in 40% and 41% yield, respectively.

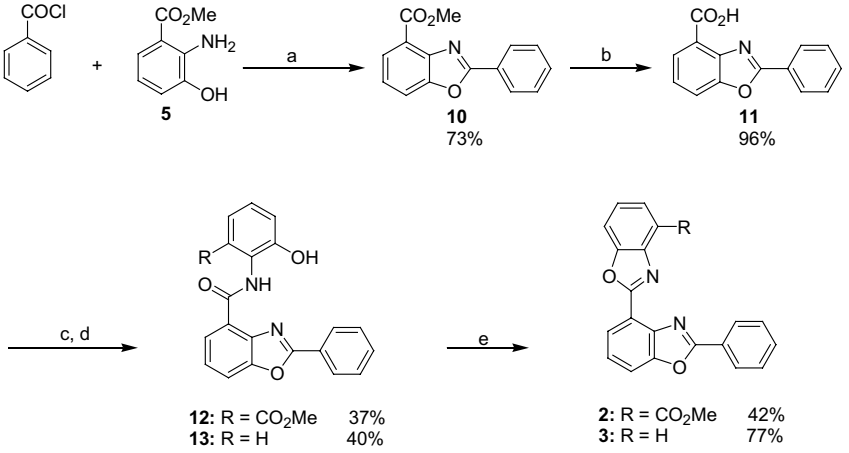
For compounds **2** and **3**, benzoyl chloride was reacted with **5** in refluxing *m*-xylene to provide benzoxazole **10** in 73% yield in a single step (Scheme 2).[21] Hydrolysis of **10** in refluxing 5 M NaOH/THF gave corresponding acid **11** in 96% yield. The acid was activated using oxalyl chloride and coupled with either **5** or 2-aminophenol to yield **12** and **13**, respectively. Refluxing the resulting amides with *p*-toluenesulfonic acid in toluene afforded the ring-closed deprotected products **2** and **3** in 42% and 77% yield, respectively.

The activity of UK-1 and analogs **1**–**3** against human topo IIα were evaluated by determining their abilities to interfere with the relaxation of supercoiled plasmid DNA.[22] The assay monitors the DNA products after 30 min incubation of supercoiled DNA with topo II in the absence and the presence of each compound.[23] The DNA products are separated on an agarose gel and visualized by fluorescence. The results from these studies are shown in Figure 3. A sample agarose gel from the assay is shown in Figure 3A. This assay is capable of determining if the compounds are topo II poisons.[24] As described earlier, poisons stabilize the cleavable complex and prolong the covalent association between the enzyme and DNA. When this stabilized complex is treated with SDS followed by digestion with proteinase K, the DNA will not be religated, resulting in the appearance of linear DNA. If a compound simply interferes with the enzyme, either by preventing DNA binding[11] or dissociation after completing the strand passage[12], no linear DNA would be observed, but there would be an increase in supercoiled DNA (relative to drug free control). In the case of UK-1 (Fig. 3A), no linear DNA is observed, but a concentration dependent increase in the amount of supercoiled DNA is seen.[25] Similar results were observed for compounds **1** and **2**, suggesting these compounds are inhibitors but not poisons.

The gel data were used to compare the effectiveness of each compound, using the ratio of fluorescence intensities for supercoiled DNA and relaxed circular DNA in each lane. Figure 3B shows the graphical presentations of the amount of supercoiled DNA as a function of drug concentration for all the compounds. From these graphs, IC_{50} values for UK-1, **1**, and **2** were determined to be 32, 20, and 38 μM, respectively.[26] Notably, compound **3** showed no measurable activity. Based on these results, the order of activity is **1** > UK-1 > **2** ≫ **3**.[27]

The results from the topo II assay as shown in Figure 3A suggest UK-1 is a catalytic inhibitor of the enzyme but not a topo II poison.[28] Unfortunately, the assay does not distinguish the remaining possible mechanisms of inhibition. As previously described, compounds can interfere with either DNA binding or enzyme dissociation, either of which would provide the observed results. The fact that UK-1 has been shown to bind DNA would tend to suggest that the former mechanism of topo II inhibition is operative. It is, however, impossible to rule out the possibility that UK-1 is capable of deactivating the enzyme during catalysis without stabilizing the cleavable complex.

UK-1 has shown cytotoxicity at submicromolar concentrations against two cancer cell lines (HL60 and PC-3, with IC_{50} values of 0.32 and 0.4 μM, respectively).[17] These IC_{50} values for live cell studies are much lower than those observed for topo II inhibition, reported



Scheme 2. Reagents and conditions: (a) TEA, pyridinium *p*-toluene sulfonate, *m*-xylene, reflux; (b) 5 M NaOH, THF, reflux; (c) $(ClCO)_2$, DMF, CH_2Cl_2; (d) **5** or 2-aminophenol, pyridine, CH_2Cl_2; (e) TsOH, toluene, reflux.

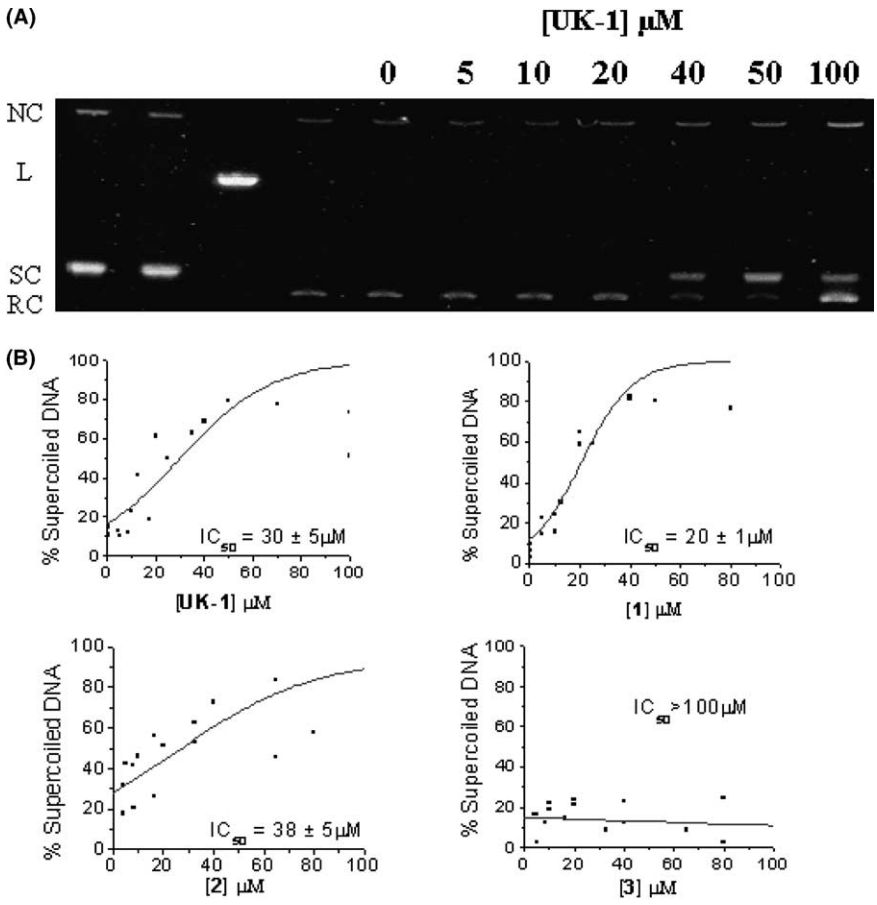


Figure 3. (A) An example of an agarose gel, showing concentration dependent inhibition of topo II-mediated relaxation of supercoiled pBR322 by UK-1. Abbreviations: Supercoiled (SC), nicked circular (NC), linear (L), and relaxed circular (RC) DNA. Controls were run in the first four lanes. Lane 1 contained plasmid DNA only, Lane 2 contained plasmid DNA with 1% DMSO, 1% SDS, and proteinase K, lane 3 contained linearized DNA, lane 4 contained plasmid DNA and topo II, and lanes 5–11 contained 1% DMSO with or without UK-1 (the final concentration of UK-1 is shown on the top of each lane). (B) The quantified data for the % supercoiled DNA present in each lane. Data from three separate assays were plotted and IC_{50} values were obtained by fitting the pooled data.[23]

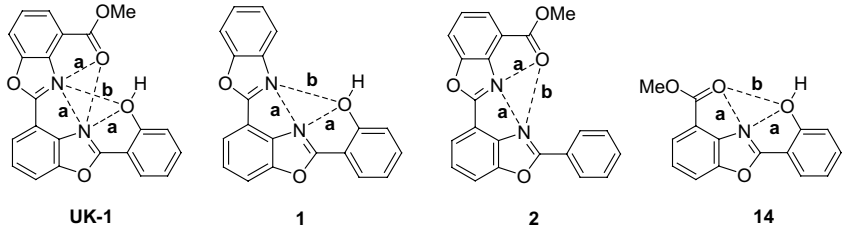


Figure 4. A unique structural motif for UK-1 and analogs based on molecular modeling. Distances indicated by **a** varied between 2.875 and 3.045 Å and those indicated by **b** varied between 4.524 and 4.727 Å for all the structures shown.[30]

here. The reason for the apparent discrepancy is still unclear. It is possible that topo II might not be the primary cellular target of UK-1. Alternatively, it is possible that UK-1 interferes with multiple cellular processes and cytotoxicity is enhanced by multiple mechanisms. It has been observed with topoisomerase I inhibitors such as Hoechst dyes that the mode of inhibition changes between low drug concentration ($<1.0\,\mu M$) and high drug concentration ($>1.0\,\mu M$).[29] At low concentration, the ligands exhibit topo I poisoning activity, inducing DNA cleavage, while at high concentration, the ligands act as catalytic supressors by preventing association of enzyme with DNA. Such a change in mode of inhibition could significantly alter the apparent cytotoxicity of each ligand. Although no evidence for such behavior has been presented for topo II inhibitors, work is currently underway to explore such a possibility for UK-1 and analogs.

Our data show that the removal of either the methyl ester or *ortho*-hydroxyl group does not alter the anti-topo II activity significantly. However, the activity is

dramatically decreased when *both* functional groups are deleted (even at 80 μM, no inhibition is observed for **3**). It is notable that UK-1 and analogs **1** and **2** all possess a triangular arrangement of Lewis-basic sites comprised of heterocyclic nitrogen atoms, an ester oxygen atom, and/or a phenolic hydroxyl group (Fig. 4). Truncated UK-1 analog **14**, reported by Kumar et al., possesses a similar arrangement of basic sites and shows cytotoxicity at similar concentrations to that of the parent compound (although anti-topo II activity was apparently not evaluated).[17] Molecular mechanics-minimized structures for all these compounds point to a very well-defined arrangement of the basic sites, which effectively defines an isosceles triangle that is 2.875–3.045 Å on two sides and 4.524–4.727 Å on the remaining side. Our data argue that this unique structural motif is necessary for enzyme inhibition.

We would like to suggest that the role of these groups involves metal ion chelation, which has been shown to be important for DNA binding and correlated to cytotoxicity.[17] For magnesium, this type of chelation is observed in chlorophylls, where Mg^{2+} is coordinated to the four nitrogens in the porphine core structure.[31] It is possible that UK-1 and the active analogs described here form similar square planar complexes, with a donor site from DNA occupying the fourth coordination site. It is important to point out that the anti-topoisomerase activity of UK-1 could involve direct interaction of the drug with the enzyme. By a similar scenario, then, it is possible that binding with enzyme-associated magnesium could also lead to inhibition (with functionality from the enzyme occupying a coordination site).

This work demonstrates that UK-1 does not inhibit human topoisomerase II by stabilizing the cleavable complex and identifies a minimal structural motif necessary for activity. Magnesium binding experiments and cytotoxicity studies, currently in progress, should establish a more clear relationship between magnesium binding, topo II inhibition, and cytotoxicity.

Acknowledgements

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23. Human topoisomerase II alpha was purchased from USB Corporation. Supercoiled pBR322 plasmid DNA was purchased from New England Biolabs. Reaction buffers were freshly prepared according to the manufacturer recommended concentrations. All drugs were diluted in dimethyl sulfoxide to give 10 mM stock solutions. Each solution was further diluted with DMSO to give the desired final concentration, and each reaction contained 1% DMSO. The assay was carried out by incubating 2 units (0.045 μg) of human topo IIα, 0.2 μL of appropriate ligand, and 0.111 μg of supercoiled pBR322 DNA in a total volume of 20 μL assay buffer (10 mM Tris–HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 15 μg/mL BSA, 1 mM ATP). The sequence of addition was water, reaction buffer, DNA, ligand, and then enzyme. Reactions were incubated at 37 °C for 30 min and stopped by the addition of 2.2 μL 10% SDS and 1 μL of 1.4 mg/mL Proteinase K solution. The samples were incubated at 37 °C for an additional hour before subjecting to electrophoresis in 1.5% agarose gel in TAE (40 mM Tris–acetate, pH 8.3, 2 mM EDTA) containing 0.5 μg/mL ethidium bromide. Gels were run at 40 V for 6 h and destained for at least 1 h before visualizing on a Typhoon 9200 (Amersham Biosciences). The bands were quantified using ImageQuant Version 5.2 (Molecular Dynamics). The data were plotted and analyzed with Origin version 6.0 (Mircrocal) imbedded DoseResp Pharmacology curve fitting.
24. The assay was first carried out with known topo II poison etopside (VP-16) to evaluate the efficiency of the assay. Under the same assay conditions, a 15% increase in linear DNA was observed at 100 μM drug concentration.
25. The linear DNA standard was obtained by treating supercoiled pBR322 DNA with *Eco*RI (Sigma).
26. IC_{50} values were obtained the by fitting the data with the Dose Response function in Microcal Origin 6.0.

$$Y = \frac{\text{Fluorescence intensity of supercoiled DNA}}{\text{Fluorescence intensity of supercoiled DNA + relaxed DNA}}$$

$$Y = A1 + \frac{A2 + A1}{1 + 10^{(\log X0 - X)p}}$$

Y = fraction of supercoiled DNA, X = ligand concentration, $A1$ = bottom asymptote, $A2$ = top asymptote.

27. The slopes of these plots at low ligand concentrations suggest a similar order of efficacy; the values are 0.043, 0.023, 0.010 for 1, UK-1, and 2, respectively. We do not believe that the lack of activity of three results from poor solubility of this compound under the assay conditions based on the fact that all reaction mixtures were visually homogeneous.
28. It should be noted that at concentration higher than 80 μM, a decrease in the intensity of supercoiled DNA was often observed. The drops in the relative concentration of supercoiled DNA range from 5% to 40%. The reason behind this phenomenon is still unclear. It is also unclear why complete inhibition is not observed at high ligand concentration.
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