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Original article

# DNA targeting of two new antitumour rebeccamycin derivatives

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**Abstract**

In the course of a medicinal chemistry program aimed at discovering novel tumour-active rebeccamycin derivatives targeting DNA and/or topoisomerase I, a series of analogues with the sugar residue linked to the two indole nitrogens was recently developed. Two promising drug candidates in this staurosporine–rebeccamycin hybrid series were selected for a DNA-binding study reported here. The DNA interaction of the cationic indolocarbazole glycosides MP059 bearing a *N*,*N*-diethylaminoethyl side chain and MP072 containing a sugar bearing an amino group was compared with that of the uncharged analogue MP024. The results show that the addition of a cationic substituent, either directly on the indolocarbazole chromophore or on the carbohydrate residue, significantly reinforces the interaction of the drugs with nucleic acids. The two cationic molecules MP059 and MP072 recognise preferentially sequences containing GpT·ApC and TpG·CpA steps but they do not inhibit topoisomerase I, in contrast to the parent uncharged derivative MP024 which stimulates DNA single strand breaks by topoisomerase I. The cytotoxic activity of the indolocarbazole derivatives bearing positively charged groups is one order of magnitude higher than that of the neutral compound MP024. The high cytotoxic potential can be attributed to the enhanced DNA binding and sequence recognition capacity of the cationic compounds. The study provides useful information for further structure–activity relationship studies in the indolocarbazole series.


*Keywords:* Anticancer drug; Indolocarbazole; DNA intercalation; Topoisomerase I; Cytotoxicity

## 1. Introduction

The antitumor antibiotics rebeccamycin and staurosporine (Fig. 1) are structurally related but functionally different [1–4]. Rebeccamycin, isolated from cultures of *Saccharothrix aerocolonigenes*, is mainly considered as a DNA-damaging agent acting via an inhibition of topoisomerase I. Staurosporine, first obtained from various strains of actinomycetes but also produced by different marine organisms, possesses strong inhibitory activity against kinases, in particular protein kinase C (PKC). The two types of activities seem to be mutually exclusive since rebeccamycin is inactive against PKC whereas staurosporine fails to bind to DNA and is inactive against topoisomerase I. Therefore, these two naturally occurring alkaloids represent two distinct branches of the indolocarbazole family of anticancer agents which also include the related antibiotics K252a (furanose) and AT2433-B1 (diglycoside) (Fig. 1) [5]. Over the past 10 years, a significant number of synthetic derivatives has been designed and a few of them, typified by the lead compounds NSC655649 and J-107088 (rebeccamycin series) and PKC412 and UCN-01 (staurosporine series), have entered clinical trials to evaluate their antitumor potential [4].

The pyranose sugar moiety of staurosporine is attached to the two indole nitrogens of the indolocarbazole chromophore via an *N*-glycosidic and an *N*,*O* ketal bonds, forming thus a cyclic rigid structure, referred to as a closed form. In contrast, the methoxy-

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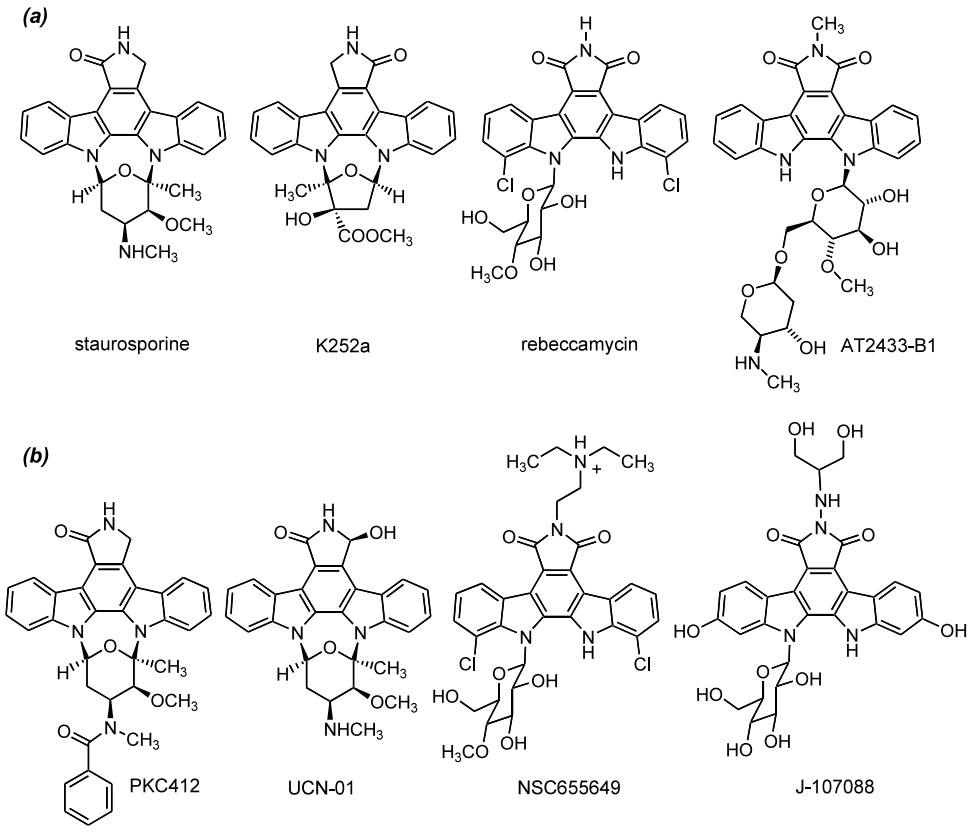


Fig. 1. (a) Naturally occurring and (b) synthetic indolocarbazoles.

glucose residue of rebeccamycin is linked to the planar chromophore only by a *N*-glycosidic bond. Nevertheless, the apparent open form is maintained in a single 'pseudo-closed' conformation due to an intramolecular hydrogen bond between the pyranose oxygen and the adjacent indole NH [6]. This hydrogen-bonded conformation is essential for the biological activity. For example, methylation of the indole NH drastically alter the orientation of the sugar with respect to the chromophore, thereby reducing considerably the cytotoxic activity and the capacity of the drug to bind to DNA and topoisomerase I [7,8]. Attempts have been made to design rebeccamycin analogs with a glycoside residue rigidly attached to the indolocarbazole platform via the two indole nitrogens, to obtain novel structures resembling the staurosporine type. To this end, rebeccamycin was converted to MP003 (Fig. 2) harbouring a rigid tetrahydropyrazine ring. This hemisynthetic product was found to be inactive against PKC but it binds to DNA and interferes with topoisomerase I activity [9]. This product was obtained by (i) a selective tosylation of the 2′-OH group on the sugar residue of rebeccamycin in the presence of *p*-toluenesulfonyl chloride and potassium carbonate, followed by (ii) a treatment of the resulting *O*-tosyl ester with sodium azide to generate the cyclic product which was then (iii) dechlorinated with ammonium formate and palladium on activated carbon to afford the desired staurosporine-like compound MP003.

The promising biological data obtained with MP003 have encouraged the synthesis of a novel series of rebeccamycin derivatives bearing a carbohydrate moiety rigidly held to the indolocarbazole nucleus. Among the various compounds recently obtained, two of them proved to be particularly interesting due to their high cytotoxic potential. Indeed, compounds MP059 and MP072 (Fig. 2) were found to be ten times more cytotoxic to HT29 colon carcinoma and A549 non-small-cell lung carcinoma cells than the parent uncharged compounds MP003 and MP024 [10]. These two novel derivatives containing a diethylaminoethyl side chain on the nitrogen imide (MP059) or an amino group on the sugar residue (MP072) are expected to bind to DNA more tightly than the parent uncharged compounds. To verify this hypothesis, we compared the DNA binding properties of the three compounds MP024, MP059 and MP072 by means of complementary biochemical and biophysical methods to evaluate the DNA affinity, mode of binding and sequence specificity.

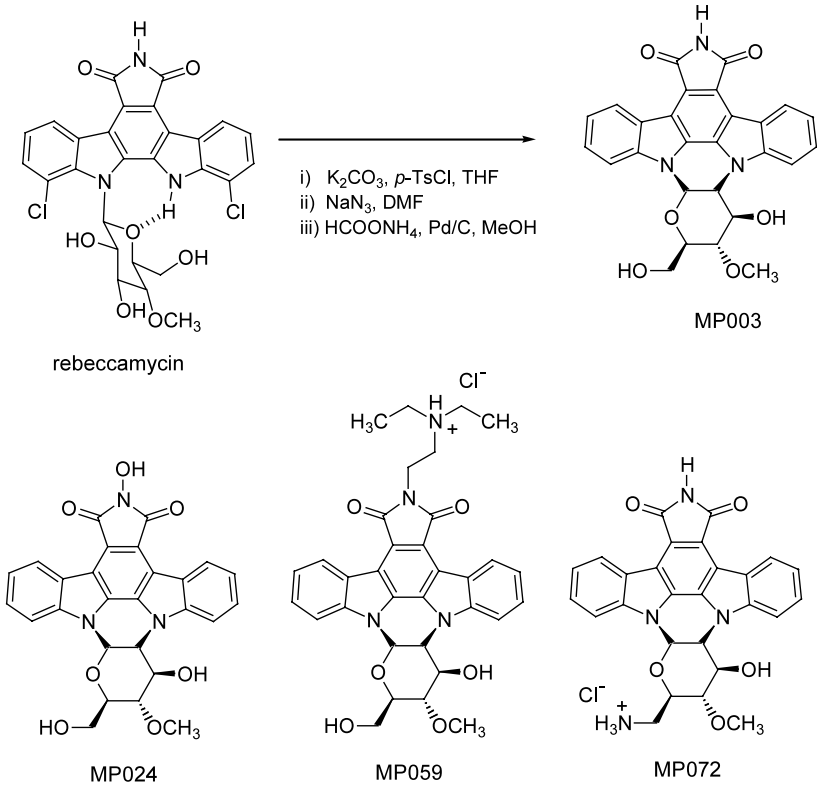


Fig. 2. Top: synthetic procedure used for the conversion of rebeccamycin into a cyclic derivative (see [9] for the details). The intramolecular H-bond in rebeccamycin is schematised. Bottom: structures of the compounds used in this study.

## 2. Biophysical and biochemical results

### 2.1. Relative DNA binding strength

Due to problems of solubility with MP003, the analogue MP024 with a OH group on the nitrogen imide ring was used as a reference uncharged molecule. The absorption spectra of the three studied drugs in the absence and presence of DNA are shown in Fig. 3. Binding of MP059 and MP072 to DNA induces marked hypochromic and bathochromic shifts of the absorption band of the indolocarbazole chromophore centred at 320 nm whereas the spectrum of MP024 was not affected in the presence of DNA. This is the first indication that the addition of the cationic substituent, either on the sugar residue (MP072) or on the nitrogen imide (MP059) reinforces considerably the DNA binding strength. Melting temperature ($T$m) measurements performed with calf thymus DNA and the polynucleotide poly(dAT)$_2$ also indicate that the two amino-substituted compounds exhibit a considerably higher affinity for DNA compared with the uncharged analogue. With poly(dAT)$_2$, the $\Delta T$m values ($\Delta T\mathrm{m} = T\mathrm{m}^{\mathrm{complex}} - T\mathrm{m}^{\mathrm{DNA}}$) exceed 30 °C with MP059 and MP072 which stabilise duplex DNA against heat denaturation at least six times more strongly than MP024. A marked difference is also observed with calf thymus DNA which has a higher GC content (42% GC) (Table 1). In this case, the neutral compound MP024 shows little or no effect on the $T$m of DNA whereas the two cationic analogues induce a large shift, $>10$ °C, of the $T$m value. The effect is most pronounced with MP059 indicating that its diethylaminoethyl side chain plays a significant role in the drug–DNA interaction.

### 2.2. DNA binding mode

Electric linear dichroism (ELD) was used to investigate the mode of drug binding to DNA. In these experiments the DNA is oriented by in an electric field and the respective orientation of the molecules bound to DNA is probed using a linearly polarised light. The ELD spectra of the drugs bound to calf thymus DNA are shown in Fig. 4a, together with the dependence of the reduced dichroism $\Delta A/A$ as a function of the DNA–drug ratio (Fig. 4b) and the electric field strength (Fig. 4c). The reduced dichroism $\Delta A/A$ is strongly negative in the drug absorption band, which indicates an orientation of the chromophore perpendicular to the helix axis (or the electric field direction). The negative reduced dichroism values measured for the three compounds at 320 nm are similar to those measured for DNA alone at 260 nm. This indicates that the indolocarbazole chromophore of these ligands is oriented parallel to the DNA base pairs, as expected for an intercalative binding.

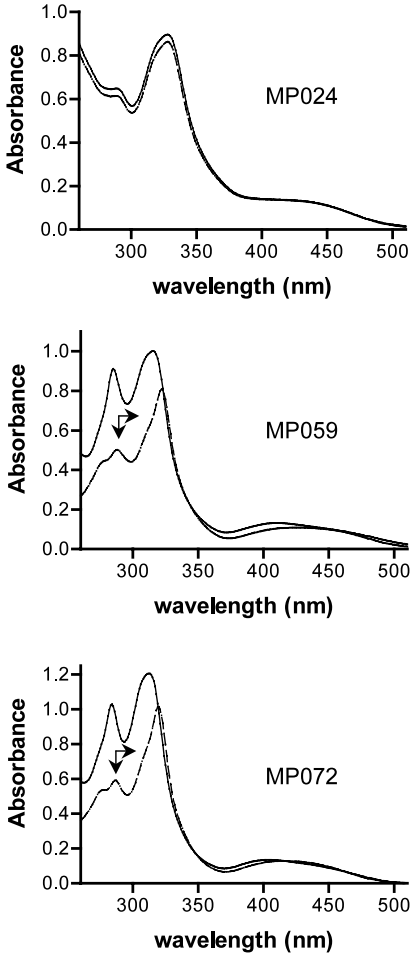


Fig. 3. Absorption spectra of the indolocarbazoles in the absence (solid line) and presence (dashed line) of calf thymus DNA. DNA titrations of the drugs were performed in 1 mM sodium cacodylate buffer at pH 7.0. To 3 mL of drug solution at 20 μM were added aliquots of a concentrated calf thymus DNA solution. The spectra of the complexes corresponded to a phosphate–DNA–drug ratio of 20.

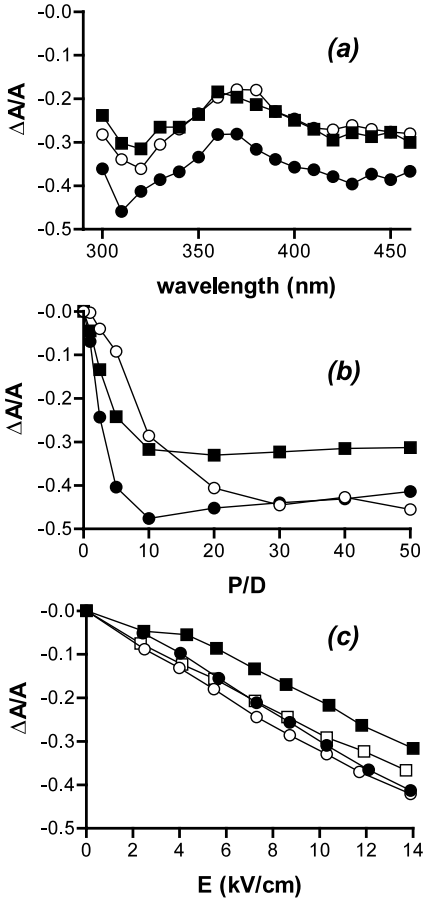


Fig. 4. ELD measurements. The graphs show the dependence of the reduced dichroism $\Delta A/A$ on (a) the wavelength, (b) the phosphate–DNA–drug ratio and (c) the electric field strength for (■) MP059, (●) MP072 and (○) MP024 bound to calf thymus DNA. Conditions: (a) 13.7 kV $cm^{-1}$, $P/D = 20$ (200 μM DNA, 10 μM drug), (b) 320 nm, 13.7 kV $cm^{-1}$, (c) 320 nm for the DNA–drug complexes and 260 nm for DNA alone (□). All measurements were performed at room temperature (20 °C) in 1 mM sodium cacodylate buffer, pH 7.0.

Table 1
DNA binding and cytotoxicity

| | $\Delta T$m (°C) [a] | | $IC_{50}$ (μM) [b] | |
|---|---|---|---|---|
| | poly(dAT)$_2$ | CT DNA | HT29 | A549 |
| MP003 | 2.8 | 0 | 2.5 | 2.0 |
| MP024 | 4.6 | 1 | 3.0 | 3.1 |
| MP059 | 38.9 | 15.8 | 0.2 | 0.3 |
| MP072 | 31.1 | 11.3 | 0.2 | 0.2 |

[a] Variation in melting temperature ($\Delta T\mathrm{m} = T\mathrm{m}^{\mathrm{complex}} - T\mathrm{m}^{\mathrm{DNA}}$). Tm 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 DNA, at 260 nm with a heating rate of 1 °C $min^{-1}$.

[b] Drug concentration that inhibits cell growth by 50% after incubation in liquid medium for 72 h [10].

### 2.3. Sequence selectivity

The drugs were 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 fragments is presented in Fig. 5. On the autoradiogram, several regions where the cleavage by the enzyme is decreased in the presence of the drugs can be discerned with MP059 but not with MP024. The neutral compound does not alter the nuclease profile whereas the cationic ligand recognises preferentially certain sequences. MP072 also interferes with the activity of DNase I but for drug concentrations $>10$ μM, the DNase I cleavage becomes totally inhibited due to non specific binding of the drug to DNA. MP059 thus presents a sharper sequence selectivity than MP072. The positions of the footprints, presumptive of drug binding sites, were located by a densitometric analysis of the gel.

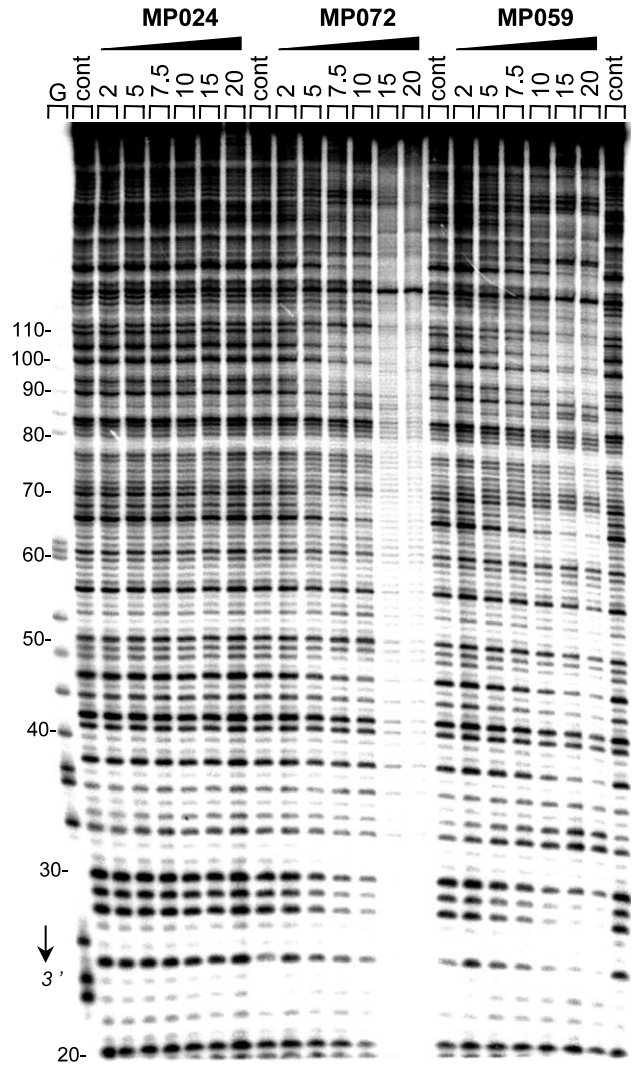


Fig. 5. DNase I footprinting with the 265-mer PvuII–EcoRI restriction fragment cuts from plasmid pBS in the presence of the indolocarbazole derivatives at concentrations increasing from 2 to 20 μM. The DNA was 3′-end labelled at the EcoRI site with [α-$^{32}$P]dATP in the presence of AMV reverse transcriptase. 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 lane 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 shown in Fig. 6 compare the profiles obtained with the three indolocarbazoles. The binding sites identified with MP059 and MP072 coincide with sequences containing GpT (ApC) and TpG (CpA) steps. Pure AT or GC tracts are generally not recognised and appear as regions of enhanced DNase I cleavage. The footprinting profile seen with MP059 bears a remarkable resemblance to that determined previously for a rebeccamycin derivative containing a methoxyglucose residue and substituted at the imide nitrogen with a hydroxyl group [11].

### 2.4. Effects on DNA topoisomerases

A relaxation assay using supercoiled plasmid DNA was used to evaluate the effects of the compounds on the catalytic activity of human topoisomerases I and II. A set of data is presented in Fig. 7. With topoisomerase I, an increase of the band corresponding to nicked DNA was observed with the neutral compound MP024 and the reference inhibitor camptothecin whereas no enhancement of topoisomerase I-mediated single strand breaks was noted with MP059 and MP072 (Fig. 7a). MP024 can be considered as a rebeccamycin-type topoisomerase I poison whereas the two cationic analogues inhibit DNA relaxation by topoisomerase due to strong DNA binding but they do not stimulate DNA cutting by the enzyme. In the experiments performed without ethidium bromide in the gels (Fig. 7b), a typical unwinding of supercoiled DNA is seen with MP024 but there is no gel retardation. On the contrary, the two cationic analogues considerably reduce the electrophoretic mobility of the DNA, reflecting thus their tight interaction with nucleic acids. This can be taken as an additional proof that the DNA binding capacity of the rebeccamycin derivative has been considerably increased by adding a cationic substituent. The drugs do not promote DNA cleavage by topoisomerase II. No linear DNA species was observed in the presence of the three indolocarbazoles, in contrast to what is observed with the reference drug etoposide which produces a high level of double strand breaks (data not shown).

## 3. Discussion

The uncharged molecule MP024 has been characterised as a conventional topoisomerase I poison capable of stabilising DNA–topoisomerase I covalent complexes, thereby promoting DNA single strand breaks. In a previous study, we showed that topoisomerase I inhibition also contributes to the cytotoxic activity of the analogous compound MP003 [9]. But these two compounds bind only weakly to double stranded DNA and for this reason, it was decided to incorporate a functional group susceptible to promote their DNA association. The addition of a cationic substituent is a facile strategy frequently used to facilitate molecular contacts between a polyanion like DNA and small molecules. We have shown previously that the DNA binding affinity and sequence selectivity of rebeccamycin can be enhanced by replacing the glucose residue with a 2′-aminoglucose moiety [12]. The same strategy applied here to the staurosporine-type compound reveals unambiguously that MP072 bearing a 6′-amino group interacts with DNA much more tightly than the uncharged derivative MP024. The 6′-OH → $NH_2$ sub-

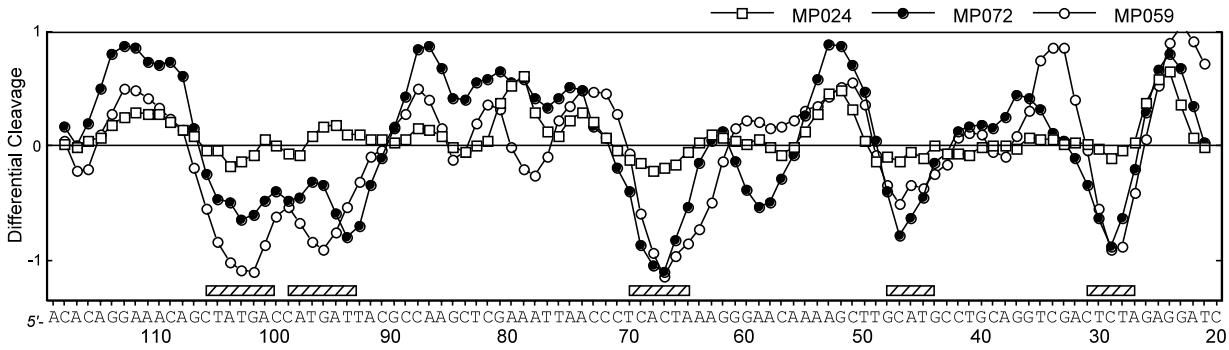


Fig. 6. Differential cleavage plots comparing the susceptibility of the 265-mer DNA fragment to DNase I cutting in the presence of the indolocarbazoles (10 μM each). Negative values correspond to a ligand-protected site and positive values represent enhanced cleavage. Vertical scales are in units of 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 3-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 neighbours. Only the region of the restriction fragments analysed by densitometry is shown. The dashed bar indicates the main site of reduced cleavage by DNase I in the presence of the drugs.

stitution facilitates the anchorage of the drug on DNA sequences containing predominantly GpT·ApC and TpG·CpA steps but this is at the expense of topoisomerase I inhibition since the cationic drug has lost its capacity to stimulate DNA cleavage by the enzyme.

The second option chosen to increase the DNA binding capacity of MP024 consisted to append a diethylaminoethyl side chain on the F-ring nitrogen imide. This side chain is identical to that found in the drug NSC655649 (Fig. 1), also known as BMS 181176,

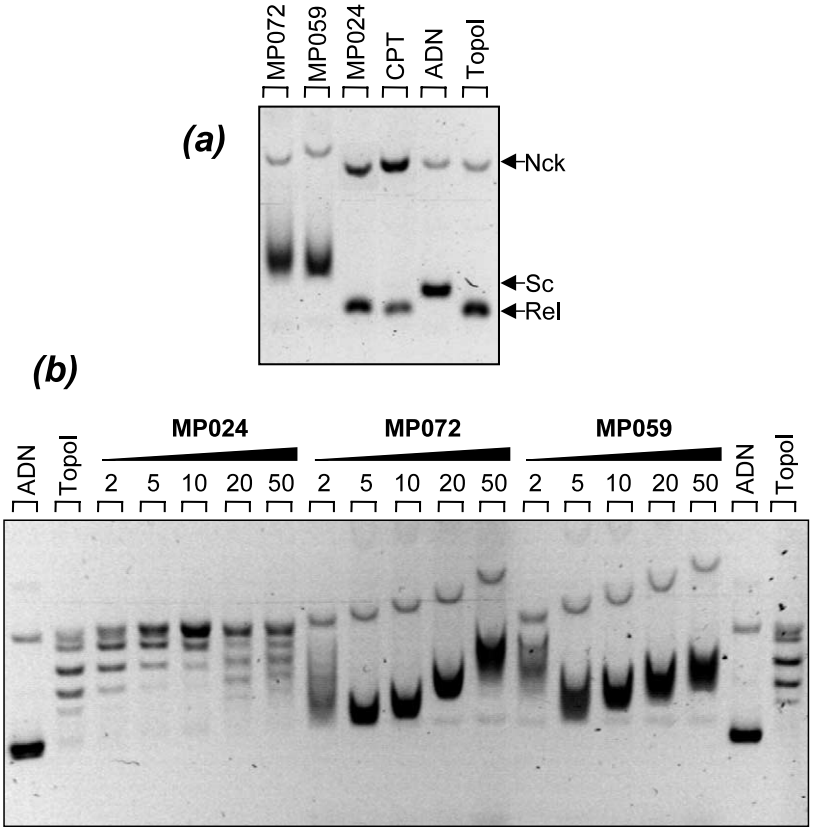


Fig. 7. Effect of the test compounds on the relaxation of plasmid DNA by human topoisomerase I. Native supercoiled pKMp27 DNA (0.5 μg) (lane DNA) was incubated with 4 units topoisomerase I in the absence (lane TopoI) or presence of a given drug at the indicated concentration (μM). In (a) the drugs were used at 50 μM including camptothecin (CPT). Reactions were stopped with sodium dodecylsulfate and treatment with proteinase K. DNA samples were stained with ethidium bromide (1 μg $mL^{-1}$) (a) before or (b) after electrophoresis on 1% agarose gels. The gels were photographed under UV light. Nck, nicked; Rel, relaxed; Sc, supercoiled DNA.

which has been evaluated in phase I clinical trials in both adult and pedriatric patients [13,14] and is now undergoing phase II clinical trials for the treatment of ovarian and hepatobiliary cancers [15]. The enhanced DNA affinity of MP059 compared with MP024 thus mimics the gain of DNA binding that distinguishes rebeccamycin and NSC655649 [16].

The two methods used to convert the neutral derivative into a cationic analogue gave satisfactory results. Both MP059 and MP072 exhibit enhanced affinity for DNA and this translate at the biological level by a 10-fold higher cytotoxic index. The loss of the DNA topoisomerase poisoning activity is apparently not an obstacle for the design of cytotoxic molecules in this series. On the basis of the footprinting data, the strategy that consists to link an aminoalkyl side chain on the indolocarbazole chromophore appears more interesting than the replacement of the neutral sugar with an aminosugar residue. The footprints are better resolved with MP059 than with MP072 which binds non specifically to DNA when the drug concentration is raised. In conclusion, the present study validates the design strategy that consisted to increase the DNA binding capacity and hence the cytotoxic activity of the rebeccamycin–staurosporine hybrid type compounds. The design of additional aminoalkyl analogues of MP059 is now considered as a profitable route for the discovery of anticancer drugs in the indolocarbazole glycoside series.

## 4. Experimental protocols

### 4.1. Drugs, chemicals and biochemicals

The synthesis of the drugs used in this study has been recently reported [9,10]. Drugs were dissolved in DMSO at 5 mM and these stock solutions were kept at −20 °C and freshly diluted with water to the desired concentration immediately prior to use. Etoposide and camptothecin were purchased from Sigma. Calf thymus DNA and the double-stranded polymer poly(dAT)$_2$ were obtained from Pharmacia. Calf thymus DNA was deproteinised with sodium dodecyl sulphate (protein content $<0.2\%$). The nucleoside triphosphate labelled with [$^{32}$P](α-dATP) was obtained from Amersham (3000 Ci mmol$^{-1}$). 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.

### 4.2. 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$^{-1}$. 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.

### 4.3. Electric linear dichroism (ELD)

Measurements were performed with a computerised optical measurement system using the procedures previously outlined [17]. 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$^{-1}$. 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 [18].

### 4.4. Purification and radiolabelling of DNA restriction fragment

The 265 bp DNA fragment was prepared by 3′-[$^{32}$P]-end labelling of the EcoRI–PvuII double digest of the plasmid pBS (Stratagene) using α-[$^{32}$P]-dATP (3000 Ci mmol$^{-1}$) 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 labelled DNA was resuspended in 10 mM Tris adjusted to pH 7.0 containing 10 mM NaCl.

### 4.5. DNase I footprinting, electrophoresis and quantitation by storage phosphor imaging

Experiments were performed essentially as previously described [19]. Briefly, reactions were conducted in a total volume of 10 μL. Samples (3 μL) of the labelled 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 $mL^{-1}$ in the reaction mixture. After 3 min, the reaction was stopped by freeze drying. Lyophilised samples were 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 3MM 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 analysed 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).

### 4.6. DNA relaxation experiments

Supercoiled pKMp27 DNA (0.5 μg) was incubated with four units 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 $mL^{-1}$. DNA samples were then added to the electrophoresis dye mixture (3 μL) and electrophoresed in a 1% agarose gel containing ethidium bromide (1 μg $mL^{-1}$), at room temperature for 2 h at 120 V. Gels were washed and photographed under UV light [20].

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