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SHORT COMMUNICATION

Stimulation of Topoisomerase II-Mediated DNA Cleavage by an Indazole Analogue of Lucanthone

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ABSTRACT. Lucanthone is an antitumour drug used as an adjuvant in radiation therapy. The drug intercalates into DNA and inhibits topoisomerase II. An indazole analogue of lucanthone (IA-5) was examined for its ability to modulate topoisomerase II–DNA cleavable complex formation *in vitro*. The drug contains a methyl-benzothiopyranoindazole chromophore instead of the methyl-thioxanthenone nucleus of lucanthone. Using a radiolabelled linear plasmid DNA as a substrate, both lucanthone and the indazole analogue were shown to promote the cleavage of DNA by human topoisomerase II. Sequencing experiments with different restriction fragments indicated that the indazole drug promoted DNA cleavage primarily at sites having a C on the 3′ side of the cleaved bond (−1 position). By contrast, in the same sequencing methodology lucanthone exerted a much weaker effect on topoisomerase II. The sequence selectivity of IA-5 is reminiscent of that of the anticancer drug mitoxantrone and its anthrapyrazole analogue losoxantrone, which is structurally close to IA-5. Binding to DNA and topoisomerase II inhibition are two distinct processes contributing separately to the cytotoxic activity of the indazole drug. BIOCHEM PHARMACOL **58**;8:1307–1312, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. lucanthone; topoisomerase II; DNA cleavage; antitumour agents

A large number of DNA-intercalating agents are used in cancer chemotherapy. Drugs such as actinomycin, daunomycin, mitoxantrone, and amsacrine, which are extensively used in the clinic, exert their cytotoxic effects via an interaction with nucleic acids. Intercalation into DNA is essential to their antitumour activity, but is not the sole determinant. In addition, these drugs interfere with the breakage–ligation reaction catalysed by DNA topoisomerase II. This ubiquitous and vital enzyme is considered as one of the most important molecular targets for the aforementioned drugs [1, 2]. The list of compounds which can interfere with topoisomerase II function has expanded considerably over the past five years [see Ref. 3 for a comprehensive review]. Most of them, but not all, behave as typical DNA-intercalating agents. Topoisomerase II inhibition is not the privilege of anticancer agents. Antibacterial quinolones, antiviral compounds, and several antiparasite drugs can also function as topoisomerase II poisons [4]. Among the DNA intercalators which exhibit antiparasite properties and act at the topoisomerase II level, there is the drug lucanthone (also known as Miracil D or Nilodin, Fig. 1), which has been used extensively for the treatment of schistosomal infections [5]. Recently, it was reported that lucanthone, which can be used as a radiosensitising agent in cancer radiotherapy [6–8], is a specific topoisomerase II inhibitor capable of trapping enzyme–DNA covalent complexes [9].

This observation prompted us to investigate the interaction between topoisomerase II and an indazole analogue of lucanthone, IA-5† (Fig. 1). This compound possesses a methyl-benzothiopyranoindazole chromophore instead of the methyl-thioxanthenone nucleus of lucanthone, but otherwise bears the same diethylaminoethylamino side chain which is positively charged under physiological conditions. Both IA-5 and lucanthone intercalate into DNA. The helix-unwinding angle associated with their intercalative binding is ~15° [10, 11]. Interestingly, IA-5 and lucanthone share the rare property of intercalating preferentially at AT-rich sequences in DNA [11], whereas the vast majority of intercalators either exhibit little or no sequence preference or bind selectively to GC sequences. AT selectivity is much more pronounced with IA-5 than with lucanthone, most likely as a result of increased affinity for double-stranded DNA [11]. IA-5 and related benzothiopyranoindazole derivatives were originally designed as antitumour agents. The anticancer properties of IA-5 itself are superior to those of lucanthone [12], and analogues are still being developed in an effort to confer higher antitumour activity [13–15]. Moreover, the same benzothiopyranoindazole chromophore occurs in the compound CI-958 [16] and its derivatives, which have a broad spectrum of antitumour activity and are considerably less cardiotoxic than the

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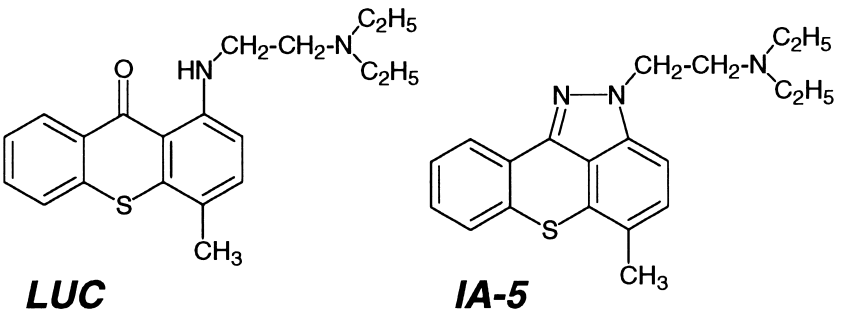
† *Abbreviations:* IA-5, benzothiopyranoindazole analogue of lucanthone; and TBE, Tris-borate-EDTA.



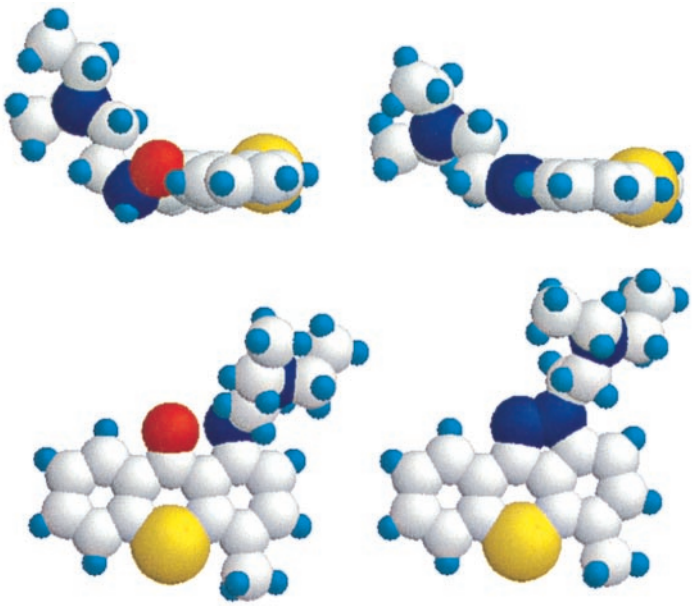


FIG. 1. Structure of lucanthone (LUC) and its indazole analogue (IA-5). An energy-minimised structure of the drugs is shown. The HyperChem™ 5.01 and Alchemy 2000® softwares were used to construct the structures. Colour code: S, yellow; N, dark blue; O, red; H, light blue.

clinically used anthracyclines [17]. It is also worth mentioning that the indazole analogues resemble the anthrapyrazole [18] and the pyrazoloacridine [19] series of anticancer agents. Consequently, elucidation of the topoisomerase II inhibition properties of IA-5 may furnish useful information concerning the likely effects on topoisomerase II of several structurally related antitumour drugs.

MATERIALS AND METHODS

Chemicals and Biochemicals

Samples of lucanthone and IA-5 were kindly supplied by Dr M.J. Waring (Dept. of Pharmacology, Cambridge, U.K.). Restriction enzymes, phosphatase, and T4 polynucleotide kinase were from Boehringer. γ-[^{32}P]-ATP (3000 Ci/mmol) was purchased from Amersham.

DNA Purification and Labelling

The 117-mer and 265-mer fragments from plasmid pBS (Stratagene) were prepared by 5′-[^{32}P]-end labelling of the *Eco*RI/alkaline phosphatase-treated plasmid using γ-[^{32}P]-ATP and T4 polynucleotide kinase followed by treatment with *Pvu*II. Similarly, the 155-mer and 178-mer fragments were prepared by 5′-end labelling of the *Eco*RI-*Hin*dIII and *Eco*RI-*Pvu*II digests, respectively, of plasmid pLAZ3 [20]. In each case, the digestion products were separated on a 6% polyacrylamide gel under native conditions in TBE-buffered solution (89 mM Tris-Borate pH 8.3, 1 mM EDTA). After autoradiography, the band of DNA was excised, crushed, and soaked in water overnight at 37°. The DNA was precipitated with ethanol.

Topoisomerase II–DNA Cleavage Reaction

The cleavage reaction mixture contained 20 mM Tris–HCl pH 7.4, 60 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM ATP, 500 cps of γ-[^{32}P]-pKM27 DNA, and the indicated drug concentrations. The reaction was initiated by the addition of human topoisomerase II (10 units in 20 μL reaction volume, p170 form from TOPO-

gen) and allowed to proceed for 30 min at 37°. Reactions were stopped by adding SDS to a final concentration of 0.25% and proteinase K to 250 μg/mL, followed by incubation for 30 min at 50°. Five microlitres of loading buffer (30 mM EDTA, 15% [w/v] sucrose, 0.1% electrophoresis dye) were added to each sample prior to loading onto a 1% agarose gel in TBE-buffered solution containing 0.1% SDS.

Sequencing of Topoisomerase II-Mediated DNA Cleavage Sites

Each reaction mixture contained 2 μL of 5′ [^{32}P]-end labelled DNA, 6 μL of water, 2 μL of 10X topoisomerase II buffer, and 10 μL of drug solution at the indicated concentration. After at least 15 min incubation to ensure equilibration, the reaction was initiated by addition of 20 units human topoisomerase II. Samples were incubated for 40 min at 37° prior to adding SDS to 0.25% and proteinase K to 250 μg/mL to dissociate the drug–DNA–topoisomerase II cleavable complexes. The DNA was precipitated with ethanol and then resuspended in 5 μL of formamide–TBE loading buffer, denatured at 90° for 4 min, then chilled in ice for 4 min prior to loading onto the gel. DNA cleavage products were resolved by polyacrylamide gel electrophoresis under denaturating conditions (8% acrylamide—8 M urea). After electrophoresis, gels were soaked in 10% acetic acid for 10 min, transferred to Whatman 3MM paper, and dried under vacuum at 80°. A Molecular Dynamics 425E PhosphorImager was used to collect and analyse the data.

RESULTS

To test the topoisomerase II inhibitory properties of IA-5, we studied its effect on purified human topoisomerase II using a ^{32}P-labelled *Eco*RI-*Ava*I restriction fragment of pKM27 as a substrate. The DNA cleavage products were analysed by neutral agarose gel electrophoresis. A phosphorimage of a typical gel obtained after treatment of the 4 kbp DNA substrate with topoisomerase II in the presence of lucanthone and IA-5 at concentrations ranging from 1 to 50 μM is shown in Fig. 2. A prominent cleavage site was detected with both drugs, indicating that lucanthone and the indazole analogue stabilise DNA–topoisomerase II covalent complexes. The effect seen with IA-5 was slightly more pronounced than with lucanthone (compare the 10-μM lanes) and differed from that seen with the well-established topoisomerase II inhibitors amsacrine and etoposide. This first set of experiments evidences that the two drugs function effectively as topoisomerase II poisons. Moreover, the cleavage profiles shown in Fig. 2 suggested that IA-5 and lucanthone can induce site-specific topoisomerase II-mediated DNA cleavages. This prompted us to sequence the cleavage sites.

To investigate the sequence selectivity of drug-induced topoisomerase II cleavage, we performed the same experiments as in Fig. 2 but using short DNA substrates. Four restriction fragments were employed: the 117- and 265-bp

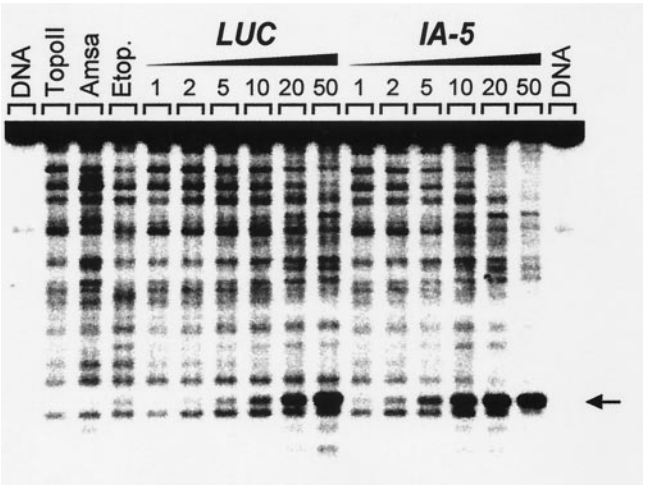


FIG. 2. Drug-induced double-stranded cleavage of DNA by topoisomerase II. The 5′-end labelled 4 kbp *Eco*RI-*Ava*I restriction fragment from plasmid pKMp27 (lane labelled DNA) was incubated with purified human topoisomerase II in the absence (lane TopoII) or presence of increasing concentrations of the test drug (μM as indicated). Reactions were carried out for 45 min at 37° then stopped with SDS–proteinase K treatment. Double-stranded DNA fragments were analysed on a 1% alkaline agarose gel containing 0.1% SDS in TBE buffer. The known toposiomerase II inhibitors amsacrine (Amsa) and etoposide (Etop.) were used at 50 μM. The right arrow points to the major site of topoisomerase II cleavage stimulated by the test drugs.

fragments from plasmid pBS, and the 157- and 178-bp fragments from plasmid pLAZ3 (Fig. 3). With IA-5, we had no difficulty in sequencing a population of topoisomerase II cleavage sites. Surprisingly, however, only a very few cleavage sites were detected with lucanthone in these assays, whatever the experimental conditions employed. Three weak cutting sites can be seen on the 117-mer fragment in the presence of lucanthone, but the effect is weak compared to what can be achieved with IA-5 (Fig. 3). There is no doubt that the indazole analogue is more potent than lucanthone at inhibiting topoisomerase II.

From the gels shown in Figure 3 and a few others, it was possible to accurately localise 29 cutting sites for IA-5. The sequences of the cleaved sites were aligned relative to the broken DNA phosphodiester bond. Table 1 reports the base distribution from positions −3 to +3 for the 29 strongest topoisomerase II-mediated cleavage sites stimulated by the drug. A weak but noticeable base preference was detected. The majority of cutting sites have a C at positions −1 and +1, i.e. on the 3′ and 5′ sides of the breaks, respectively. The preference for a G at positions −2 and +2 may also be deduced, but the frequency is weak. Although only a limited number of topoisomerase II cleavage sites stimulated by IA-5 were located at nucleotide resolution, the data appear sufficient to suggest that the drug exhibits a preference for a cytosine at the 3′ terminus of the cutting site.

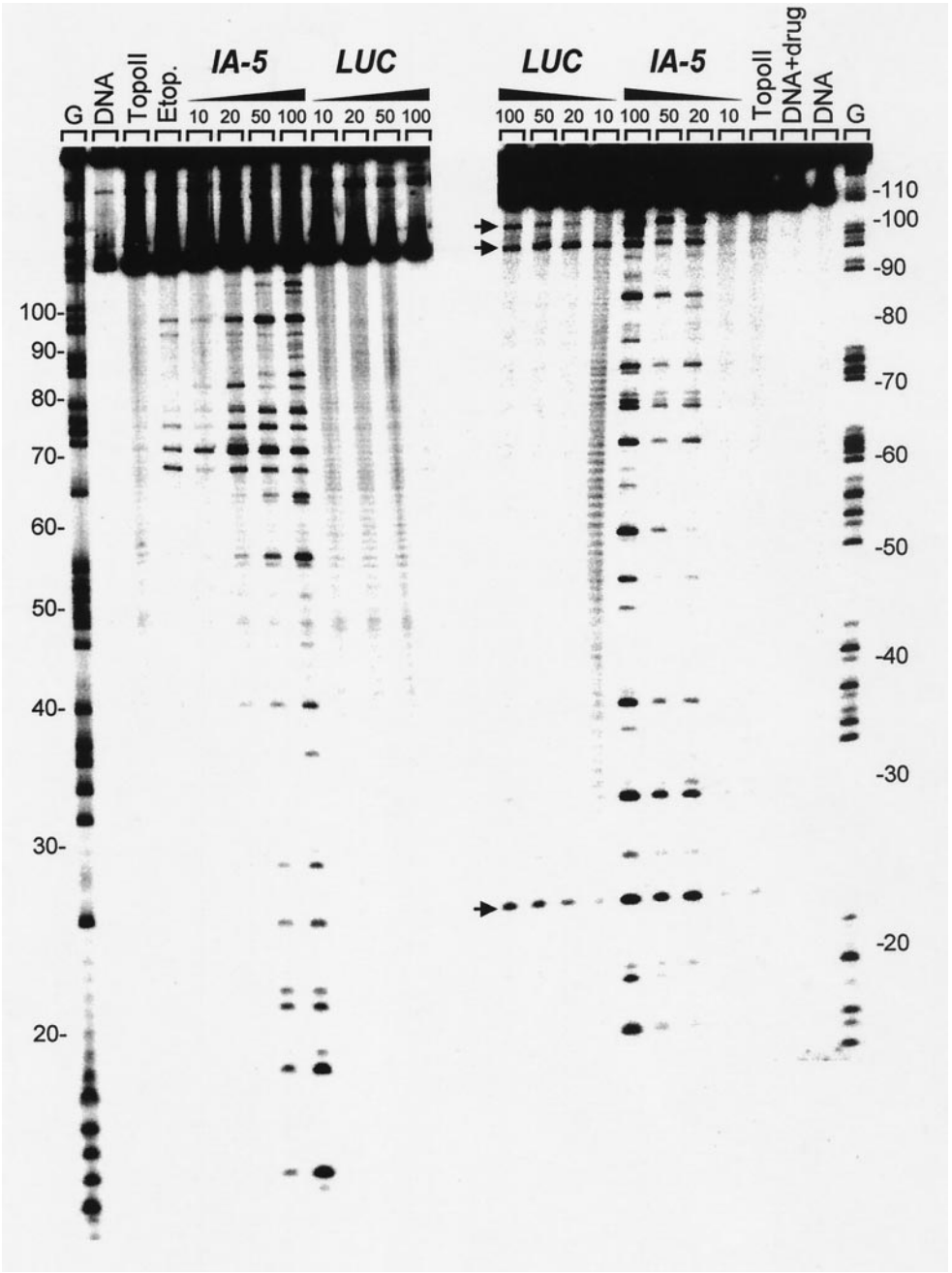


FIG. 3. Sequence analysis of the cleavage sites stimulated by IA-5. The gels show the results obtained with the 178-mer (left) and 117-mer (right) DNA fragments. In both cases, the 5′-end labelled DNA was incubated in the absence (lane Topo II) or presence of the test drug at the indicated concentration (μM). Etoposide (Etop.) was used at 10 μM. Topoisomerase II cleavage reactions were analysed on an 8% denaturing polyacrylamide gel as described in Materials and Methods. Numbers at the side of the gels refer to the nucleotide position of cleavage sites. The short arrows point to the three sites of cleavage stimulated by lucanthone. The lane marked DNA+drug refers to IA-5 at 100 μM incubated with the DNA without topoisomerase II.

DISCUSSION

The results presented above demonstrate that the indazole analogue of lucanthone behaves as a topoisomerase II inhibitor. The drug stimulates DNA cleavage by the enzyme with a weak preference for sites having a C on the 3′ side of the cleaved bond. The selectivity of IA-5 thus differs from that reported with anthracyclines such as doxorubicin, which induces cleavage preferentially at sites having an A at the 3′-DNA termini of the topoisomerase II cleavage sites [21]. However, the preference for a C at the −1 position has been observed with various anticancer drugs including the epipodophyllotoxins, etoposide and teniposide, and the anthraquinone derivative, mitoxantrone [22]. Interestingly, a preference for a C(−1) was also reported

TABLE 1. Base frequencies at DNA cleavage sites induced by topoisomerase II in the presence of IA-5

Position	Base frequencies (%)				Pyr/ Pur	(weak)
5′ → 3′	A	G	C	T	ratio	preference
−3	13.8	31	27.6	27.6	1.2	–
−2	24.1	37.9	24.1	13.8	0.6	(G)
−1	13.8	17.2	44.8	24.1	2.2	C ←
+1	27.6	17.2	37.8	17.2	1.2	(C)
+2	31.0	37.9	10.3	20.7	0.45	(G)
+3	31.0	17.2	31.0	20.7	1.1	–

A panel of 29 strong cleavage sites was considered.

with losoxantrone (DuP 941), an anthrapyrazole analogue of mitoxantrone [23]. IA-5 and losoxantrone both contain a structurally close four-ring planar chromophore.

As mentioned in the introduction, IA-5 and lucanthone were found to bind selectively to AT-rich sequences in DNA in the absence of topoisomerase II [11]. In contrast, mitoxantrone and anthrapyrazole derivatives exhibit a slight preference for GC-rich sequences [18]. In fact, mitoxantrone itself intercalates preferentially into 5′-(A/T)CG and 5′-(A/T)CA sites on DNA [24]. Therefore, there is no obvious correlation between the preferential drug-binding sites on DNA inferred from footprinting experiments and the sites of drug-stimulated DNA cleavage by topoisomerase II. In fact, this is not the only study that has failed to find a correlation between the sequence selectivity of drug binding to (protein-free) DNA and effects on topoisomerase II. For example, daunomycin binds preferentially to (A/T)GC and (A/T)CG triplets [25], whereas daunomycin-induced topoisomerase II strand breaks can occur at many types of sites not necessarily encompassing the aforementioned triplets. The presence of an adenine residue at position −1 relative to the cleavage site is the only requirement for doxorubicin-stabilises cleavage of DNA by topoisomerase II [21, 26]. Many other examples may be cited. In nearly all cases, it seems that the known sequence selectivity of drug binding to DNA has little to do with the location of drug-induced topoisomerase II breaks. Binding to DNA and topoisomerase inhibition may be viewed as two distinct molecular processes contributing separately to the cytotoxic activity.

Given the lack of correspondence between drug binding to naked DNA and topoisomerase II-mediated cleavage sites, the interaction of IA-5 with DNA may be considered essentially as repository in nature [2]. However, the drug–DNA interaction may have indirect effects on topoisomerase II cleavage. The higher stimulation of DNA cleavage by IA-5 compared to lucanthone may be a consequence of their different abilities to distort the DNA structure. Chemical probing experiments of drug–DNA complexes (without topoisomerase) showed that the distortion of the double helix emanating from the intercalative drug binding was very different for lucanthone and its indazole analogue. Stacking deformations are reduced with benzothiopyranoindazoles compared to thioxanthenones, but the side chain of IA-5 would project more deeply into the minor groove of DNA compared to the lucanthone side chain [11]. It will be of interest to probe the extent of deformation of the double helix in the drug–DNA–topoisomerase II ternary complexes.

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