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INDOLOCARBAZOLE POISONS OF HUMAN TOPOISOMERASE I: REGIOISOMERIC ANALOGUES OF ED-110

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Abstract: All four "symmetrical" regioisomers of ED-110, an indolocarbazole derivative having potent activity against human topoisomerase I (Topo I) were synthesized. The isomer containing hydroxyl groups in the 3- and 9-positions was approximately ten-fold more active against Topo I, and 5- to 35-fold more active against human solid tumor cell lines in vitro, relative to ED-110.

Introduction

Topoisomerase I (Topo I) has generated significant interest as a target for the development of anticancer drugs,[1,2] following the discovery that Topo I was the target of camptothecin. Topotecan, a semi-synthetic analogue of camptothecin, was the first antineoplastic agent approved for clinical use that targets Topo I. Topo I poisons generally act via stabilization of the covalent enzyme-DNA intermediate, often referred to as the "cleavable complex", thus interrupting cell division and eventually leading to cell death. Very few specific Topo I poisons are known.

In recent years, indolocarbazoles of microbial origin were identified which act as Topo I poisons, with potencies similar to camptothecin. BE-13793C (Figure 1), isolated from a streptomycete, was reported to induce TopoI-associated DNA damage.[3] A semi-synthetic derivative, ED-110, prepared via enzymatic glycosylation of BE-13793C, possessed enhanced anti-Topo I activity and increased water solubility.[4] ED-110 exhibited cytotoxicity against a variety of tumor cell lines in vitro, as well as in vivo antitumor activity in xenotransplanted nude mice.[5] Another semi-synthetic derivative, NB-506, possessed potent anti-Topo I activity, as well as antitumor activity in vitro and in vivo.[6] A phase I clinical trial of NB-506 showed reduction of tumor-specific markers in ovarian and breast cancer patients clinically resistant to taxol therapy.[6]

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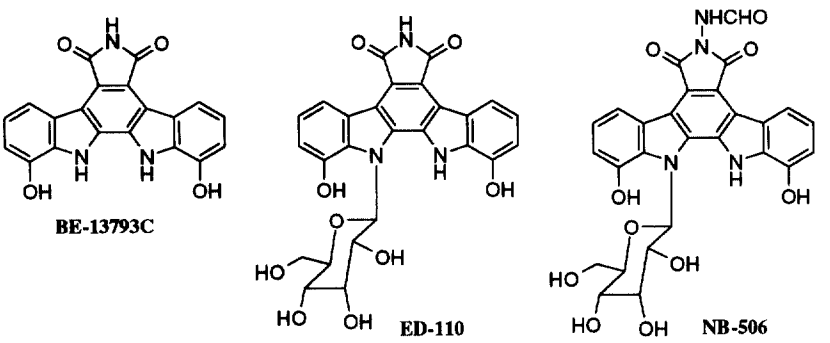


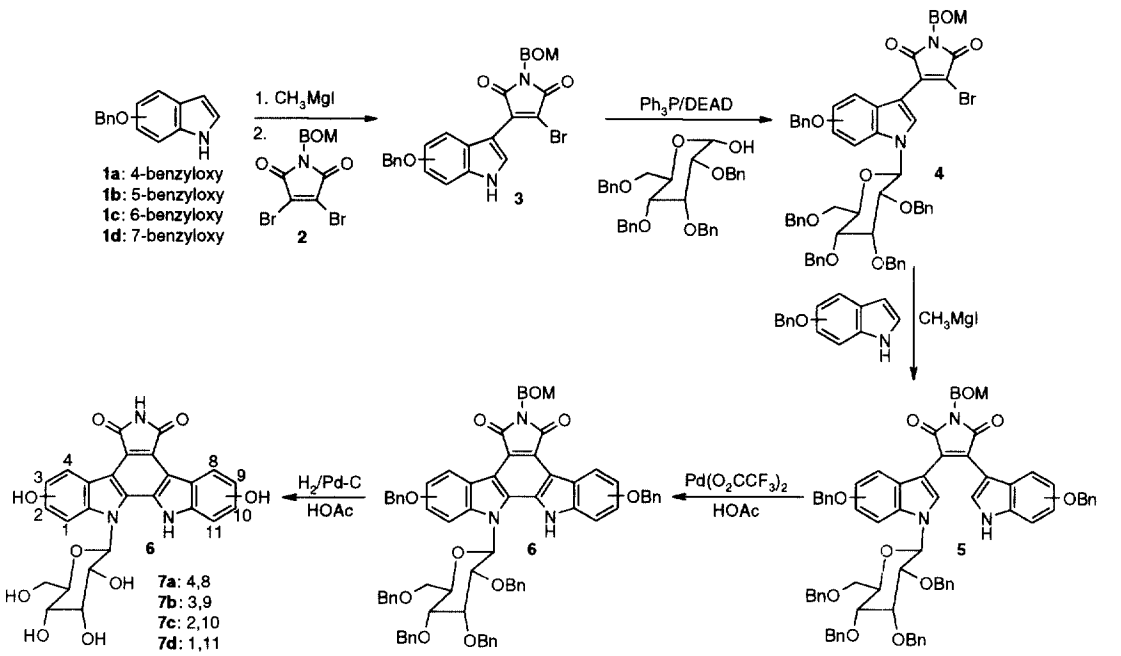
Figure 1

Total syntheses of ED-110 and NB-506 have been reported.[7] The goal of our studies was to prepare synthetic derivatives of ED-110, and to examine the effects of modifying the positions of the benzenoid hydroxyl groups on Topo I activity and tumor cell growth inhibition. It is known that removal of the hydroxyl groups or replacement with chlorine atoms decreases biological activity.[8] Though syntheses of indolocarbazole analogues having mono- and di-hydroxy substituents at various ring positions have been reported,[9,10] no results regarding the effects of positioning of the hydroxyl groups on biological activity have been published. To serve as a pilot study, we chose to prepare the four "symmetrical" analogues of ED-110, in which the two indole units comprising the indolocarbazole core were identical. The results of our study are presented herein.

Chemistry

The four regioisomers of ED-110, with respect to positioning of the hydroxyl groups, were synthesized as illustrated in Scheme 1. Benzyl-protected hydroxyindoles **1a-1d** were prepared from the corresponding benzyl-protected nitrocresols using the method of Batcho and Leimgruber,[11] except for **1b**, which was prepared via benzylation of commercially available 5-hydroxyindole.

Treatment of *N*-(benzyloxymethyl)dibromomaleimide (**2**, prepared via protection of 3,4-dibromomaleimide[12] with benzyloxymethyl chloride[13]), with the magnesium halide salts of indoles **1** provided the monoindolylmaleimides **3a-3d**. Introduction of the glucose moiety via Mitsunobu reaction[10] (PPh_3, DEAD) with tetra-*O*-benzyl-α-D-glucose afforded the glycosylated intermediates **4a-4d**. Introduction of the second indole unit, again using the magnesium halide salts of indoles **1**, afforded bisindolylmaleimides **5a-5d**. Oxidative cyclization of **5** using Pd(II) trifluoroacetate[7] in acetic acid afforded indolocarbazoles **6a-6d**. Hydrogenolysis of **6** in acetic acid (3 atm H_2, 10% Pd-C) provided the desired analogues **7a-7d**.



Scheme 1

Results and Discussion

The indolocarbazoles **7** were evaluated for activity against human TopoI, measured by inhibition of enzyme-catalyzed relaxation of pBR322 supercoiled plasmid DNA.[14] The concentrations of indolocarbazole that imparted inhibition of Topo I-mediated relaxation of pBR322 DNA by 50% relative to inhibitor-free controls (IC_{50}) are reported for each of the four analogues in Table 1.

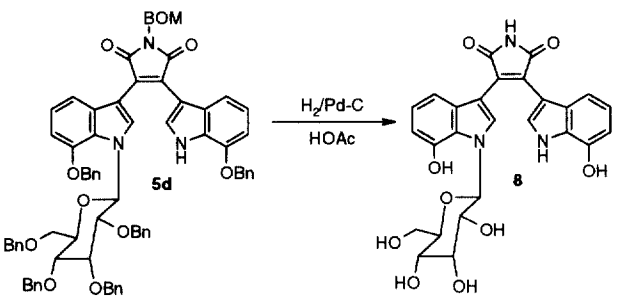
Table 1. Inhibition of human topoisomerase I by indolocarbazole derivatives **7a-7d**

Compound	IC_{50} (μM)
7a	36
7b	1.5
7c	2.3
7d (ED-110)	13

The observed activity of ED-110 (**7d**) against human Topo I-induced relaxation of pBR322 was consistent with data previously reported for assays using P388/S Topo I (isolated from P388/S murine leukemia cells). In our study, an average IC_{50} value of 13 μM was observed for ED-110 (mean of three trials). The regioisomer **7a**, which consists of the same indolocarbazole core as ED-110, but with the two phenol moieties shifted to the

4- and 8-positions, exhibited decreased activity relative to ED-110, approximately three-fold less active. However, the two analogues having the dihydroxyl moieties in the 2,10- and 3,9-positions (compounds **7b** and **7c**) respectively, possessed activity superior to ED-110. Analogue **7c** was approximately 6-fold more potent than ED-110, while analogue **7b** possessed activity approximately 10-fold superior to ED-110, representing an order of magnitude increase in anti-Topo I potency. Analogues **7b** and **7c** increased distortion of the DNA bands during electrophoretic separation on 1% agarose gel, indicating these two materials may intercalate DNA to a greater degree than the other two analogues, for which such distortion was not observed.

Previously, in research targeted toward inhibition of protein kinases, bis-indolylmaleimides were prepared which exhibited inhibition of protein kinases with potencies similar to their parent indolocarbazoles, but with greater selectivity.[15] We thus prepared the bis-indolylmaleimide analogue of ED-110 (**8**), illustrated in Scheme 2. Hydrogenolysis of **5d** in acetic acid afforded **8**, without competitive reduction of the maleimide double-bond. Compound **8** was evaluated for inhibition of Topo I, and found to be completely inactive up to a concentration of 400 μM.



Scheme 2

In vitro antitumor activity was evaluated in three human solid tumor lines, using the MTT cytotoxicity assay.[16] The HT-29 colon, OVCAR-3 ovarian, and DU-145 prostate cell lines were utilized to assess activity against a broad array of tumors. ED-110 (**7d**) was previously shown to vary greatly in its toxicity against different cell lines.[17] Results of the in vitro antitumor assays are summarized in Table 2.

The four indolocarbazole analogues (**7**) exhibited a broad spectrum of toxicity against the three tumor lines evaluated. ED-110 (**7d**) was active at concentrations similar to those previously reported. **7d** was moderately active against the HT-29 colon line (IC_{50} > 10 μM), consistent with previous data regarding poor activity against DLD-1 and WiDr colon cell lines.[17] The compound was active against the ovarian line (IC_{50} = 0.92 μM) and moderately active against the prostate line (IC_{50} = 2.3 μM).

Compound **7a** followed the same trends, showing weak activity against the colon line, potent activity against the ovarian line, and moderate activity against the prostate line. Compounds **7b** and **7c**, which were the

two most active analogues in the Topo I assay, were also the most active analogues in the in vitro antitumor assay. All compounds exhibited the weakest activity against the colon line. The bis-indolylmaleimide **8**, which was inactive against Topo I, was also devoid of activity against the tumor cells at a concentration as high as 10 μM.

Table 2. IC_{50} values (μM) for compounds **7** and **8** against human tumor cell lines in vitro (average of three trials)

Compound	HT-29 (colon)	OVCAR-3 (ovarian)	DU-145 (prostate)
7a	1.3	0.44	0.95
7b	0.84	0.19	0.067
7c	9.7	0.19	0.12
7d (ED-110)	>10	0.92	2.3
8	No activity	No activity	No activity

Analogue **7b**, which was the most active compound against Topo I, was also the most active compound in the in vitro antitumor study. **7b** was active against the colon line (IC_{50} = 0.84 μM), the ovarian line (IC_{50} = 0.19 μM), and very active against the prostate line (IC_{50} = 0.067 μM).

The differential cytotoxicity of the indolocarbazole analogues may be a result of differing levels of catalytically active Topo I across cell lines. Indeed, baby hamster kidney cells transfected with human Topo I, and which overexpress catalytically active Topo I, were hypersensitive to camptothecin.[18] In contrast, tumor cell lines resistant to camptothecin contained decreased levels of Topo I relative to the corresponding sensitive cell lines.[19] In clinical tumor samples, colorectal and prostate tumors contained elevated levels of Topo I relative to surrounding normal tissue, while kidney tumors did not.[20] These data could explain the hypersensitivity of the DU-145 prostate line to compound **7b**.

The results presented above demonstrate that subtle changes in the structure of indolocarbazoles can result in significant attenuation of biological activities, including Topo I poisoning and in vitro antitumor activity. Further studies in the structure-activity relationships of this class of potential antitumor agent are being pursued.

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14.**Topoisomerase I assay.** The assay mixture contained 40 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol, 0.5 mM EDTA, 15 μg/mL BSA, 7.5 mM $MgCl_2$, 80 mM KCl, 0.25 μg pBR322 plasmid DNA and 1 unit Topo I, in a total volume of 20 μL. Test drug was added, dissolved in DMSO, with a final DMSO concentration of approximately 5%. Reactions were allowed to proceed for 30 min at 37 °C, then the reaction was terminated by the addition of proteinase K in 1% SDS (10 mg/mL, 2 μL). After 30 min at 37 °C, the samples were cooled to 0 °C and treated with EtOH (75 μL) and 5 M NaCl (2.2 μL). The reaction vessels were cooled on dry ice for 60 min, then the DNA pelleted by centrifugation (16,000 x *g*, 10 min). The supernatent was discarded, and the DNA pellet resuspended in 18 μL reaction buffer, diluted with 2 μL loading buffer, and then added to appropriate lanes of a 1% agarose gel made up with 1X TPE buffer containing 2 μg/mL chloroquine. Gels were run for 15 h at 15 V, then stained with 0.5 μg/mL ethidium bromide. After destaining in H_2O for 30 min, gels were photographed over a UV light box with Polaroid 665 positive/negative film, and negatives were scanned with a UMAX Super Vista S-12 scanner, using Adobe and NIH imaging software. The IC_{50} values were assigned as the drug concentration which prevented TopoI-mediated relaxation of pBR322 plasmid DNA by 50% relative to controls in the absence of enzyme and inhibitor, measured by densitometry of the band area of the unreacted plasmid and comparison with controls. Each IC_{50} value was determined in triplicate, and are reported in Table 1 as the mean of three trials, having standard deviations ranging from 17% to 36% of the means. Prior to use, the activity of each stock of human Topo I (Topogen, Inc., Columbus, OH) was evaluated and normalized by titrating with pBR322 supercoiled plasmid DNA. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the complete relaxation of 0.25 μg pBR322 DNA in 30 min under the above conditions at 37 °C. As another measure of control, it was verified that one unit of Topo I was completely inhibited by 100 μM camptothecin.
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