Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 68 (2004) 1911–1922
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Novel antitumor indenoindole derivatives targeting DNA and topoisomerase II

Christine Bal[a], Brigitte Baldeyrou[a], Florence Moz[a], Amélie Lansiaux[a], Pierre Colson[b], Laurence Kraus-Berthier[c], Stéphane Léonce[c], Alain Pierré[c], Marie-Françoise Boussard[d], Anne Rousseau[d], Michel Wierzbicki[d], Christian Bailly[a,*]

[a]*INSERM U-524 et Laboratoire de Pharmacologie Antitumorale du Centre Oscar Lambret, IRCL, Lille 59045, France*
[b]*Biospectroscopy and Physical Chemistry Unit, Department of Chemistry and Natural and Synthetic Drugs Research Center, University of Liege, Sart-Tilman, 4000 Liege, Belgium*
[c]*Division Recherche Cancérologie, Institut de Recherches Servier, 125 Chemin de Ronde, 78290 Croissy sur Seine, France*
[d]*Division Chimie E, Institut de Recherches Servier, 11 rue des Moulineaux 92150, Suresnes*

Received 23 May 2004; accepted 12 July 2004

Abstract

We have identified a novel series of indenoindole derivatives endowed with potent cytotoxic activities toward cancer cells. Five compounds containing a 8-[2-(dialkylamino)ethoxy]-2,3-dimethoxy-5H-10H-indeno[1,2-*b*]indol-10-one-*O*-propynyl-oxime core substituted with a phenyl, furanyl, or a methyl substituent on the propynyl side chain have been synthesized and their mechanism of action was investigated using a panel of complementary biophysical and biochemical techniques. The compounds were shown to intercalate into DNA with a preference for AT-rich sequences. They have no effect on topoisomerase I but they strongly stimulate DNA cleavage by topoisomerase II. Their capacity to stabilize topoisomerase II–DNA covalent complexes is comparable to that of the reference drug etoposide. The nature and orientation of the substituent on the propynyl chain modulate the DNA binding and topoisomerase II inhibitory properties of the compounds and, apparently, there is a correlation between the cytotoxic potential and the molecular action at the DNA–topoisomerase II level. The growth of human K562 leukemia cells is strongly reduced in the presence of the indenoindoles (IC_{50} in the 50 nM range) which maintain a high cytotoxic activity toward the adriamycin-resistant K562adr cells line in vitro. The low resistance indexes measured with the indenoindoles (RRI = 10–30) compared to adriamycin (RRI = 1000) suggest that our new compounds are weakly or not sensitive to drug efflux mediated by glycoprotein-P and/or multidrug resistance (MDR) protein pumps. Finally, we also show that these indenoindoles arrest K562 cells in the G2/M phase of the cell cycle and promote apoptosis, as indicated by the appearance of internucleosomal DNA cleavage. One compound in the series was tested for in vivo antitumor activity against the colon 38 model and at 25 mg/kg it showed 100% complete tumor regression in the treated mice, without significant body weight loss. Altogether, the results reported here establish that our indenoindole derivatives represent a novel interesting series of DNA-targeted cytotoxic agents.

Keywords: Anticancer drug; DNA intercalation; Topoisomerase II; Cytotoxicity; P-glycoprotein; Cell cycle

1. Introduction

Some of the most active drugs currently available for the treatment of cancers hold topoisomerase II as their primary target [1,2]. This is the case for etoposide, which is front-line therapy for small cell lung cancer and a variety of leukemia and lymphomas, and for doxorubicin, which is also one of the most commonly prescribed anticancer drugs. These two drugs exploit the potentially lethal nature of topoisomerase II by stabilizing a covalent protein–DNA complex which is normally transient during the catalytic cycle of the enzyme. The accumulation of this covalent complex in rapidly dividing cancer cells results in the

* Corresponding author. Tel.: +33 320 16 92 18; fax: +33 320 16 92 29.
E-mail address: bailly@lille.inserm.fr (C. Bailly).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.07.008

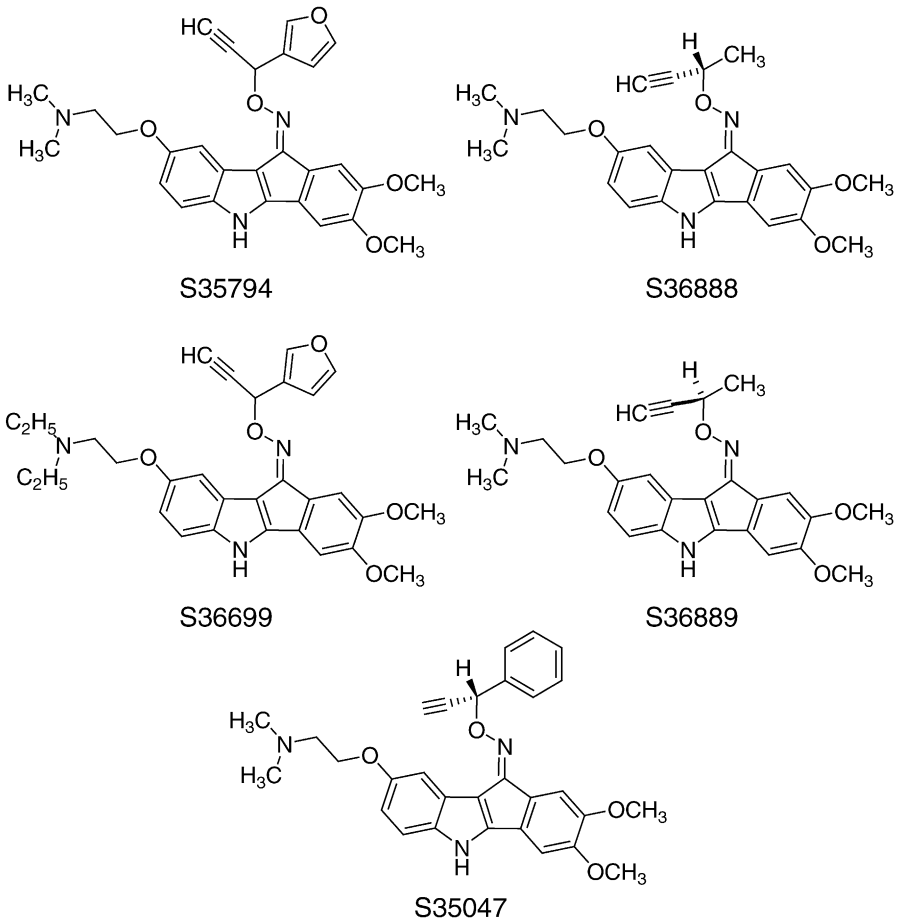


Fig. 1. Structures of the indenoindole derivatives used in this study.

formation of multiple DNA strand breaks and these genotoxic damages activate the cell death machinery.

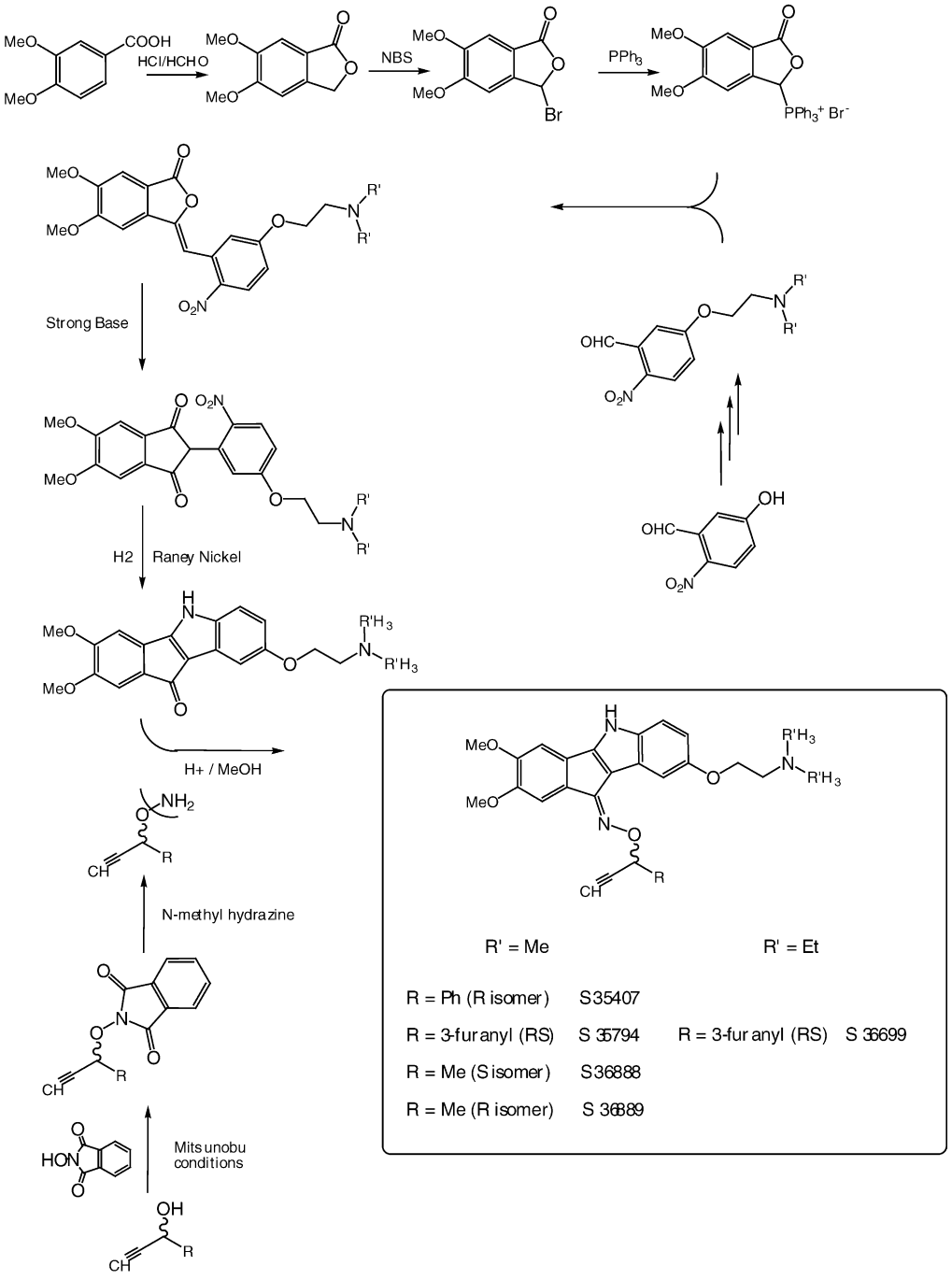
Epipodophyllotoxins such as etoposide, and anthracyclines like doxorubicin, have been extensively characterized as topoisomerase II–DNA stabilizing factors or topoisomerase II "poisons" as they are usually termed. But over the past two decades a large variety of anticancer agents with a similar mechanism of action has been reported. Topoisomerase II poisons include mitoxantrone, bisantrene, ellipticine, intoplicine, amonafide, azatoxin, TAS103, quinoxaline compounds (e.g. XK469), anilinoacridines (e.g. amsacrine and DACA), pyridoacridines (e.g. ascididemine), anthracenediones, pyrroloquinolines and many other heterocyclic synthetic and natural products belonging to a wide range of chemical classes [3–6]. This chemical diversity reflects distinct possibilities to block the DNA cleavage activity of topoisomerase II at several sites along the receptor DNA sequence and/or the different structural states of the covalent complex which can be trapped by small molecules. The development of novel topoisomerase II poisons is also legitimized by the knowledge that drugs from different chemical families, which share a common target, generally exhibit different spectra of anticancer activity [7].

Here we report a novel family of topoisomerase II poisons structurally unrelated to all topoisomerase II inhibitors known so far. We have identified the 5H-10H-indeno[1,2-*b*]indole chromophore as a favorable structure for stabilization of DNA–topoisomerase II complexes. The five compounds studied here possess the same 8-[2-(dialkylamino)ethoxy]-2,3-dimethoxy-5H-10H-indeno[1,2-*b*]indol-10-one-*O*-propynyl-oxime core and differ by a phenyl, a furanyl or a methyl substitutent on the propynyl chain (Fig. 1). Specifically, compounds S35794 and S36699 bear a dimethyl- or diethyl-aminoethoxy chain, respectively. The two enantiomers S36888 and S36889 both contain a methyl group on the propynyl chain whereas S35407 is a phenyl-substituted analogue of the active enantiomer S36888. This short, exploratory series of compounds has been prepared at the Servier Institute in the course of a medicinal chemistry program aimed at discovering novel anticancer agents. A battery of spectroscopic, biochemical and cellular techniques were deployed to unravel the mechanism of action of these compounds. The potent cytotoxic activities of these compounds may be a consequence of their capacity to intercalate into DNA and to induce topoisomerase II-dependent DNA breaks.

2. Materials and methods

2.1. Drugs

The general procedure for the synthesis of the drugs is mentioned in the corresponding patent [8]. An outline of the synthetic process is given in Scheme 1. Briefly, the synthesis starts with the reaction of dimethoxybenzoic acid with formaldehyde to form a lactone derivative which is then α-brominated and added to triphenyl phosphine prior to coupling with the appropriate aminoalcoxy nitrobenzaldehyde derivative. Rearrangement of the resulting benzylidene lactone with a strong base gives a nitrophenyl indane-1,3-dione which is then catalytically reduced and cyclized in a one-step procedure to afford the aminoalcoxy indeno(1,2-*b*)indol-10-one derivative. Final treatment with the correctly substituted *O*-propargyl hydroxylamine under acidic conditions leads to the expected oxime derivative with an overall yield of 20–40%. Drugs were dissolved in DMSO at 5 mM. The stock DMSO solutions of drugs were



Scheme 1.

kept at −20 °C and freshly diluted with water to the desired concentration immediately prior to use.

2.2. Chemicals and biochemicals

Etoposide and adriamycine were purchased from Sigma. Calf thymus DNA and the double stranded alternating polymers poly(dAT)$_2$ and poly(dGC)$_2$ were obtained from Pharmacia. Calf thymus DNA was deproteinized with sodium dodecyl sulphate (protein content <0.2%). The nucleoside triphosphate labelled with [^{32}P](α-dATP) was obtained from Amersham (3000 Ci/mmol). Restriction endonucleases and AMV reverse transcriptase were purchased from Roche and used according to the supplier's recommended protocol in the activity buffer provided. All other chemicals were analytical grade reagents.

2.3. Absorption spectra and melting temperature studies

Melting curves were measured using an Uvikon 943 spectrophotometer coupled to a Neslab RTE111 cryostat. For each series of measurements, 12 samples were placed in a thermostatically controlled cell-holder, and the quartz cuvettes (10 mm pathlength) were heated by circulating water. The T_m measurements were performed in BPE buffer pH 7.1 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA). The temperature inside the cuvette was measured with a platinum probe; it was increased over the range 20–100 °C with a heating rate of 1 °C/min. The ''melting'' temperature T_m was taken as the mid-point of the hyperchromic transition. The Uvikon 943 spectrophotometer was also used to record the absorption spectra. Titrations of the drug with DNA, covering a large range of DNA phosphate/drug ratios (P/D), were performed by adding aliquots of a concentrated DNA solution to a drug solution at constant ligand concentration (20 μM). DNA blanks at the same nucleotide concentrations were prepared concomitantly and used as a reference in the recording of absorption spectra.

2.4. Circular dichroism

CD spectra were recorded on a J-810 Jasco dichrograph. Solutions of drugs, nucleic acids and their complexes (1 ml in 1 mM sodium cacodylate buffer, pH 7.0) were scanned in 1 cm quartz cuvettes. Measurements were made by progressive dilution of drug–DNA complex at a high P/D (phosphate/drug) ratio with a pure ligand solution to yield the desired drug/DNA ratio. Three scans were accumulated and automatically averaged.

2.5. Electric linear dichroism (ELD)

Measurements were performed with a computerized optical measurement system using the procedures previously outlined [9]. All experiments were conducted with a 10 mm pathlength Kerr cell having 1.5 mm electrode separation. The samples were oriented under an electric field strength varying from 1 to 14 kV/cm. The drug under test was present at 10 μM concentration together with the DNA at 200 μM concentration unless otherwise stated. This electro-optical method has proved most useful to determine the orientation of the drugs bound to DNA. It has the additional advantage that it senses only the orientation of the polymer-bound ligand: free ligand is isotropic and does not contribute to the signal [10].

2.6. Purification and radiolabeling of DNA restriction fragment

The 265 bp DNA fragment was prepared by 3′-[^{32}P]-end labeling of the *Eco*RI-*Pvu*II double digest of the plasmid pBS (Stratagene) using α-[^{32}P]-dATP (3000 Ci/mMol) and AMV reverse transcriptase. 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 °C. This suspension was filtered through a Millipore 0.22 μm filter and the DNA was precipitated with ethanol. Following washing with 70% ethanol and vacuum drying of the precipitate, the labeled DNA was resuspended in 10 mM Tris adjusted to pH 7.0 containing 10 mM NaCl.

2.7. DNAse I footprinting, electrophoresis and quantitation by storage phosphor imaging

Experiments were performed essentially as previously described [11]. Briefly, reactions were conducted in a total volume of 10 μl. Samples (3 μl) of the labeled DNA fragments were incubated with 5 μl of the buffered solution containing the ligand at appropriate concentration. After 30 min incubation at 37 °C to ensure equilibration of the binding reaction, the digestion was initiated by the addition of 2 μl of a DNAse I solution whose concentration was adjusted to yield a final enzyme concentration of about 0.01 U/ml in the reaction mixture. After 3 min, the reaction was stopped by freeze drying. Samples were lyophilized and resuspended in 5 μl of an 80% formamide solution containing tracking dyes. The DNA samples were then heated at 90 °C for 4 min and chilled in ice for 4 min prior to electrophoresis.

DNA cleavage products were resolved by polyacrylamide gel electrophoresis under denaturating conditions (0.3 mm thick, 8% acrylamide containing 8 M urea). After electrophoresis (about 2.5 h at 60 W, 1600 V in Tris–borate–EDTA buffered solution, BRL sequencer model S2), gels were soaked in 10% acetic acid for 10 min, transferred to Whatman 3 MM paper, and dried under vacuum at 80 °C. A Molecular Dynamics 425E PhosphorImager was used to collect data from the storage screens exposed to dried gels overnight at room temperature. Base

line-corrected scans were analyzed by integrating all the densities between two selected boundaries using ImageQuant version 3.3 software. Each resolved band was assigned to a particular bond within the DNA fragments by comparison of its position relative to sequencing standards generated by treatment of the DNA with dimethylsulphate followed by piperidine-induced cleavage at the modified guanine bases in DNA (G-track).

2.8. DNA relaxation experiments

Supercoiled pKMp27 DNA (0.5 μg) was incubated with 4 U human topoisomerase I or II (TopoGen Inc.) at 37 °C for 1 h in relaxation buffer (50 mM Tris pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA) in the presence of varying concentrations of the drug under study. Reactions were terminated by adding SDS to 0.25% and proteinase K to 250 μg/ml. DNA samples were then added to the electrophoresis dye mixture (3 μl) and electrophoresed in a 1 % agarose gel containing ethidium bromide (1 μg/ml), at room temperature for 2 h at 120 V. Gels were washed and photographed under UV light [12].

2.9. Cell cultures and survival assay

K562 and K562adr [13] cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI 1664 medium, supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml). The cytotoxicity of the compounds was assessed using a cell proliferation assay developed by Promega (CellTiter 96® AQueous one solution cell proliferation assay). Briefly, 2×10^4 exponentially growing cells were seeded in 96-well microculture plates with various drug concentrations in a volume of 100 μl. After 72 h incubation at 37 °C, 20 μl of the tetrazolium dye were added to each well and the samples were incubated for a further 3 h at 37 °C. Plates were analyzed on a Labsystems Multiskan MS (type 352) reader at 492 nm.

2.10. Cell cycle analysis

For flow cytometry analysis of DNA content, 10^6 K562 or K562(adr) cells in exponential growth were treated with the test compound for 24 h and then washed three times with citrate buffer. Cells were washed in PBS, fixed in 70% cold ethanol and placed at −20 °C overnight. Then, cells samples were washed in PBS, treated with RNAse A (0.5 mg/ml, 10 min) and stained with propidium iodide (200 μl PI at 125 μg/ml) for 30 min at room temperature in the obscurity. Samples were analyzed on a Becton Dickinson FACScan flow cytometer using the LYSYS II software which is also used to determine the percentage of cells in the different phases of the cell cycle. PI was excited at 488 nm, and fluorescence analyzed at 620 nm (Fl-3).

2.11. DNA fragmentation

K562 cells at a density of about 5×10^5 cells/ml were treated with various concentrations of the drugs for 24 h and then collected by centrifugation at $2500 \times g$ for 5 min. The resultant cell pellets were resuspended in PBS buffer containing 5 mM $MgCl_2$ and lysed in 500 μl of Tris–EDTA buffer containing 0.1% SDS and proteinase K (1.5 mg/ml) overnight at 37 °C. After two successive extractions with phenol/chloroform, the aqueous layer was transferred to a new centrifuge tube. The DNA was precipitated with ethanol, resuspended in water (100 μl) and treated with RNAse A (100 μg/ml) for 2 h at 37 °C. Electrophoresis was performed in 1% agarose gel in Tris–borate buffer at about 12 V/cm for approximately 4 h. After electrophoresis, the gel was stained with ethidium bromide (1 mg/ml), washed and photographed under UV light.

2.12. Antitumor activity

Female B6D2F1 mice were purchased from Iffa Credo (Lyon, France). They weighed 20–22 g (6–8 weeks of age) at the start of the experiments. Mice received proper care and maintenance in accordance with institutional guidelines. The murine Colon 38 adenocarcinoma was provided by the Division of Cancer Treatment, Tumor Repository, NCI (Frederick, Md.). Briefly, fragments of approximately 50 mg were grafted s.c. into B6D2F1 mice on day 0 [14]. S36888 was prepared in 0.1% tween 80, then diluted with water just before the administration to animals at 0.1 ml/10 g body weight. S36888 was administered i.v. on days 10, 17 and 24 at a dose of 12.5 or 25 mg/kg, the optimal dose was defined as the most active non-toxic dose, i.e. body weight loss less than 20% and absence of early death. Animals were weighted and Colon 38 tumors measured twice a week and tumor volumes (V_t) were calculated using the following formula: V_t = length (mm) × width (mm^2).

3. Results

3.1. DNA interaction

Addition of DNA induces marked changes of the absorption spectra of the compounds, with large bathochromic and hypochromic shifts, as illustrated in Fig. 2 for S36699 and S36889. With this later compound, the absorbance at the absorption maxima at 285 and 330 nm decreases significantly and the red-shift amounts to about 10 nm. Similar spectral changes were observed for the five compounds, reflecting in each case the interaction of the indoindole ring system with DNA base pairs. Thermal denaturation analyses, carried out with calf thymus DNA, gave monophasic melting curves and the ΔT_m values ($(T_m = T_m^{complex} - T_m^{DNA})$ are collated in Table 1. All the compounds stabilize duplex DNA against heat denatura-

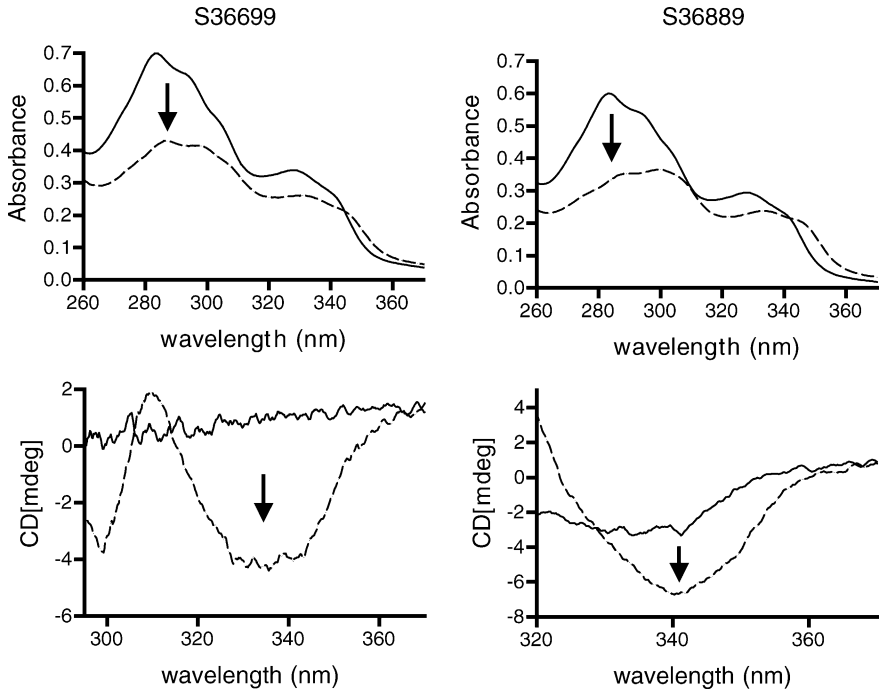


Fig. 2. Absorption (top) and circular dichroism (bottom) spectra of S36699 and S36889 (10 μM) in the absence (solid lines) and presence (dashed lines) of calf thymus DNA. DNA titrations of the drugs were performed in 1 mM sodium cacodylate buffer at pH 7.0. To 3 ml of drug solution at 20 μM were added aliquots of a concentrated calf thymus DNA solution. The spectra of the complexes corresponded to a phosphate-DNA/drug ratio of 20. Arrows point to the spectral changes induced by adding DNA.

tion, with (T_m ranging from 4 to 10 °C. The highest (T_m value was obtained with S35794 which binds to the DNA double helix about two times more tightly than the phenyl derivative S35407.

Circular dichroism (CD) and electric linear dichroism (ELD) were used to investigate the mode of drug binding to DNA. With both S36889 and S36699 (Fig. 2) a negative CD band develops in the 340 nm region upon binding of the drug to DNA. In the ELD experiments, performed to define the orientation of the compounds with respect to the DNA helix, the DNA is oriented by in an electric field and the respective orientation of the molecules bound to DNA is probed using a linearly polarized light. The ELD spectrum of compound S36888 bound to calf thymus DNA is shown in Fig. 3, together with the dependence of the reduced dichroism $\Delta A/A$ as a function of the electric field strength (inset in Fig. 3). The reduced dichroism (A/A is strongly negative in the drug absorption band which indicates an orientation of the chromophore perpendicular to the helix axis (or electric field direction). This indicates that the indenoindole chromophore of these ligands is oriented parallel to the DNA base pairs, as expected for an intercalative binding. The negative reduced dichroism values measured for the five compounds at 330 nm are even more negative than that measured for DNA alone at 260 nm. The larger negative amplitudes observed with the drug–DNA complexes compared to unbound DNA likely arises from the drug-induced unwinding and stiffening of the double helix which facilitate the orientation of DNA in the electric field. Overall, the CD and ELD measurements indicate that the drugs behave as typical DNA intercalating agents. S36888, as well as its analogues, binds equally well to the synthetic polynucleotides poly(dAT)$_2$ and poly(dGC)$_2$ (Fig. 3). Roughly identical negative $\Delta A/A$ values were recorded with both polymers, suggesting that the drug forms similar complexes with the AT and GC sequences. Nevertheless, a slight preference for the AT sequences was evidenced in the footprinting experiments.

The drug was incubated with a ^{32}P-radiolabelled DNA restriction fragment of 265 bp and the complexes were subjected to limited digestion by the endonuclease DNAse I. A typical electrophoresis gel used to resolve the DNA

Table 1
DNA binding and cytotoxicity

	ΔT_m [a] (°C)	IC_{50} [b] K562	IC_{50} [b] K562adr	RRI[c]
S35794	10.8	0.047	1.337	28.4
S36699	7.0	0.194	1.868	9.6
S36888	9.0	0.038	0.478	12.6
S36889	4.8	0.125	3.332	26.7
S35407	4.1	0.046	1.028	22.3
Adriamycin	–	0.005	5.03	1006

[a] Variation in melting temperature ($\Delta T_m = T_m^{complex} - T_m^{DNA}$). T_m measurements were performed in BPE buffer pH 7.1 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA) using 10 μM drug and 20 μM calf thymus DNA, at 260 nm with a heating rate of 1 °C/min.

[b] Drug concentration that inhibits cell growth by 50% after incubation in liquid medium for 72 h. Each drug concentration was tested in triplicate.

[c] The relative resistance index (RRI) is the ratio between the K562adr IC_{50} value and the K562 IC_{50} value.

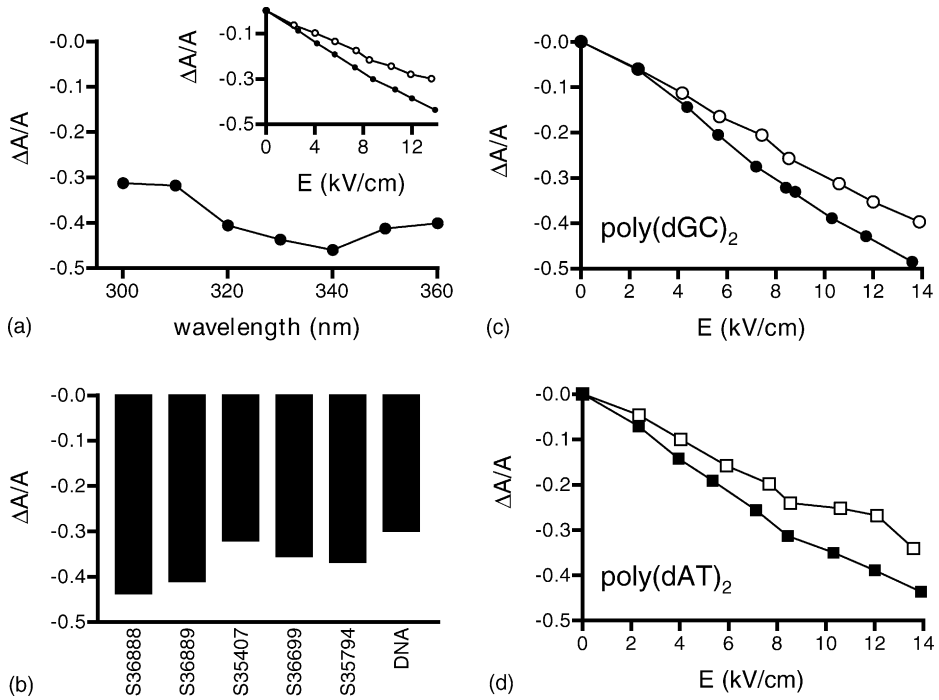


Fig. 3. Electric linear dichroism measurements. Panel (a) shows the dependence of the reduced dichroism $\Delta A/A$ on the wavelength and (inset) the electric field strength for S36888 bound to calf thymus DNA. Conditions: (a) 13.6 kV/cm, P/D = 20 (200 μM DNA, 10 μM drug) (inset) 330 nm, for the DNA-S36888 complex (●) and 260 nm for the DNA alone (○). Panel (b) indicates the reduced dichroism (A/A at 330 nm) of the complexes between calf thymus DNA and the test compounds. In each case, (A/A was measured at 330 nm and 13.6 kV/cm. Panels (c) and (d) refer to the interaction of S36888 with poly(dAT)$_2$ and poly(dGC)$_2$. The graphs show the dependence of the reduced dichroism on the electric field strength for the drug–polynucleotide complexes (●,■) or the polynucleotide alone (○,□). All measurements were performed at room temperature (20 °C) in 1 mM sodium cacodylate buffer, pH 7.0, at a DNA/drug ratio of 20.

fragments is presented in Fig. 4A. On the autoradiogram, a few regions where the cleavage by the enzyme is decreased in the presence of the drugs can be discerned. A densitometric analysis of the gel was performed in order to identify the position of the footprints, presumptive of drug binding sites, and to compare the sequence selectivity of the different compounds. The differential cleavage plots shown in Fig. 4B compare the profiles obtained with the isomeric compounds S36888 and S36889. Obviously, they both bind preferentially to the same sequences, mainly those rich in AT-bp. A preferential binding site encompassing the sequence 5′-AAATTAA has been identified (dashed box in Fig. 4). A preferential binding to AT sequences is uncommon for intercalating agents.

3.2. Topoisomerases inhibition

A relaxation assay using supercoiled plasmid DNA was used to evaluate the effects of the compounds on the catalytic activity of topoisomerases I and II. A set of data is presented in Fig. 5. The drugs do not promote DNA cleavage by topoisomerase I. The amount of nicked DNA species remains weak in the presence of the indenoindole derivatives, in contrast to what is observed with the reference drug camptothecin which produces a high level of single strand breaks (left part in Fig. 5A). In the relaxation assay, a slight increase of the band corresponding to nicked DNA was generally observed with the drugs but this observation was not confirmed when using a ^{32}P-labelled DNA substrate analyzed on sequencing gel (data not shown). In sharp contrast, a potent inhibition of topoisomerase II was detected. In this case, the reference drug was the epipodophyllotoxin derivative etoposide which greatly stimulates DNA cleavage by topoisomerase II to generate a substantial amount of linear DNA molecules (Fig. 5B and C). A massive increase of the band corresponding to linear DNA (double strand breaks) was also seen with S36888 and S35794 which appear to be equally potent as etoposide to inhibit topoisomerase II. The stabilization of the topoisomerase II–DNA complexes was observed with all five drugs but with varying potency. In term of topoisomerase II inhibition, the drugs rank in the order S36888, S35794 > S35407 > S36889, S36699. With no doubt, these indenoindole derivatives promote the cleavage of DNA by the enzymes and therefore they must be considered as topoisomerase II poisons. It is worth mentioning also that at a high concentration (20 μM) compounds S36699 and S35407 both strongly inhibit topoisomerase II-mediated DNA cleavage (Fig. 5C) whereas they differ in term of DNA binding (Table 1). They must probably interact with DNA in a different manner (e.g. different orientation of the intercalated

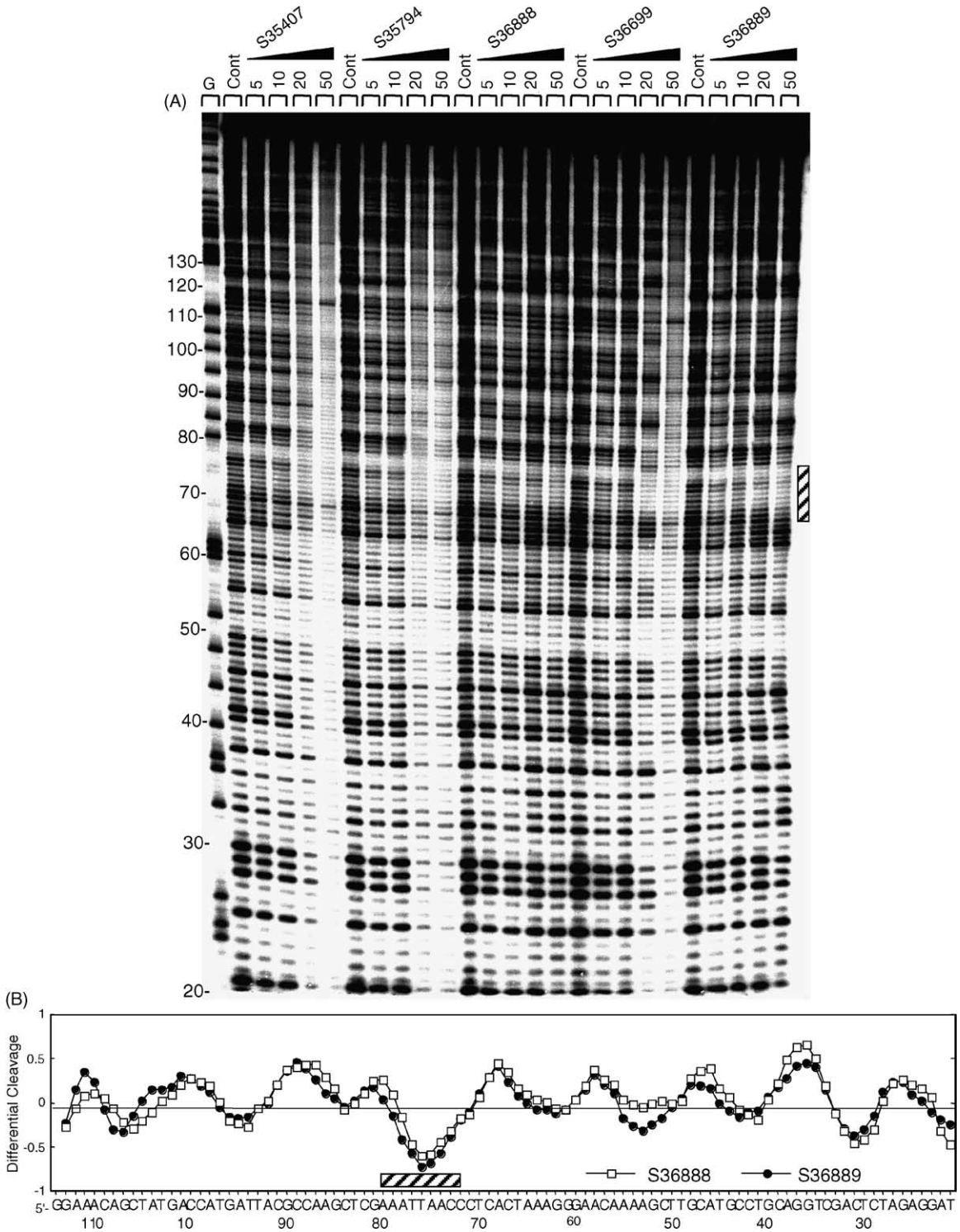


Fig. 4. Sequence selective binding. The gel in (A) shows DNAse I footprinting with the 265-mer *Pvu*II-*Eco*RI restriction fragment cuts from plasmid pBS in the presence of the indenoindole derivatives at concentrations increasing from 5 to 50 μM. The DNA was 3′-end labelled at the *Eco*RI site with [α-^{32}P]dATP in the presence of AMV reverse transcriptase. The products of nuclease digestion were resolved on an 8% polyacrylamide gel containing 7 M urea. Control tracks (Cont) contained no drug. Guanine-specific sequence markers obtained by treatment of the DNA with dimethylsulfate followed by piperidine were run in the lanes marked G. Numbers on the left side of the gel refer to the standard numbering scheme for the nucleotide sequence of the DNA fragment. The differential cleavage plots in (B) compare the susceptibility of the 265-mer DNA fragment to DNAse I cleavage in the presence of S36888 and S36889 (20 μM each). Negative values correspond to a ligand-protected site and positive values represent enhanced cleavage. Vertical scales are in units of ln(fa)-ln(fc), where fa is the fractional cleavage at any bond in the presence of the drug and fc is the fractional cleavage of the same bond in the control, given closely similar extents of overall digestion. Each line drawn represents a three-bond running average of individual data points, calculated by averaging the value of ln(fa)-ln(fc) at any bond with those of its two nearest neighbors. Only the region of the restriction fragments analyzed by densitometry is shown. The dashed bar indicates the main site of reduced cleavage by DNAse I in the presence of the drugs.

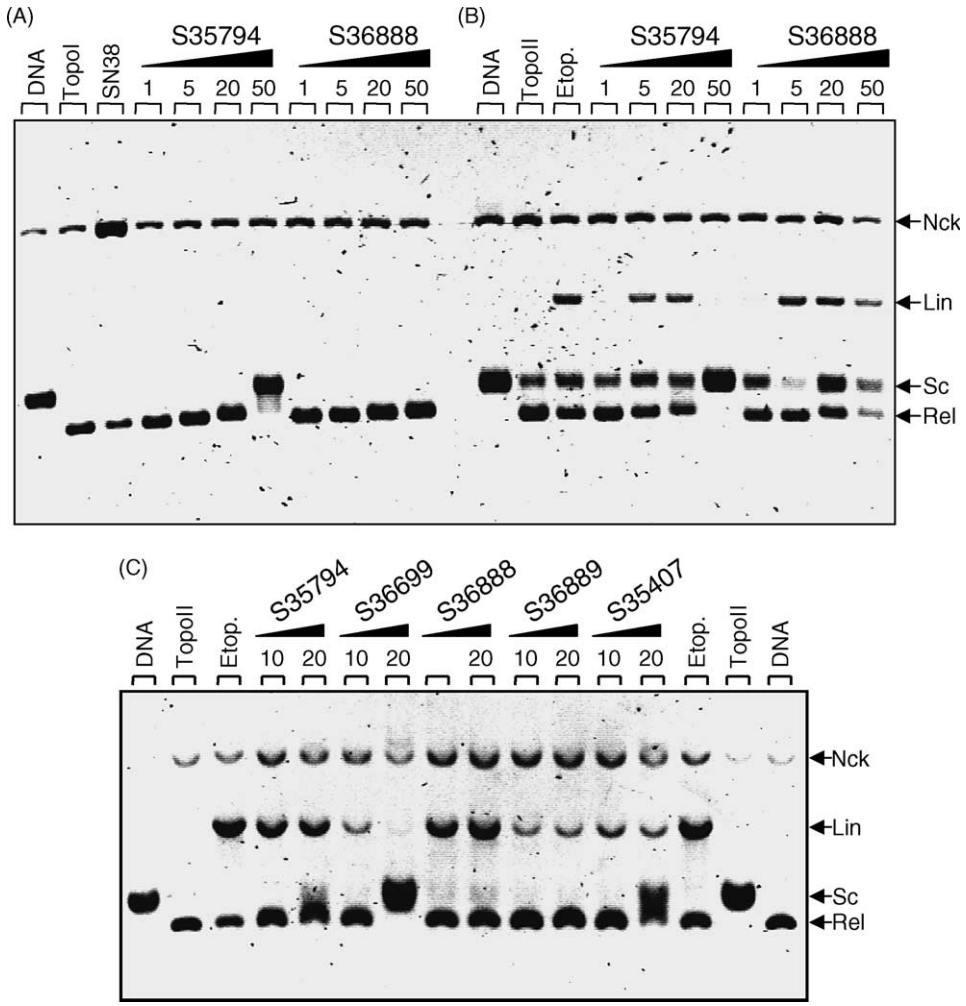


Fig. 5. Effect of increasing concentrations of the test compounds on the relaxation of plasmid DNA by human topoisomerases I (A) and II (B and C). Native supercoiled pKMp27 DNA (0.5 μg) (lane DNA) was incubated with 4 U topoisomerase in the absence (lane Topo) or presence of a given drug at the indicated concentration (μM). The camptothecin derivative SN38 and etoposide (lane Etop.) were used at 50 μM. Reactions were stopped with sodium dodecylsulfate and treatment with proteinase K. DNA samples were separated by electrophoresis on agarose gels containing ethidium bromide (1 μg/ml). The gels were photographed under UV light. Nck, nicked; Lin, linear; Rel, relaxed; Sc, supercoiled DNA.

chromophore) so as to perturb differently the positioning of the enzyme on its DNA substrate.

3.3. Cytotoxicity and cell cycle effects

The cytotoxicity of the compounds was assessed by a cell growth inhibition assay using two human leukemia cell lines, K562 and K562adr, respectively sensitive and resistant to the antitumor drug adriamycin [13]. The K562adr cell line shows classical multidrug resistance (MDR) phenotype with overexpression of the P-glycoprotein. Under the experimental conditions used (3 days continuous exposure) all five compounds proved quite cytotoxic with IC_{50} values in the 40–50 nM range for S36888, S35407 and S35794. The two other molecules S36889 and S36699 are slightly less cytotoxic, with IC_{50} values in the 120 and 200 nM range, respectively (Table 1). Unsurprisingly, the drugs were less potent at inhibiting the growth of adriamycin-resistant cells compared to the parental cells. However, the relative resistance index (RRI in Table 1) did not exceed 30 at most with S35794 whereas it reaches 1000 with adriamycin. Unlike the anthracycline antitumor agent, the indenoindole derivatives seem to be poorly susceptible to the overexpression of efflux pumps. Apparently, the test compounds would not be substrates for the P-glycoprotein, the major drug efflux pump most commonly associated with resistance of tumor cells to antineoplastic agents. However, this hypothesis will need further investigations to validate it.

In parallel to the cytotoxicity evaluation, we studied the variations of the cell cycle profile upon treatment of the leukemia cells with the test compounds. Drug-induced cell cycle perturbations were observed with both K562 and K562(adr) cells. As shown in Fig. 6a, treatment of the K562adr cells with the different drugs for 24 h led to profound changes of the cell cycle profiles. The flow

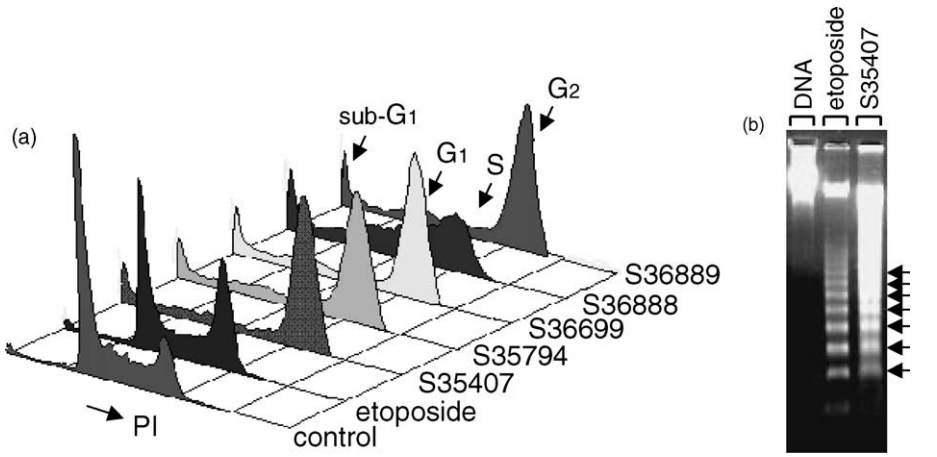


Fig. 6. (a) Cell cycle analysis of K562adr human leukemia cells treated for 24 h with the different compounds (5 μM each). Etoposide was used at 25 μM. Cells were analyzed with the FACScan flow cytometer. (b) Agarose gel electrophoresis of DNA extracted from K562 cells treated with 25 μM etoposide or 5 μM S35047 for 24 h. DNA was stained with ethidium bromide after electrophoresis on a 1% agarose gel and then visualized under UV light. Oligonucleosome-sized DNA fragmentation can be seen in drug-treated cells (arrows).

cytometric analysis of propidium iodide-labeled cells indicates that the treatment with 5 μM S35407 induces a massive accumulation of cells in the G2 + M phases. The G2 + M cell population increases considerably (from 18% in the control up to 88 %) while the G1- and S-phase cell populations decrease. In contrast, with the parental cell line, the cells accumulated in the S phase (data not shown). Roughly similar trends were observed with the four other drugs, with S36888 being slightly less efficient than the other analogues at inducing a G2 block. Moreover, in all cases, we detected a small proportion of cells with hypodiploid DNA contents (sub-G1) suggesting that the compounds induce apoptosis in this human cell line (Fig. 6a). The DNA of K562(adr) cells treated with the drugs was extracted and its integrity was analyzed by electrophoresis on agarose gels. The genomic DNA of the drug-treated cells was found to be fragmented, with a pronounced typical internucleosomal cleavage (Fig. 6b). Like etoposide, S35407 and its analogues produce patterns of internucleosomal fragmentation, indicating that they promote the apoptotic cell death of K562 cells.

3.4. In vivo antitumor activity

The most cytotoxic compound in the series, S36888 was tested in vivo against the C38 colon adenocarcinoma model. Mice bearing C38 tumors were treated i.v. with S36888 at 12.5 or 25 mg/kg at days 10, 17 and 24 and tumor volumes were compared in control versus drug-treated mice (Fig. 7). Significant tumor regressions were observed at the low dose and remarkably, complete cure were seen at the higher dose of 25 mg/kg. All animals in the treated group (6/6) were tumor-free even after up to 85 days of post-tumor inoculation. Moreover, no apparent toxicity was observed; the animals showed no significant body weight loss.

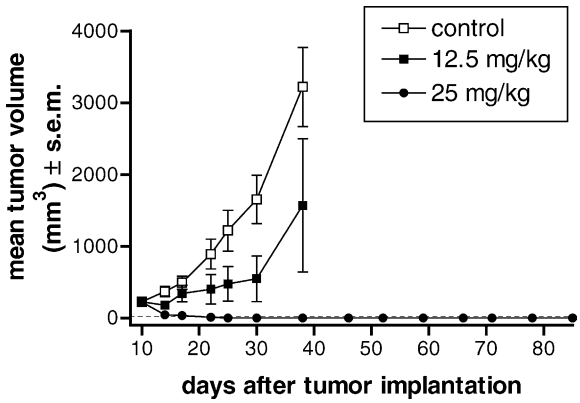


Fig. 7. Antitumor efficacy of S36888 against murine C38 adenocarcinoma. Mice were treated with S36888 at the indicated doses on days 10, 17 and 24, and monitored for 10 weeks. Each point represents the mean ± S.E.M. of tumor volume.

4. Discussion

In the course of a medicinal chemistry program aimed at identifying novel cytotoxic agents, chemists at the Servier Institute synthesized a series of indenoindole derivatives and the biological evaluation of these compounds had revealed potent cytotoxic activities against a variety of cancer cell lines in vitro (Servier, internal unpublished data). A priori, no specific molecular target could be envisioned for these compounds but given the planar structure of the indenoindole aromatic nucleus and the presence of a cationic side chain, we considered the possibility that these compounds might interact with DNA and perhaps function as topoisomerase inhibitors. The results reported here validate this hypothesis. We have identified DNA and topoisomerase II as potential targets for the indenoindole derivatives. These synthetic compounds potently stabilize double stranded DNA against

heat denaturation and there is clear spectroscopic evidences that they intercalate into DNA. In parallel, we show that these compounds are efficient at stabilizing topoisomerase II–DNA covalent complexes. The extent of double stranded DNA breaks formed with S36888 is comparable to that observed with etoposide which is one of the most potent topoisomerase II-targeted anticancer drugs known so far. There is apparently a correlation between topoisomerase II inhibition and cytotoxicity in this series but not with DNA binding. The methyl substituted compound S36888 is very potent against topoisomerase II but its DNA binding capacity is lower than that of S35794 with a furan-3-yl substituted propynyl chain. S36888 and S35794, which are both potent inhibitors of topoisomerase II, are very cytotoxic whereas S36889 is less active against topoisomerase II and also much less cytotoxic than its analogs. With only five molecules studied here, it is not possible to define clear structure–function relationships but however, through this preliminary work we can approach some of the pharmacological properties of these indenoindole compounds which are (i) DNA intercalators recognizing preferentially AT-rich sequences, (ii) inhibitors of topoisomerase II with a potency comparable to that of etoposide, (iii) potent cytotoxic agents inducing apoptosis, (iv) active against adriamycin-resistant cells expressing Pgp and, most importantly, (v) potent antitumor agents. S36888 is remarkably efficient against the C38 adenocarcinoma tumors and apparently devoid of toxic side effect. The antitumor activity of this compound is currently investigated further with different tumor models.

The AT sequence preference of the indenoindoles is surprising and interesting as the vast majority of DNA intercalators usually display no sequence preference or they bind selectively to GC-rich sites or alternating purine–pyrimidine tracts. A few AT selective compounds have been reported in the past, such as benzothiopyranoindazoles [15] and thiophenes [16] for examples, but this is not a frequent situation with intercalating drugs. This property may well contribute to the cytotoxic action via the recruitment of specific AT-binding proteins [17].

Over the past 10 years, a large number of topoisomerase II inhibitors have been reported and a few of them have revealed interesting antitumor activities against hematologic and solid malignancies [18]. These inhibitors are usually grouped in two categories depending on their mechanism of enzyme inhibition [19]. Catalytic inhibitors, which do not stabilize topoisomerase II–DNA covalent complexes, either bind to the enzyme or to the DNA so as to prevent the protein–nucleic acid interaction (e.g. aclarubicin) or they stabilize post-cleavage complexes (e.g. bisdioxopiperazines). Some of these compounds look promising [20] and may reveal clinical utility in the near future [21,22]. In contrast, the so called ''poisons'' which trap topoisomerase II–DNA covalent complexes have shown a major clinical utility. In this category, etoposide and doxorubicin as well as ellipticine and amsacrine, are the best known members but many other chemical families of topoisomerase II poisons have been described: acridines, anthraquinones, imidazoacridininones, naphthalimides, and makaluvamines to cite only a few of them. However, despite this chemical diversity, the good old drug etoposide (used in the clinic for more than 15 years now) is still viewed as the most efficient topoisomerase II poison. Efforts to develop novel potent poisons have met a limited success. In this regard, the indenoindole derivatives presented here, which have a novel chemical structure, are of great interest due to their potent capacity to stimulate topoisomerase II-mediated DNA cleavage. In addition these compounds seem to be weakly or not sensitive to protein-mediated drug efflux. They maintain a marked cytotoxic activity against adriamycin-resistant K562adr cells, probably because they are not removed by Pg-P proteins and/or MDR-associated pumps, unlike the anthracyclines [13]. This property might turn into a selective advantage to combat chemoresistant tumors. Further investigations are going on to define more precisely the mechanism of action and the antitumor profiles of these indenoindoles.

The structure of the studied compounds can be divided in two parts. The planar indenoindole system likely represents the DNA interaction domain forming appropriate contacts with the double helix, via stacking interactions with the bases and possibly groove or phosphate interaction with the dialkyamino side chain. The propynyl chain, with its furanyl, methyl or phenyl substituent may represent a secondary pharmacophoric domain for binding to the enzyme. Such a decoupling of topoisomerase II inhibitors into two portions forming a sort of bridge to maintain the DNA–topoisomerase II complex in its covalent state has been proposed for a number of drugs [23]. In this model, the respective position of the two pharmacophores is essential and it is not surprising to see, as it is the case here, that moving one domain with respect to the other (e.g. change in the orientation of the methyl and propynyl substituents) has a significant impact on the extent of topoisomerase II inhibition and on the cytotoxic response [24]. On the light of their interesting pharmacological profile, a more extensive medicinal chemistry program has been engaged to consolidate the series and identify lead candidates for the development of antitumor agents based on the indenoindole skeleton.

Acknowledgements

This work was done under the support of research grants (to C.B.) from the Association pour la Recherche sur le Cancer and (to P.C.) from the Actions de Recherches Concertées contract 95/00-93. Support by the ''Actions intégrées Franco-Belge, Programme Tournesol'' is acknowledged.

References

[1] Burden DA, Osheroff N. Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme. Biochim Biophys Acta 1998;1400:139–54.

[2] Walker JV, Nitiss JL. DNA topoisomerase II as a target for cancer chemotherapy. Cancer Invest 2002;20:570–89.

[3] Capranico G, Zunino F. Antitumor inhibitors of DNA topoisomerases. Curr Pharm Des 1995;1:1–14.

[4] Wang HK, Morris-Natschke SL, Lee KH. Recent advances in the discovery and development of topoisomerase inhibitors as antitumor agents. Med Res Rev 1997;17:367–425.

[5] Denny WA, Baguley BC. Dual topoisomerase I/II inhibitors in cancer therapy. Curr Top Med Chem 2003;3:339–53.

[6] Gatto B, Leo E. Drugs acting on the beta isoform of human topoisomerase II (p180). Curr Med Chem Anti-Canc Agents 2003;3: 173–85.

[7] Pommier Y, Fesen MR, Goldwasser F. Topoisomerase II inhibitors: the epipodophyllotoxins, *m*-AMSA, and the ellipticine derivatives. In: Cancer Chemotherapy and Biotherapy. . second ed. Philadelphia: Lippincott-Raven Publishers; 1996.

[8] Wierzbicki M, Boussard MF, Rousseau A, Atassi G, Hickman J, Pierré A, et al. Indenoindoline derivatives, process for their preparation, and pharmaceutical compositions containing them, for use in the treatment of cancer. European Patent Appl. EP 1266887 A2, 2002.

[9] Houssier C. Investigating nucleic acids, nucleoproteins, polynucleotides, and their interactions with small ligands by electro-optical systems. In: Molecular Electro-Optics. New York: Plenum Publishing Corporation; 1981.

[10] Colson P, Bailly C, Houssier C. Electric linear dichroism as a new tool to study sequence preference in drug binding to DNA. Biophys Chem 1996;58:125–40.

[11] Bailly C, Waring MJ. Comparison of different footprinting methodologies for detecting binding sites for a small ligand on DNA. J Biomol Struct Dyn 1995;12:869–98.

[12] Bailly C. DNA relaxation and cleavage assays to study topoisomerase I inhibitors. Methods Enzymol 2001;340:610–23.

[13] Principe P, Faussat-Suberville AM, Coulomb H, Marie JP, Braquet P. Flow cytometric monitoring of anthracycline accumulation after antineoplastic ether phospholipid treatment. Anticancer Drugs 1994;5: 329–35.

[14] Kraus-Berthier L, Guilbaud N, Leonce S, Parker T, Genissel P, Guillonneau C, et al. Comparison of the pharmacological profile of an olivacine derivative and a potential prodrug. Cancer Chemother Pharmacol 2002;50:95–103.

[15] Bailly C, Waring MJ. Preferential intercalation at AT sequences in DNA by lucanthone, hycanthone, and indazole analogs. A footprinting study. Biochemistry 1993;32:5985–93.

[16] Wilson WD, Wang Y-H, Kusuma S, Chandrasekaran S, Yang NC, Boykin DW. Binding strength and specificity in DNA interactions: the design of AT specific intercalators. J Am Chem Soc 1985;107:4989–95.

[17] Hurley LH. DNA and its associated processes as targets for cancer therapy. Nature Rev Cancer 2002;2:188–200.

[18] Clapp JM, Hande KR. Topoisomerase II inhibitors. Cancer Chemother Biol Response Modif 2002;20:125–49.

[19] Wilstermann AM, Osheroff N. Stabilization of eukaryotic topoisomerase II–DNA cleavage complexes. Curr Top Med Chem 2003;3:321–38.

[20] Larsen AK, Escargueil A, Skladanowski A. Catalytic topoisomerase II inhibitors in cancer chemotherapy. Pharmacol Therapeut 2003;99: 167–81.

[21] Kruczynski A, Ricome C, Waud WR, Hill BT. In vivo antitumor activity of F 11782, a non-intercalating dual catalytic inhibitor of topoisomerases I and II, against a panel of human tumor xenografts. J Exp Ther Oncol 2002;2:219–27.

[22] Sargent JM, Elgie AW, Williamson CJ, Hill BT. Ex vivo effects of the dual topoisomerase inhibitor tafluposide (F11782) on cells isolated from fresh tumor samples taken from patients with cancer. Anticancer Drugs 2003;14:467–73.

[23] Gatto B, Capranico G, Palumbo M. Drugs acting on DNA topoisomerases: recent advances and future perspectives. Curr Pharm Des 1999;5:195–215.

[24] Capranico G, Guano F, Moro S, Zagotto G, Sissi C, Gatto B, et al. Mapping drug interactions at the covalent topoisomerase II–DNA complex by bisantrene/amsacrine congeners. J Biol Chem 1998;273: 12732–9.