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Short Communication

EFFECTS OF TWO DISTAMYCIN-ELLIPTICINE HYBRID MOLECULES ON TOPOISOMERASE I AND II MEDIATED DNA CLEAVAGE: RELATION TO CYTOTOXICITY

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Abstract—Two distamycin–ellipticine conjugates were examined for their ability to modulate topoisomerase I and topoisomerase II-DNA cleavable complex formation *in vitro*. Hybrid molecules Distel (1+) and Distel (2+) both contain a DNA-intercalating chromophore and a tris-pyrrole element capable of binding within the minor groove of DNA. The two drugs differ only in the nature of the side chain attached to the distamycin moiety. The monocationic hybrid Distel (1+) is a dual topoisomerase I and II inhibitor with characteristics differing from those of the parent compounds distamycin and ellipticine. By contrast, the biscationic hybrid Distel (2+) exerts no significant effects on either topoisomerase I or II. The cytotoxic properties of the two drugs towards P388 leukaemic cells sensitive and resistant to camptothecin correlate with topoisomerase inhibitory properties but not with DNA-binding properties.

Key words: distamycin; ellipticine; topoisomerases; antitumour agents

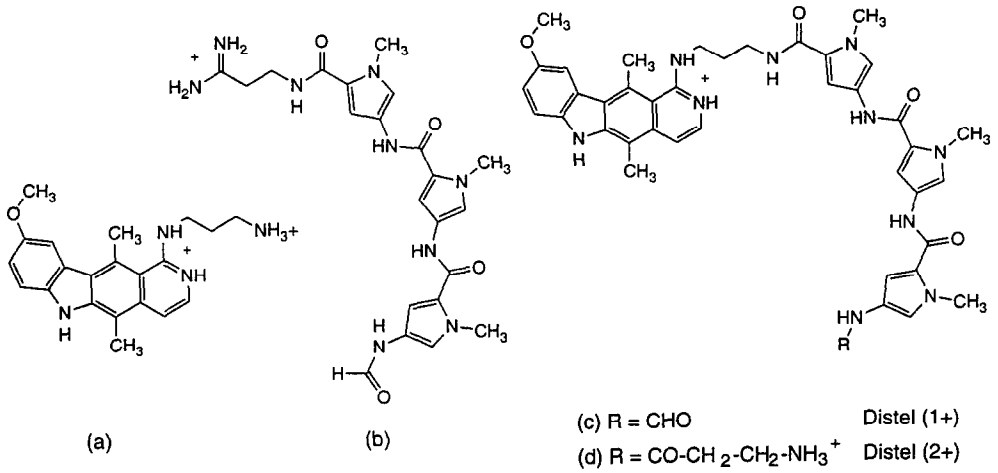

Fig. 1. Chemical structure of the ellipticine derivative (a), distamycin (b), and the distamycin-ellipticine hybrids Distel (1+) (c) and Distel (2+) (d).

The chemotherapeutic potential of DNA topoisomerase inhibitors has been clearly established in recent years [1, 2]. Topoisomerases have been implicated as the intracellular targets of a wide range of antitumour, antibacterial and antiviral agents [3]. The antitumour properties of several clinically used drugs (e.g. amsacrine, doxorubicin, actinomycin D, mitoxantrone) are believed to result from their ability both to bind to DNA and to interfere with the catalytic activities of topoisomerases [4, 5]. Therefore, designing drugs able to bind to specific DNA sequences as well as to inhibit topoisomerase activities is considered a worthwhile goal in the search for new antitumour agents. Over the last few years we have developed series of "combilexins" or intercalator-minor groove binder hybrid molecules which react at the genome level via sequence-specific binding to DNA and inhibition of topoisomerases [6]. Recently, we studied two composite molecules

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containing a DNA-intercalating ellipticine chromophore linked to the DNA-minor groove binder distamycin (Fig. 1). The two distamycin–ellipticine conjugates, Distel (1+) and Distel (2+) differ only in the nature of the side chain attached to the distamycin moiety. In addition to interacting with nucleic acids [7–9], we suspected that these hybrid ligands could interact with topoisomerases since ellipticine derivatives are potent inhibitors of topoisomerase II [10–13] and distamycin modulates the activities of topoisomerases I and II [14–16]. To shed light on the mechanism of drug action we have compared the effect of the conjugates Distel (1+) and Distel (2+) on topoisomerase I and II-DNA cleavable complex induction *in vitro*.

Materials and methods

Drugs and chemicals. m-AMSA* was obtained from Substantia Laboratories (Courbevoie, France) and camptothecin from Sigma Chemical Co. (La Verpillière, France). The synthesis of the Distel compounds has been described previously [8]. Drugs were dissolved in dimethylsufoxide at 1 mM and then further diluted with water.

Topoisomerase I and II DNA cleavage reaction. Topoisomerase I and topoisomerase II were purified from calf thymus using an adaptation of procedures described previously [17]. The cleavage reaction mixture contained 20 mM Tris HCl pH 7.4, 60 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol (plus 10 mM $MgCl_2$ and 1 mM ATP for topoisomerase II), 2×10^4 dpm of $\alpha[^{32}P]$-pBR322 DNA and the indicated drug concentrations. The reaction was initiated by the addition of topoisomerase I or II (20 units in 20 μL reaction volume) and allowed to proceed for 10 min at 37°. Reactions were stopped by adding SDS to a final concentration of 0.25% and proteinase K to 350 μg/mL, followed by incubation for 30 min at 50°. For topoisomerase I, samples were denatured by the addition of 10 μL denaturing loading buffer consisting of 0.45 M NaOH, 30 mM EDTA, 15% (w/v) sucrose, 0.1% bromocresol green prior to loading on a 1% agarose gel in 1 × TBE buffer containing 0.1% SDS. Electrophoresis was maintained at 2V/cm for 18 hr.

Cell cultures and growth inhibition assay. P388 murine leukaemia cell line was obtained from the tumour bank of the National Cancer Institute (Bethesda, MD, U.S.A.). P388CPT5 cell line resistant to camptothecin was derived from a stable clone of the P388CPT0.3 cell line obtained at the 42nd passage [18]. Both cell lines were grown in RPMI 1640 medium containing 0.01 mM 2-mercaptoethanol, 10 mM L-glutamine, 10% (v/v) foetal calf serum, 100 IU/mL penicillin, 2 μg/mL streptomycin, 50 μg/mL gentamycin and 50 μg/mL nystatin at 37° in a humidified atmosphere containing 5% CO_2. Experiments were carried out with exponentially growing cells as previously described [18]. Briefly, 2×10^4 P388 or P388CPT5 cells were seeded in 96-well microculture plates with various drug concentrations (semi-log dilutions). After a 96-hr incubation at 37°, cells were incubated with 0.02% neutral red for 16 hr, then washed and lysed with 1% SDS. The incorporation of the dye, which reflects cellular growth and viability, was evaluated by measuring the optical density at 540 nm for each well using a titertek multiwell spectrophotometer. Each point was done in quadruplicate and all compounds were evaluated in parallel on the two cell lines.

Results

To test the topoisomerase inhibitor properties of the two distamycin–ellipticine conjugates we studied the effect of the hybrids and their parent compounds on both purified calf thymus topoisomerases I and II using the ^{32}P-labelled *Eco*RI-*Hind*III restriction fragment of pBR322 as a substrate. The DNA cleavage products were analysed by alkaline (for topoisomerase I) or neutral (for topoisomerase II) agarose gel electrophoresis. Autoradiographs of typical gels obtained after treatment of the 4330 bp/DNA substrate with topoisomerases I and II in the presence and absence of the test drugs at concentrations ranging from 0.1 to 10 μM are shown in Fig. 2.

Effect on topoisomerase I-mediated DNA cleavage. Purified topoisomerase I produces a characteristic cleavage pattern in the absence of drug. Similar electrophoretic profiles at 0.01, 0.1 and 1 μM and a slight inhibition of the cleavage reaction at 10 μM were observed in the presence of the biscationic hybrid, indicating that Distel (2+) has minimal, if any, effect on topoisomerase I. On the contrary, the monocationic hybrid ligand stimulates topoisomerase I-mediated DNA cleavage in a dose-dependent manner. Distel (1+) enhances the formation of a subset of topoisomerase I cleavage sites observed in the absence of drug (arrowheads). The Distel (1+)-promoted sites are clearly different from those observed with the topoisomerase I-specific inhibitor camptothecin and also differ from those produced by the parent compounds distamycin and the ellipticine derivative. At low concentration (0.1–1 μM) distamycin weakly arouses the formation of two cleavage sites while at higher concentration it inhibits topoisomerase I. These results are in agreement with the data in the literature [14–16]. The DNA cleavage by topoisomerase I in the presence of the ellipticine derivative is stimulated at a single site at low concentration (0.1 μM) but is inhibited at higher concentrations. This first set of data indicates that the two conjugates exhibit distinct effects on topoisomerase I.

Effect on topoisomerase II-mediated DNA cleavage. The pattern of DNA double-strand cleavages induced by topoisomerase II in the absence of drug is similar to that previously reported with the same DNA fragment [19]. The biscationic hybrid Distel (2+) does not stimulate DNA cleavage by topoisomerase II but rather inhibits the enzyme at high concentration (10 μM). Distamycin has very little effect on topoisomerase II though a weak stimulation of DNA cleavage is observed at low concentrations, as reported [14]. The cleavage patterns obtained with the monocationic hybrid are comparable to those observed with the ellipticine derivative: with both drugs, two closely-spaced topoisomerase II cleavage sites (depicted by arrowheads) are stimulated at low drug concentrations (0.01, 0.1 and 1 μM) and the enzyme activity is abolished at high concentrations (10 μM). The ellipticine derivative seems to be slightly more efficient than Distel (1+) in promoting topoisomerase II-mediated DNA cleavage since the appearance of the two cleavage sites is detected at 0.01 μM and 1 μM for the ellipticine derivative and Distel (1+), respectively. However, in both cases cleavage enhancement is rather modest compared to what can be achieved with the topoisomerase II-specific antileukaemic drug m-amsa.

This second set of data confirms that the two structurally related hybrids exert different effects on topoisomerases. In other words, the nature of the side chain on the distamycin moiety (the only substituent that distinguishes the two hybrids) is critical for the effect of the drugs on topoisomerases.

Cytotoxicity. The cytotoxic properties of the conjugate molecules were assessed by a cell growth inhibition assay using P388 and P388CPT5 murine leukaemia cell lines sensitive and resistant to camptothecin, respectively [18]. For comparative purposes, distamycin, the ellipticine derivative and camptothecin were tested in parallel. Under the experimental conditions used (4 days continuous exposure) the monocationic hybrid exhibited marked cytotoxic effects (Fig. 3) whereas the biscationic hybrid and distamycin proved to be totally inactive. The IC_{50} values measured for the different compounds are collated in Table

* Abbreviations: m-amsa, 4′-(9-acridinylamino)methanesulphon-*m*-anisidide; SDS, sodium dodecyl sulphate; TBE, tris-borate-EDTA.

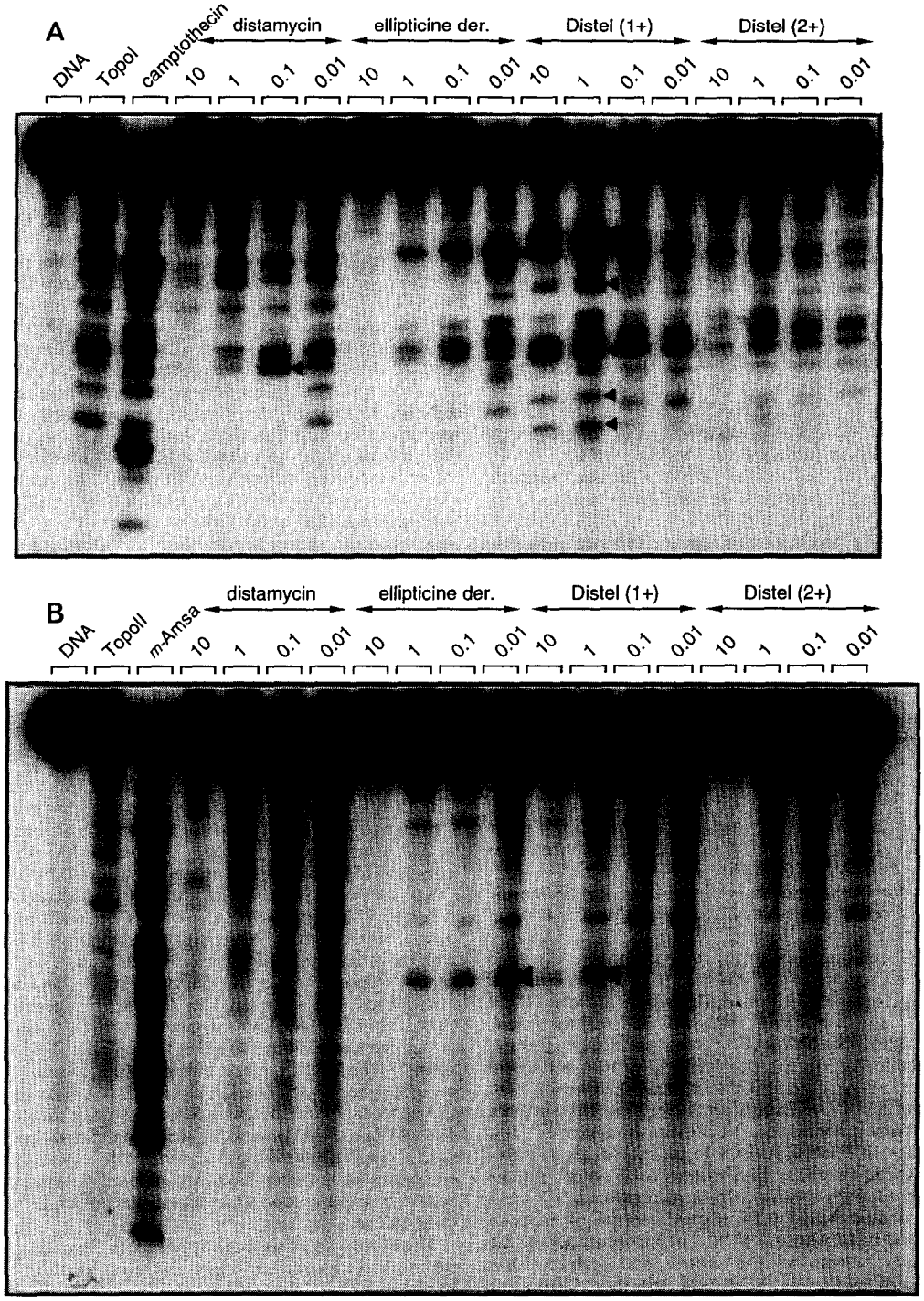

Fig. 2. Effect of the distamycin-ellipticine hybrids on (A) topoisomerase I and (B) topoisomerase II. Purified calf thymus topoisomerases I or II were incubated with the *Eco*RI-*Hind*III restriction fragment from pBR322 (^{32}P-labelled at the *Eco*RI site) in the presence and absence of the test ligands. Drug concentrations (μM) are indicated at the top of each lane. Reactions were carried out for 10 min at 37° then stopped with SDS-proteinase K treatment. (A) Single-strand DNA fragments were analysed on a 1% alkaline agarose gel in TBE buffer. Lanes DNA and TopoI refer to the radiolabelled 4330 bp/DNA substrate incubated without and with topoisomerase I, respectively. Camptothecin was used at 0.03 μM. (B) Double-strand DNA fragments were analysed on a 1% neutral agarose gel in TBE buffer. Lanes DNA and TopoII refer to the radiolabelled 4330 bp/DNA substrate incubated without and with topoisomerase II respectively. m-amsa was used at 10 μM. Arrowheads point to the major sites of topoisomerase cleavage stimulated by the drugs.

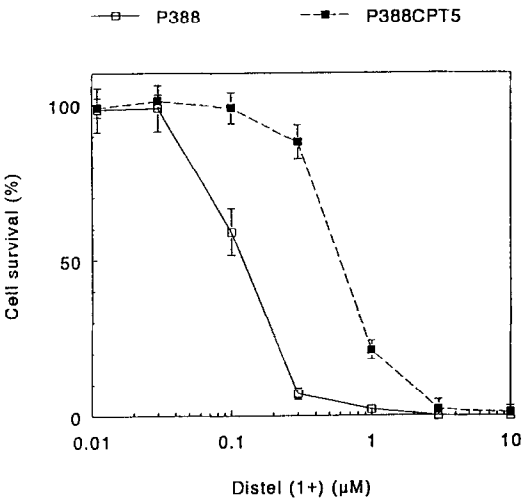

Fig. 3. Effect of Distel (1+) on the growth of P388 (□) and P388CPT5 (■) murine leukaemia cells sensitive and resistant to camptothecin, respectively.

1. For P388 cells, the IC_{50} value measured with Distel (1+) is eight-fold lower than that measured with the ellipticine derivative, prompting the conclusion that this conjugate is more active than its parent compound in this system. P388CPT5 cells exhibit marked resistance to camptothecin (87-fold), attributable to the expression of a deficient form of topoisomerase I as a result of a mutation in the topoisomerase I gene of these cells [18]. Weak cross-resistance to the ellipticine derivative (2.1-fold) was observed in P388CPT5 cells. With Distel (1+), the relative resistance index is significantly increased to 6.2 compared with the ellipticine derivative but remains considerably lower than that calculated with camptothecin. These results suggest that interference with topoisomerase I exerts a larger influence on the mechanism of action of the monocationic hybrid relative to the ellipticine derivative.

Discussion

Spectroscopic measurements have shown that the monocationic hybrid Distel (1+) is capable of binding to DNA and chromatin via a bidentate mechanism involving intercalation of the ellipticine chromophore and minor groove binding of the uncharged distamycin moiety [7]. However, footprinting studies have revealed that this conjugate is essentially sequence neutral and can accommodate many types of DNA sequences and structures. Molecular modeling has suggested that the introduction of a second positive charge onto the distamycin moiety could confer sequence-specific recognition [7]. From recent studies [9], we can state that the prediction is absolutely correct: introducing a positively-charged tail onto the distamycin entity was the right way to proceed to convert a sequence neutral ligand into a highly AT-specific DNA binder. Nevertheless, the results reported here indicate that the increased DNA-binding properties due to the newly-attached substituent are detrimental to the biological activity of the drug. Under the experimental conditions specified above, Distel (2+) is inactive whereas Distel (1+) exhibits cytotoxic properties notably superior to those of the ellipticine derivative. The different cytotoxic properties of the two hybrids are not attributable to a difference in cellular penetration since the bischarged conjugate enters easily and accumulates in the cell nuclei (and nucleoli) as reported previously with Distel (1+) and the ellipticine derivative [8]. It is plausible that the cytotoxicity is connected with the drug's ability to interfere with the catalytic activities of topoisomerases. Indeed, Distel (1+) proves to be a potent topoisomerase I poison whereas Distel (2+) exerts no significant effect on this enzyme; on the other hand, P388CPT5 cells exhibit a notable cross resistance to Distel (1+) but not to Distel (2+). These observations led to the proposal that topoisomerase I constitutes a privileged target for the monocationic conjugate. Moreover, Distel (1+) moderately stimulates topoisomerase II-mediated DNA cleavage whereas Distel (2+) is essentially inactive in this respect. It is thus entirely plausible that the topoisomerase I and II poisoning observed *in vitro* takes place in cells and contributes to the cytotoxicity of the drug.

The DNA binding and topoisomerase inhibitory properties of the distamycin–ellipticine hybrid Distel (1+) are, to a limited extent, reminiscent of those of the established antitumour antibiotic actinomycin D. As for Distel (1+), actinomycin D binding to DNA involves intercalation of the phenoxazone chromophore coupled with minor groove binding of the cyclic peptides. Moreover, actinomycin D inhibits both topoisomerase I and II by trapping cleavable complexes [20, 21]. Actinomycin D and Distel (1+) can be considered as dual topoisomerase poisons, a rare characteristic shared with another ellipticine-derived antitumour drug, intoplicine [19]. The observation that Distel (1+) but not Distel (2+) constitutes a potent

Table 1. Cytotoxic properties of the distamycin–ellipticine hybrid molcules on P388 and P388CPT5 murine leukaemia cells

	IC_{50} (μM)*		
	P388	P388CPT5	Relative resistance index†
Camptothecin	0.032	2.8	87
Distamycin	>10	>10	—
Ellipticine derivative	0.8	1.7	2.1
Distel (1+)	0.105	0.65	6.2
Distel (2+)	>10	>10	—

* Drug concentration that inhibits cell growth by 50% after incubation in liquid medium for 96 hr. Each drug concentration was tested in quadruplicate, SE of each point is < 10%.

† The relative resistance index is the ratio between the P388CPT5 IC_{50} value and the P388 IC_{50} value.

topoisomerase inhibitor suggest that the terminal side chain may provide possibilities for modulation of topoisomerase activities. Our findings provide an important new basis for understanding the mechanisms of action of combilexin molecules. They also pave the way for the design of new intercalator–minor groove binding hybrid ligands endowed with superior biological properties.

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