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Enhanced Binding to DNA and Topoisomerase I Inhibition by an Analog of the Antitumor Antibiotic Rebeccamycin Containing an Amino Sugar Residue

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ABSTRACT

Many antitumor agents contain a carbohydrate side chain appended to a DNA-intercalating chromophore. This is the case with anthracyclines such as daunomycin and also with indolocarbazoles including the antibiotic rebeccamycin and its tumor active analog, NB506. In each case, the glycoside residue plays a significant role in the interaction of the drug with the DNA double helix. In this study we show that the DNA-binding affinity and sequence selectivity of a rebeccamycin derivative can be enhanced by replacing the glucose residue with a 2′-aminoglucose moiety. The drug-DNA interactions were studied by thermal denaturation, fluorescence, and footprinting experiments. The thermodynamic parameters indicate that the newly introduced amino group on the glycoside residue significantly enhanced binding to DNA by increasing the contribution of the polyelectrolyte effect to the binding free energy, but does not appear to participate in any specific molecular contacts. The energetic contribution of the amino group of the rebeccamycin analog was found to be weaker than that of the sugar amino group of daunomycin, possibly because the indolocarbazole derivative is only partially charged at neutral pH. Topoisomerase I-mediated DNA cleavage studies reveal that the OH→NH_2 substitution does not affect the capacity of the drug to stabilize enzyme-DNA covalent complexes. Cytotoxicity studies with P388 leukemia cells sensitive or resistant to camptothecin suggest that topoisomerase I represents a privileged intracellular target for the studied compounds. The role of the sugar amino group is discussed. The study provides useful guidelines for the development of a new generation of indolocarbazole-based antitumor agents.

Indolocarbazoles represent an important class of antitumor agents (Prudhomme, 1997). Antibiotics such as staurosporine (Fig. 1) and K252a, which have the two indole nitrogens linked to the carbohydrate residues, are potent inhibitors of protein kinases, in particular, protein kinase C. This subgroup also includes the synthetic derivative 7-hydroxy-staurosporine, known as UCN-01 (Fig. 1), which is undergoing clinical trials (Akinaga et al., 1991; Shao et al., 1997). Another series of indolocarbazole derivatives has the sugar residue attached to only one indole nitrogen. This second subgroup is typified by the antibiotic rebeccamycin (Fig. 1), which has very little effect on protein kinase C, but, unlike the compounds of the first series, it is a DNA-binding agent and an inhibitor of topoisomerase I (Nettleton et al., 1985; Bush et al., 1987). Numerous synthetic derivatives have been designed to confer higher DNA-binding and anti-topoisomerase I activities. A few synthetic compounds, such as NB-506 (Fig. 1), are presently in clinical trials (Arakawa et al., 1995).

Over the last few years, we have screened more than 80 rebeccamycin derivatives for topoisomerase I inhibition and biological activity. The structure-activity relationships are not yet fully elucidated but at least three important rules were established. First, the two chlorine atoms on the indolocarbazole chromophore of rebeccamycin are detrimental to the interaction with DNA and, consequently, to the biological activity. These bulky chloro substituents prevent the drug from intercalating into DNA. Second, a variety of substituents can be added on the imide nitrogen without disrupting the drug-DNA complex. For example, polar formyl-amino or bis(hydroxyethyl)methylamino groups as well as nonpolar

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ABBREVIATIONS: DMSO, dimethyl sulfoxide; bp, base pair; pBS, pBluescript; AMV, avian myeloblastosis virus; DNase I, deoxyribonuclease I (EC 3.1.21.1).

methyl groups can be tolerated without loss of the topoisomerase I poisoning effect. Third, and this is probably the most important point, the sugar residue is absolutely required to ensure tight interaction with DNA. Analogs of rebeccamycin lacking the methoxyglucose residue are much less active than their glycosylated counterparts. Moreover, the stereochemistry of the sugar is essential. Indolocarbazoles linked to the carbohydrate via a β-glycoside linkage are potent inhibitors of topoisomerase I, whereas the α-analogs completely failed to inhibit the enzyme, most likely as a result of their greatly reduced affinity for DNA. Both the chemical nature and the isomeric form of the sugar residue are essential to the interaction with DNA and to the biological activity (for the structure-activity relationships, see Rodrigues-Pereira et al., 1996; Anizon et al., 1997, 1998; Bailly et al., 1997, 1998a; Prudhomme, 1997; Moreau et al., 1998).

A number of antitumor antibiotics are equipped with carbohydrate residues that contribute significantly to the interaction with DNA. For example the aryltetrasaccharide domain of the enediyne antibiotic calicheamycin γ_1^I plays a critical role in the recognition of specific sequences and contributes positively to the DNA-cleaving activity as well as to the inhibition of transcription by DNA polymerase II (Nicolaou et al., 1992; Paloma et al., 1994; Ikemoto et al., 1995; Kumar et al., 1997a). Neocarzinostatin and esperamicin, two other enediyne antitumor antibiotics, also contain aminoglycoside side chains that play a functional role in the recognition and/or cleavage of DNA sequences (Kumar et al., 1997b; Myers et al., 1997).

The anthracycline antibiotics all bear one or two glycosyl side chains that come to lie in the grooves of DNA when the chromophore is intercalated. The amino sugar residue of the prototype anthracycline daunomycin is needed for specific binding of the drug to (A/T)GC and (A/T)CG triplets (Chaires et al., 1990; Bailly et al., 1998b). Thermodynamic studies revealed that a significant energetic penalty results from the removal of the daunosamine sugar part attached to the daunomycinone chromophore (Chaires, 1996). Although the amino group at position 3′ on the sugar is not essential for cytostatic and topoisomerase II-targeting activities (Capranico et al., 1994), it is important for DNA binding for more than just its positive charge. According to several high-resolution NMR and crystal structures, the amine participates in hydrogen-bonding interactions with DNA bases (Chaires, 1990; Frederick et al., 1990). The replacement of the 3′-amine with a hydroxyl group results in a marked decrease of the affinity for DNA. The binding constant of doxorubicin for DNA is more than 80 times stronger than that of hydroxyrubicin, and the total favorable energetic contribution of the amine is approximately 2.5 kcal/mol (Chaires, 1996).

With these data in mind, we postulated that the addition of an amino group on the methoxy-sugar residue of rebeccamycin-type compounds should permit tighter binding to DNA and might be beneficial to their biological activity. The present study reports the synthesis and biological activity of a new rebeccamycin derivative possessing an amino group at position 2′. To evaluate the influence of the newly introduced amino group, we compared the DNA-binding and topoisomerase I-poisoning activities of the two drugs shown in Fig. 2. The substitution of the hydroxyl group with an amino group at position 2′ of the sugar moiety is the only difference between the two test molecules, and the rest of the molecule is identical. Such a substitution produces a significant increase in hydrophilicity and basicity as well as a change in steric hindrance, all of which could affect DNA binding. We provide clear evidence that the OH→NH_2 substitution significantly enhances the DNA-binding affinity without any loss of the activity against topoisomerase I.

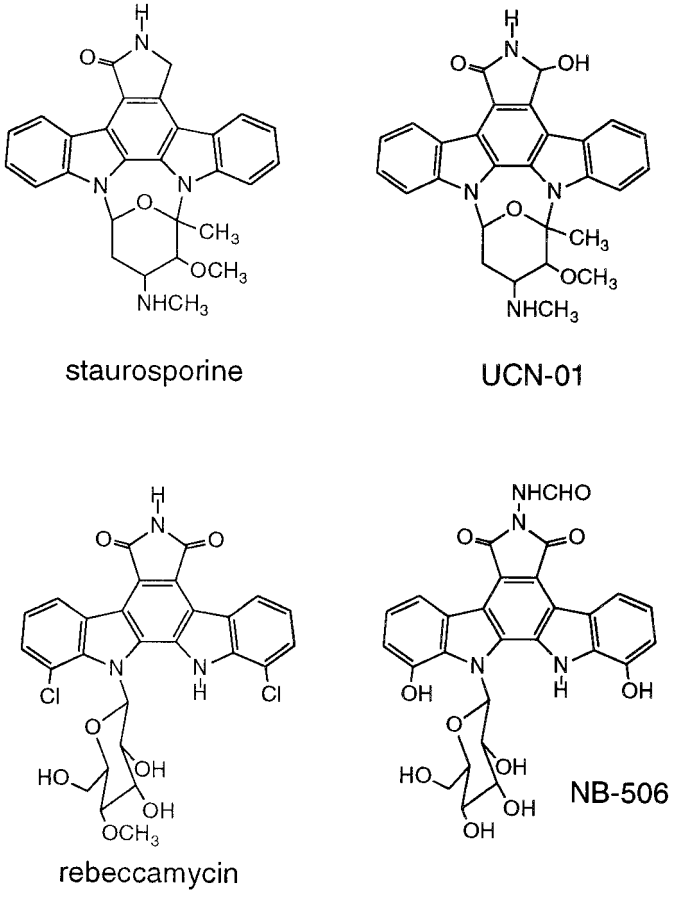

Fig. 1. Indolocarbazoles. Structure of the protein kinase C inhibitors—the antibiotic staurosporine and the synthetic derivative UCN-01—and structure of the toposiomerase I inhibitors—the antibiotic rebeccamycin and the synthetic derivative NB-506.

Materials and Methods

Synthesis of Compound (2). Most indolocarbazole derivatives that we studied previously were obtained by hemisynthesis from the microbial metabolite rebeccamycin. In contrast, the amino sugar derivative **2** was obtained by total synthesis by coupling of a sugar possessing a protected amine function to an indolocarbazole aglycone (Fig. 3). The amino group of commercial D-glucosamine hydrochloride was protected as a phtalimide by treatment with sodium methoxide followed by reaction with phtalic anhydride, and then the intermediate was treated with pyridine and acetic anhydride (Lemieux et al., 1976). The protected sugar was coupled to the *N*-methylmaleimide indolocarbazole (Brenner et al., 1988). Coupling was performed with *N*-methylmaleimide indolocarbazole trimethylsilylated at one of the indole nitrogens in the presence of trimethylsilyltriflate in 1,2-dichloromethane according to a method described for the synthesis of nucleosides (Chu et al., 1990). Reaction of the coupling product **C** with hydrazine hydrate followed by an acidic treatment led to diamine **2** as a monohydrochloride. The chemical shifts of the protons of the two amino groups were 5.04 ppm in dimethyl sulfoxide (DMSO) and in the signal of water for the free amine and 8.20 and 5.0 (broad s) for the hydrochloride. For the

corresponding aglycone bearing an amino function only on the imide nitrogen, the chemical shift of the protons of the amino group in the same solvent was 5.00 ppm and the hydrochloride could not be formed (Rodrigues-Pereira et al., 1995). Therefore, the signal at 5.04 ppm for compound **2** is attributed to the free amine on the imide nitrogen.

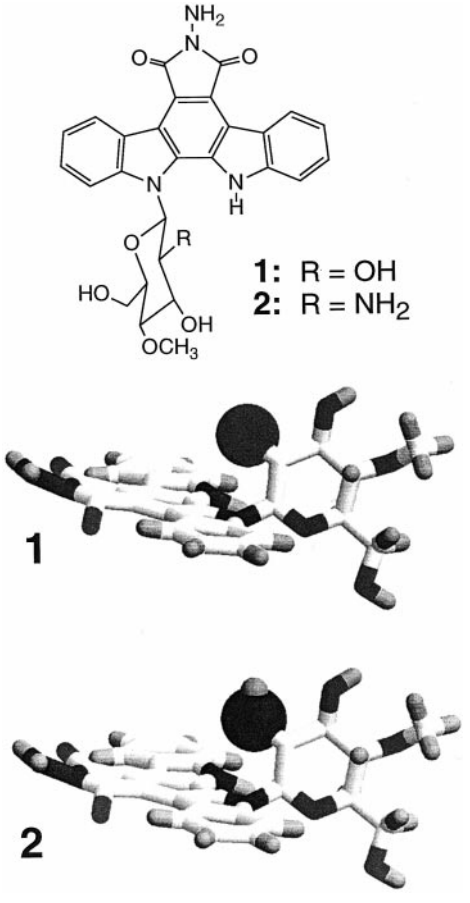

Fig. 2. Structure of the drug used in this study. An energy-minimized structure of the drugs is shown. The software HyperChem 5.01 and Alchemy 2000 were used to construct the structures.

Chemistry. Infrared (IR) spectra were recorded on a Perkin-Elmer 881 spectrometer (ν in cm^{-1}). NMR spectra were performed on a Bruker AC 400 (^1H: 400 MHz; ^{13}C: 100 MHz) (chemical shifts δ in ppm, and the following abbreviations are used: s, singlet; d, doublet; t, triplet; m, multiplet; C tert, tertiary carbons; C quat, quaternary carbons). Mass spectrum (FAB+) was determined at CESAMO (Talence, France) on a high-resolution Fisons Autospec-Q spectrometer. Chromatographic purifications were performed by Kieselgel 60 (Merck) 0.063- to 0.200-mm column chromatography. For purity tests, thin-layer chromatography was performed on fluorescent silica gel plates (60 F_{254}; Merck).

6-Amino-12-(2-amino-β-D-glucopyrannosyl)-6,7,12,13-Tetrahydroindolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7-dione, hydrochloride (2). *N*-methylmaleimide indolocarbazole B (100 mg, 0.295 mmol) was dissolved in tetrahydrofuran (THF; 20 ml), and NaH (60% in mineral oil, 26 mg, 0.65 mmol) was added. The mixture was stirred for 45 min at room temperature, and then TMSCl [0.1 ml, 0.796 mmol, 2.7 equivalent (eq)] was added. After stirring for 30 min, THF was evaporated before addition of 170 ml of $(CH_2Cl)_2$ and *N*-phtalimido-sugar **A** (a mixture of α and β, 37:63, 270 mg, 0.583 mmol, 2 eq). Trimethylsilyl triflate (50 μl) was added, and the mixture was stirred at room temperature for 2.5 h and then poured into saturated aqueous $NaHCO_3$. After extraction with EtOAc, the organic phase was dried over $MgSO_4$ and the solvent was removed. CH_2Cl_2 was added to the residue and the mixture was filtered off. After purification by chromatography (eluent, EtOAc/CH_2Cl_2, 5:95), a mixture of **C** and few quantities of starting products that could not be separated by chromatography was obtained and used for the next step without further purification.

This mixture was dissolved in ethanol (20 ml), and hydrazine hydrate (1.5 ml) was added. After refluxing for 3 h, the solvent was removed and the residue was dissolved in AcOEt, washed with brine. The organic phase was dried over $MgSO_4$. After removal of the solvent, 1.1 N HCl (231 μl) was added to a suspension of the residue in methanol and the mixture was stirred at room temperature. A precipitate of the hydrochloride was obtained by addition of AcOEt. Filtration gave **2** as an orange solid (52.3 mg, 0.10 mmol, 33% overall yield): m.p. > 300°C. IR (KBr) ν_{CO} 1710, 1760 cm^{-1}, $\nu_{NH,\ OH}$ 3200 to 3600 cm^{-1}. High resolution mass spectrum calculated for

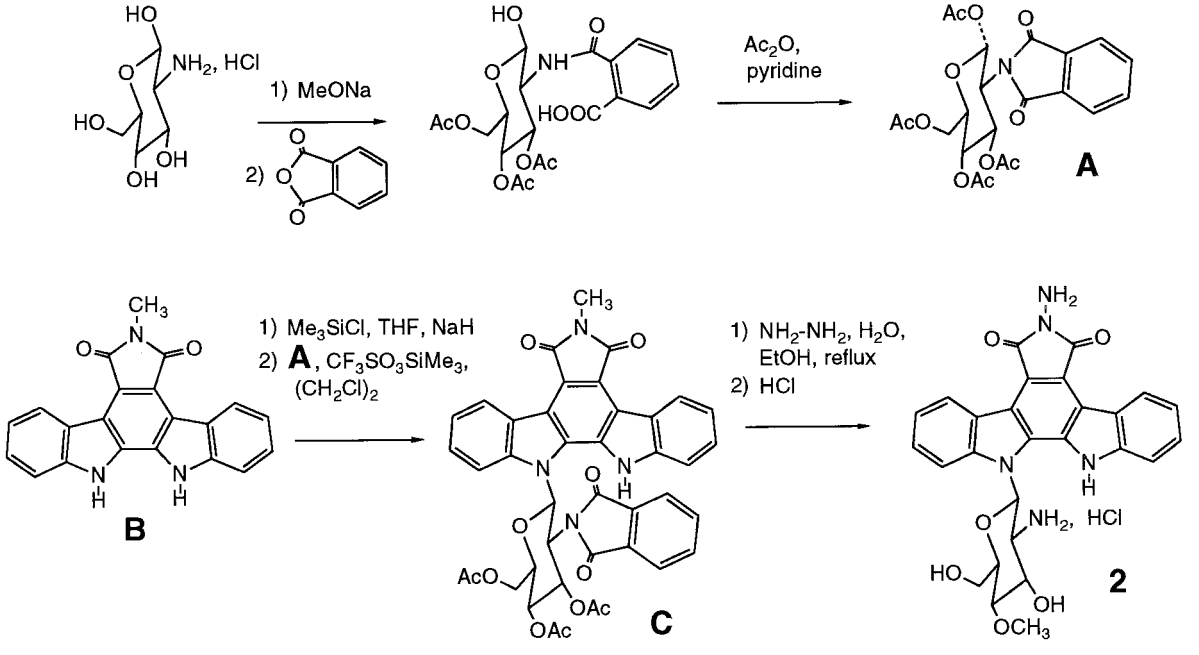

Fig. 3. Synthetic scheme.

$C_{26}H_{24}N_5O_6$ $(M+H)^+$, 502.1726, found, 502.1728. ^1H NMR (400 MHz, DMSO-d_6) spectrum of the free amine: 3.10 (1H, m), 3.57 (1H, m), 3.83 (1H, d, J = 10.9 Hz), 3.98 (1H, d, J = 9.3 Hz), 4.12 (2H, d, J = 10.3 Hz), 5.04 (2H, s, NH_2), 5.39 (1H, br s, OH), 5.56 (1H, br s, OH), 6.11 (1H, br s, OH), 6.34 (1H, d, J = 8.9 Hz), 7.42 (1H, t, J = 7.8 Hz), 7.44 (1H, t, J = 7.9 Hz), 7.61 (1H, t, J = 7.9 Hz), 7.64 (1H, t, J = 7.4 Hz), 7.74 (1H, d, J = 7.9 Hz), 8.09 (1H, d, J = 8.4 Hz), 9.15 (1H, d, J = 7.9 Hz), 9.23 (1H, d, J = 7.9 Hz), 11.68 (1H, s, N_{indole}-H). ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of the hydrochloride: 57.9 (C6′), 56.1, 67.5, 72.3, 79.0, 80.4 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 111.1, 112.5, 120.8, 121.4, 124.4, 124.5, 127.1, 127.4 (C tert arom), 116.8, 117.9, 118.4, 119.3, 121.6, 121.7, 127.7, 129.3, 140.3, 141.4 (C quat arom), 168.5, 168.7 (C═O).

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 path length) were heated by circulating water. The 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 midpoint of the hyperchromic transition.

Fluorescence Titration Experiments. The stock solutions of compounds **1** and **2** were freshly prepared at a concentration of 2 mM in DMSO and diluted into buffer solution at the desired concentration. Calf thymus DNA was purchased from Pharmacia (lot 27-4562-02) and was sonicated and purified as described earlier (Chaires et al., 1993). Before further use, the DNA was dialyzed in the appropriate buffer for 24 h, and its concentration was determined by UV absorption at 260 nm by using a molar extinction coefficient, ϵ_{260} = 12,824 cm^{-1} M^{-1}. Titration experiments were carried out in a buffer (BPE) consisting of 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.0, unless noted otherwise. Fluorescence titration data were recorded at room temperature using an I.S.S. Greg 200 fluorometer. Excitation was at 320 nm, and fluorescence emission was monitored over the range of 340 to 620 nm. Samples used for titration experiments were prepared separately at a constant drug concentration of 5 μM and DNA concentrations ranging from 0.1-μM to 1 mM base pairs (bp).

Fluorescence titration data were fit directly to get binding constants using a fitting function incorporated into FitAll (MTR Software, Toronto, Canada). Simply, the observed fluorescence is assumed to be a sum of the weighted concentrations of free and bound ligand:

$$F = F^0(C_t - C_b) + F^bC_b \quad (1)$$

where F is the apparent fluorescence at each DNA concentration, F^0 is the fluorescence intensity of free ligand, and F^b is the fluorescence intensity of the bound species. For the interaction of a ligand D with a DNA site S, it may be easily shown that:

$$Kx^2 - x(KS_0 + KD_0 + 1) + KS_0D_0 = 0 \quad (2)$$

where $x = C_b$, K is the association constant, S_0 is the total concentration, and D_0 is the total ligand concentration. Equation 2 is readily solved using the quadratic formula. Data in the form of fluorescence response F as a function of total DNA site concentration at fixed concentration of ligand then may be fit by nonlinear least-squares methods to get K, F^0, and F^b.

DNA Purification and Labeling. The plasmid pBluescript (pBS) (Stratagene, La Jolla, CA) was isolated from *Escherichia coli* by a standard SDS-sodium hydroxide lysis procedure and purified using Qiagen columns. The purified plasmid then was precipitated and resuspended in appropriate buffered medium before digestion by the restriction enzymes. The two pBS DNA fragments were prepared by 3′ ^{32}P end-labeling of the *Eco*RI-*Pvu*II double digest of the plasmid using [α-^{32}P]dATP and avian myeloblastosis virus (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-μ filter, and the DNA was precipitated with ethanol. After washing with 70% ethanol and vacuum-drying the precipitate, the labeled DNA was resuspended in 10 mM Tris, adjusted to pH 7.0, containing 10 mM NaCl.

Footprinting Experiments. Cleavage reactions by DNase I were performed essentially according to the previously detailed protocols (Bailly and Waring, 1995). Briefly, reactions were conducted in a total volume of 10 μl. Samples (3 μl) of the ^{32}P-labeled DNA fragment were incubated with 5 μl of the buffer solution containing the desired drug concentration. After a 20-min incubation at 37°C to ensure equilibration of the binding reaction, the digestion was initiated by the addition of 2 μl of DNase I (0.01 U/ml enzyme in 20 mM NaCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$, pH 7.3). At the end of the reaction time (routinely 4 min at room temperature), the digestion was stopped by freeze-drying. After lyophilization, each sample was resuspended in 4 μl of an 80% formamide solution containing tracking dyes before electrophoresis.

Topoisomerase I Inhibition

Experiments with Linear Plasmid DNA on Agarose Gels. pBR322 DNA (Boehringer Mannheim, Mannheim, Germany) was linearized with *Eco*RI and labeled with [α-^{32}P]dATP in the presence of the Klenow fragment of DNA polymerase I. The labeled DNA was then digested to completion with *Hin*dIII. The cleavage reaction mixture contained 20 mM Tris-HCl, pH 7.4, 60 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 2×10^4 dpm of [α-^{32}P]pBR322 DNA, and the indicated drug concentrations. The reaction was initiated by the addition of topoisomerase I (40 U in 20 μl of reaction volume) and allowed to proceed for 10 min at 37°C. 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°C. Samples were denatured by the addition of 10 μl of denaturing loading buffer consisting of 0.45 M NaOH, 30 mM EDTA, 15% (w/v) sucrose, and 0.1% bromocresol green before loading onto a 1% agarose gel in TBE buffer containing 0.1% SDS. Electrophoresis was conducted at 2 V/cm for 18 h.

Sequencing of Topoisomerase I-Mediated DNA Cleavage Sites. Each reaction mixture contained 2 μl of 3′ ^{32}P end-labeled DNA (~1 μM), 5 μl of water, 2 μl of 10× topoisomerase I buffer, and 10 μl of drug solution at the desired concentration (50 μg/ml). After at least 30 min of incubation to ensure equilibration, the reaction was initiated by addition of 10 U of topoisomerase I. Samples were incubated for 40 min at 37°C before adding SDS to 0.25% and proteinase K to 250 μg/ml to dissociate the drug-DNA-topoisomerase I cleavable complexes. The DNA was precipitated with ethanol and then resuspended in 5 μl of formamide-TBE loading buffer, denatured at 90°C for 4 min, and then chilled in ice for 4 min before loading onto the sequencing gel.

Growth Inhibition Assay. P388 murine leukemia cells were incubated at 37°C for 96 h in the presence of various concentrations of drug and evaluated for viability by neutral red staining as described previously (Rodrigues-Pereira et al., 1996). The concentrations of drugs giving 50% of growth inhibition (IC_{50}) were determined.

Results

Interaction with DNA. Initially, we used a thermal denaturation procedure to monitor the interaction of the drugs with DNA. Both the hydroxyl derivative **1** and its amino counterpart **2** stabilize duplex DNA against thermal denaturation. The effect is slightly more pronounced with compound **2** than with **1**. At a drug/DNA ratio of 1, the T_m of calf

thymus DNA was raised from 63.6–68.7°C with compound **2** in BPE buffer (16 mM Na^+). The stabilizing effect (ΔT_m) is 2°C lower with compound **1** (3.3 for **1** versus 5.1° for **2**). This preliminary experiment suggests that the introduction of the amino group enhances the interaction of **2** with DNA. Direct fluorescence measurements fully confirmed this belief.

We exploited the intrinsic fluorescence of the indolocarbazole chromophore to evaluate the strength of the interaction of the drugs with calf thymus DNA. The fluorescence emission at 560 nm is weak when the drug is free in solution but it is considerably enhanced when the drug is bound to DNA. This property is very useful for accurate determination of the DNA-binding affinities. Nonlinear least-squares analysis of the fluorescence titration curves shown in Fig. 4 yielded binding constants of 3.6×10^4 (M bp)$^{-1}$ and 10.6×10^4 (M bp)$^{-1}$ for compounds **1** and **2,** respectively, in low-salt BPE buffer at neutral pH. There is no doubt that the OH→NH_2 substitution significantly enhances the binding to DNA. The affinity constant of compound **2** is about 3-fold higher than that of compound **1**.

The hydroxyl derivative **1** is uncharged whereas the amino compound **2** is expected to be positively charged at neutral pH. In this case it is important to determine the variation of the binding constants as a function of the salt concentration. The charged compound must be more sensitive to the changes of the ionic strength than the neutral molecule (Record et al., 1978). We repeated the fluorescence titration experiments in the presence of increasing NaCl concentrations. The data are presented in the form of double-logarithmic plots of log K versus log [NaCl] (Fig. 5). At pH 7.0, the slope of the data for compound **1** is −0.26, i.e., very close to the theoretical value of −0.24 predicted for the binding of an uncharged intercalator to DNA (Friedman and Manning, 1984). The slope observed for compound **2** is higher, as expected. However, its value (−0.496) is less than the theoretical value of −0.88 to −1.24 predicted for a ligand bearing one positively charged group (Record et al., 1978; Friedman and Manning, 1984). The difference from the predicted value may signify that only a fraction of the drug molecules **2** are protonated (see *Discussion*). Nonetheless, these experiments confirm that introducing an amino group into the sugar residue promotes the interaction of the drug with DNA.

The binding data presented above enable the calculation of the free energy of drug binding to DNA. ΔG_{obs} was calculated from the binding constants using the standard Gibbs relation $\Delta G_{obs} = -RT \ln K$. The observed binding free energy can then be partitioned into its nonpolyelectrolyte contribution (ΔG_t) and its polyelectrolyte contribution (ΔG_{pe}) (Chaires, 1996). The ΔG_{pe} values reported in Table 1 indicate that both drugs **1** and **2** bind to DNA with a favorable polyelectrolyte contribution, although the magnitude of the contribution is greater for **2**, as expected. The values of ΔG_t are the same for **1** and **2** and are the major contributors to ΔG_{obs}. These values indicated that the DNA binding of both **1** and **2** is stabilized primarily by molecular interactions other than the polyelectrolyte effect, such as van der Waals interactions and, possibly, hydrogen-bonding interactions. Dissection of the observed binding free energy reveals that the greater affinity of **2** for DNA arises solely from the more favorable polyelectrolyte contribution resulting from the addition of the charged amine group. Binding studies also were done at pH 5.0 to test whether more acidic conditions might more fully protonate compound **2** and increase its charge. That proved to be the case. As Table 1 shows, the binding constant for **2** is higher at pH 5 than at pH 7. Figure 5 shows that the slope SK is greater at pH 5 than at pH 7, consistent with the greater net charge on the molecule resulting from full protonation.

Sequence-Selective Binding to DNA. DNase I footprinting experiments were performed using two restriction fragments from the plasmid pBS: the 117-mer and 165-mer *Eco*RI-*Pvu*II fragments radiolabeled at the 3′ end at the *Eco*RI site. As shown in Fig. 6, the main footprint detected with the 117-mer corresponds to a GC-rich sequence around nucleotide position 70. The intensity of the footprint is more

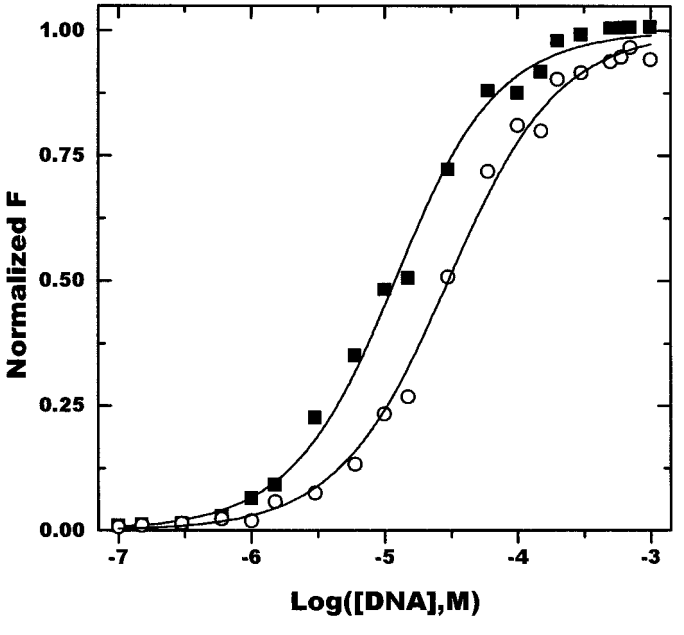

Fig. 4. Fluorescence titrations for the interaction of compounds (B) **1** and (E) **2** with calf thymus DNA. The normalized fluorescence response, $\theta = F - F_0/F_b - F_0$, is shown as a function of total DNA concentration. The concentration of ligand was kept constant at 5 μM whereas the DNA concentration varied between 1 mM and 0.1 μM bp. Curve fitting and determination of binding constants (Table 1) were carried out by using nonlinear least-squares analysis, as described in the text. The solid lines show the best-fit curves to the binding model described in the text.

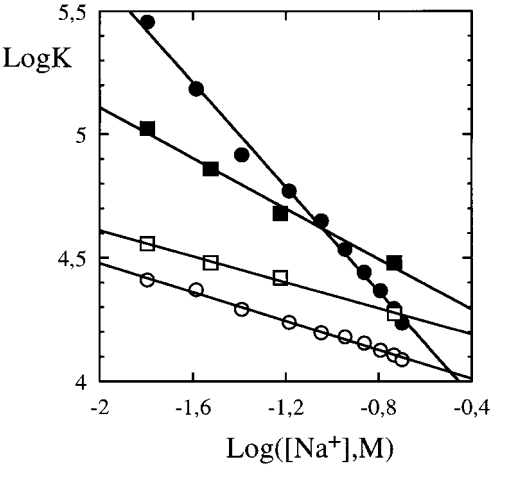

Fig. 5. Variation of the binding constant (K_a) for the interaction of compounds (○, □) **1** and (●, ■) **2** with calf thymus DNA as a function of the salt concentration. The values for the slopes ($-\delta \log K/\delta \log [Na^+]$) are given in Table 1. ●, ○: pH 5.0; ■, □: pH 7.0.

pronounced with compound **2** than with compound **1**. The same observation was made at the binding site 5′-CCTCAC with the 265-bp fragment (not shown). The data are entirely consistent with the results described recently in a detailed footprinting study with another rebeccamycin analog (Bailly et al., 1998a). Binding occurs preferentially at sequences containing GC or GT sites. The introduction of the amino group on the sugar residue enables tighter interactions at GY-containing binding sites (Y═T or C).

Topoisomerase I Inhibition. The topoisomerase I inhibitory properties of compounds **1** and **2** were examined using the ^{32}P-labeled *Eco*RI-*Hin*dIII restriction fragment of pBR322 as a substrate. The labeled DNA fragment was incubated with topoisomerase I in the presence and absence of the indolocarbazoles at concentrations ranging from 0.01 to 10 μg/ml, and the resulting DNA cleavage products were analyzed by agarose gel electrophoresis under alkaline conditions. The two drugs stimulated DNA cleavage with the same efficacy. In both cases, we determined a minimum inhibitory concentration of 0.1 μg/ml (0.18 ± 1 μM, Table 2).

TABLE 1
Binding constants and energetics of the drugs binding to DNA

Compound	pH 7.0		pH 5.0	
	1	**2**	**1**	**2**
$K/10^4$ (M^{-1})	3.6	10.6	2.8	31.8
$-\Delta G^{obs}$ (kcal/mol)	6.1	6.7	6.0	7.4
$-\delta$ log K/δ log[Na^+]	0.26	0.50	0.29	1.06
$-\Delta G_{pe}$ (kcal/mol)	0.6	1.2	0.7	2.5
$-\Delta G_t$ (kcal/mol)	5.5	5.5	5.3	4.9

Binding constant (K) and standard free energy changes (ΔG^{obs}) refer to solution conditions of 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, and 1 mM Na_2EDTA, pH 7.0, at 20°C. The polyelectrolyte contribution to the standard free energy change was calculated from the relationship ΔG_{pe} = (SK) RT ln [NaCl], where SK = δ log K/δ log[Na^+]. The thermodynamic free energy change was calculated by difference $\Delta G_t = \Delta G^{obs} - \Delta G_{pe}$.

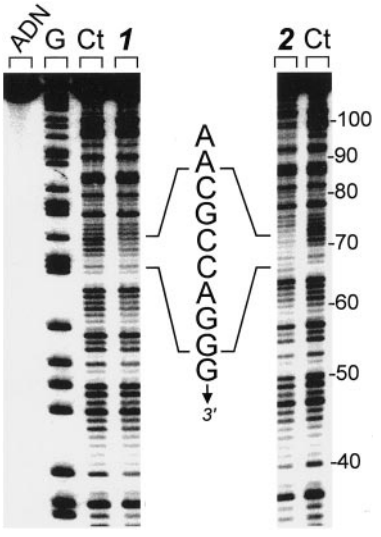

Fig. 6. DNase I footprinting with the 117-mer *Pvu*II-*Eco*RI restriction fragment of plasmid pBS in the presence of the two drugs at the indicated concentration (μM). The DNA was 3′ end-labeled at the *Eco*RI site with [α-^{32}P]dATP in the presence of AMV reverse transcriptase. The products of nuclease digestion were resolved on an 8% polyacrylamide gel containing 7 M urea. Control tracks (Ct) contained no drug. Guanine-specific sequence markers obtained by treatment of the DNA with dimethyl sulfate followed by piperidine were run in the lane marked G. Numbers on the right side of the gel refer to the standard numbering scheme for the nucleotide sequence of the DNA fragment. The sequence of a preferential binding site is indicated between the two panels.

To confirm this observation, a few topoisomerase I cleavage sites were sequenced using the restriction fragments used for the footprinting experiments. A typical example of a gel is shown in Fig. 7. Little or no significant differences were observed between the two drugs. In both cases, the position and the relative intensity of the cleavage sites are almost identical. The main cleavage sites correspond to TpG steps, in agreement with the known specificity of rebeccamycin analog (Bailly et al., 1997). We concluded that the OH→NH_2 substitution has little or no effect on the poisoning of topoisomerase I. This observation is consistent with the model for the interaction of the drug with the enzyme-DNA complex (see *Discussion*).

Cytotoxicity. The in vitro antiproliferative activity of compounds **1** and **2** was examined using the P388 leukemia cell line. The two drugs are more or less equally toxic, with IC_{50} values of 0.3 to 0.45 μg/ml (0.5–0.8 μM). The possibility that the cytotoxicity of the drugs is attributable to their action on topoisomerase I prompted us to evaluate their toxicities toward P388CPT5 leukemia cells resistant to the topoisomerase I inhibitor camptothecin (Table 2). The resistance of the P388CPT5 cell line has been attributed to the expression of a deficient form of topoisomerase I as a result of a mutation in the *top1* gene of these cells (Madelaine et al., 1993). The two drugs are much more toxic against P388 cells than to the resistant cells (Table 2), suggesting that the toxicity is, at least partially, linked to topoisomerase I inhibition. Identical resistance indexes (i.e., the ratio between IC_{50} P388CPT5 and IC_{50} P388) were calculated for compounds **1** and **2**.

Discussion

Considerable interest has been devoted by our laboratories to the study of correlations between the molecular structure and biochemical and/or biological activities of indolocarbazole drugs to obtain information at the molecular level that may be relevant to the proper design of chemotherapeutically more effective drugs (Rodrigues-Pereira et al., 1996; Anizon et al., 1997, 1998; Bailly et al., 1997, 1998a; Prudhomme, 1997; Moreau et al., 1998). The present approach has been centered on the addition of an amine group at position 2′ (modifications at 3′ and 4′ positions currently are being investigated). All of the DNA-binding data indicate that the replacement of the initial hydroxyl group with an amino group enhances the interaction of the drug with the DNA double helix. The affinity is increased by more than three times, and the sequence specificity is more pronounced.

The thermodynamic data summarized in Table 1 show that compound **2** binds to DNA with a free energy that is 0.6 kcal/mol more favorable than compound **1** at neutral pH. By

TABLE 2
Cytotoxicity and topoisomerase I inhibition properties

Compound	Topo I,[a] MIC	P388,[b] IC_{50}	P388/CPT5,[b] IC_{50}	RI[c]
		μM		
1	0.191	0.57	>25	>30
2	0.186	0.84	>25	>30

[a] Minimum inhibitory concentration producing a detectable cleavage of DNA by topoisomerase I (Topo I).
[b] P388 and P388/CPT5 leukemia cells are sensitive and resistant to camptothecin, respectively.
[c] The resistance index (RI) refers to the ratio of $IC_{50}^{P388/CPT5}/IC_{50}^{P388}$.

parsing the binding free energy into its polyelectrolyte and nonpolyelectrolyte components, the origin of the difference is revealed to be solely due to the ΔG_{pe} term. The more favorable binding free energy results only from the introduction of the charged amine group. That the ΔG_t is the same for **1** and **2** signifies that all other molecular interactions for DNA binding are essentially the same for the two compounds and that introduction of the amine neither hinders nor helps stabilize the complex in any other way apart from the polyelectrolyte effect. These findings reinforce a simple design principle for DNA-binding agents. Binding affinity may be enhanced simply by the addition of a positively charged group, which need not participate in any specific molecular interactions.

The results described here for the indolocarbazoles provide an interesting comparison with a pair of anthracycline antibiotics, doxorubicin (Adriamycin) and hydroxyrubicin (3′-deamino-doxorubicin) (Chaires et al., 1993, 1996). Doxorubicin contains an amine group (pKa = 8.4) at the 3′ position of the daunosamine moiety, whereas hydroxyrubicin has a hydroxyl group at that position. Doxorubicin is charged, whereas hydroxyrubicin is uncharged. In that case, the observed binding free energies were found to differ by more than 2.5 kcal/mol, a much greater difference than observed here for compounds **1** and **2.** Both ΔG_{pe} and ΔG_t were found to differ for doxorubicin compared with hydroxyrubicin, in contrast to the behavior observed for compounds **1** and **2** reported here. For the anthracyclines, loss of the charged amine resulted not only in a decrease in the polyelectrolyte free energy contribution, but also in the loss of free energy due to other types of molecular interactions. For the anthracyclines, the 3′ amine group lies deep in the minor groove and has been observed in some crystal structures to participate in hydrogen bond interactions with the DNA bases. Hydroxyrubicin would be unable to form such bonds. The interesting contrast in the DNA-binding thermodynamics between the anthracyclines and the indolocarbazole reflects the difference in the ability of their respective amine groups to interact with DNA. In both cases, the charged amine contributes to the nonspecific polyelectrolyte free energy, but for the indolocarbazoles, the amine evidently does not participate in any additional molecular interactions to stabilize the complex. Perhaps, also, the orientation of the amine is not favorable to enable the formation of hydrogen bonds with the DNA bases.

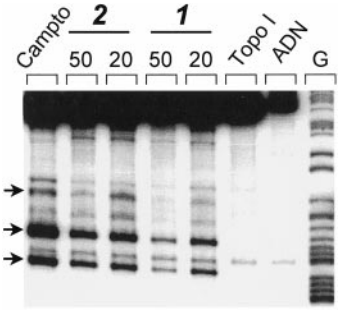

Fig. 7. Sequencing of drug-induced topoisomerase I cleavage sites. The 265-mer *Eco*RI-*Ava*I fragment from plasmid pBS was 3′ end-labeled at the *Eco*RI site with [α-^{32}P]dATP in the presence of AMV reverse transcriptase. The DNA was subjected to cleavage by human topoisomerase I in the presence of the drugs (20–50 μM, as indicated). Cleavage products were resolved on an 8% polyacrylamide gel containing 7 M urea. Guanine-specific sequence markers obtained by treatment of the DNA with dimethyl sulfate followed by piperidine were run in the lane marked G. The control track (DNA) contained no drug and no enzyme. The lane Topo I refers to the radiolabeled DNA substrate incubated with the enzyme but with no drug. Camptothecin was used at 20 μM (lane Campto). The positions of the three main topoisomerase I cleavage sites are indicated by arrows.

It is of interest that the slope ($-\delta \log K/\delta \log [Na^+]$) is less for compound **2** than expected for a DNA-binding ligand with a single, positive charge. One possible explanation is that the amine compound is only partially charged at neutral pH, so that the interaction with the negatively charged polymer is not as high as expected. This hypothesis seems plausible for several reasons. With anthracyclines, the pKa of the amino group of the daunosamine is 8.4 (Frezard and Garnier-Suillerot, 1990) and, by analogy, we anticipated that the pKa of the 2′-amino function of the amino compound **2** would be close to this value, or at least >8.0. However, preliminary UV analysis suggests that the pKa is below 8.0, probably around 7.8 (spectra not shown). It is therefore possible that the amine is not entirely protonated at pH. 7.0. The results of the experiments performed at pH 5.0 and the salt-dependence binding analysis also support this idea. According to the theory of Record et al. (1978), the slope SK of the curve in Fig. 5 is related to the charge (Z) on the ligand. The equation is:

$$(\delta \log K/\delta \log[Na+]) = -Z\Psi \equiv SK \qquad (3)$$

Ψ represents the average fraction of monovalent cation associated with each phosphate group of DNA. For B-DNA, Ψ = 0.88, meaning that the double helix retains a net charge corresponding to 12% of the total number of phosphates (see Record et al., 1978 and Chaires, 1996 for detailed explanations of the theory). Using eq. 3 we calculated that for the amino compound, Z = 0.56, suggesting that only about 50 to 60% of the molecules are positively charged under the experimental conditions used in this study at neutral pH. Upon reading carefully the literature on anthracycline derivatives, we noted that the pKa of the amino sugar group can vary significantly depending on the nature of the adjacent substituents. For example, the pKa of the amine of doxorubicin drops from 8.4 to 6.4 when an iodine atom is introduced at position 4′ (Cera and Palumbo, 1991). The hypothesis that the amino compound is partially charged at physiological pH is valid and consistent with the modest dependence of K upon

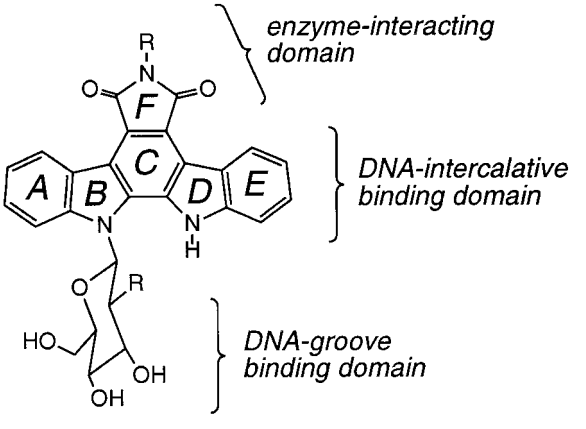

Fig. 8. Putative functional domains of rebeccamycin drugs. The planar indolocarbazole chromophore can intercalate into DNA whereas the carbohydrate residue contacts externally the DNA in the (minor) groove. Previous studies suggested that the bis-imide F ring and its substituent play a direct role in the interaction with topoisomerase I (Bailly et al., 1997).

ionic strength and increased affinity of the drug by a factor of only 3.4. It is also consistent with the significant increase in DNA-binding affinity under acidic conditions. This point deserves one further comment related to cell uptake. Addition of the amino group increases considerably the hydrophilicity of the molecule, thus facilitating the handling of the drug. However, it does not much improve the toxicity, perhaps because the cellular uptake is reduced. Once again it is worthwhile to refer to studies with anthracyclines, which have shown that, by the removal of the amino sugar group, the drug becomes more lipophilic, favoring therefore the cellular uptake by passive diffusion (Capranico et al., 1994). That the pKa of the amino compound could be around 7.8 instead of being >8.0 might be of interest from a therapeutic point of view. First, it may favor action on tumor cells rather than normal cells. High lactate production is believed to lower the pH of the cytoplasm of tumor cells compared with normal cells (Di Marco et al., 1977). Second, drug-induced apoptosis can decrease the pH of the treated cells. This effect has been detected with the topoisomerase II inhibitor etoposide (Barry et al., 1993), and very recently we have seen the same effect with topoisomerase I inhibitors such as camptothecin and rebeccamycin analogs (manuscript in preparation).

On the basis of previous studies using series of rebeccamycin analogs with or without a glycosyl residue, and also from a molecular modeling analysis, three functional domains of rebeccamycin-type drugs can be identified (Fig. 8). As indicated previously (Bailly et al., 1997), the insertion of the planar indolocarbazole chromophore between two consecutive base pairs places the appended sugar residue into the groove of the double helix, most likely the minor groove. The glycosyl residue can engage contacts with the base pair below the intercalation site. In the minor groove orientation, the 2′ substituent may form an H bond with the carbonyl group at position 2 of a pyrimidine when the drug intercalates at a GpY site. In this case, it is plausible that, for steric reasons, an amino group is more favorable than an OH group for the formation of the H bond. According to this molecular arrangement, the imide nitrogen on the F ring is supposed to protrude toward the opposite groove, where it can interact with topoisomerase I.

In conclusion, the present study indicates that the introduction of an amino group on the glycosyl residue of rebeccamycin contributes to a tighter interaction with DNA and does not prevent the drug from inhibiting topoisomerase I. It will be of interest to extend the carbohydrate domain to further reinforce the interaction with DNA and possibly to target longer sequences in DNA. By analogy with antitumor drugs like calicheamycin, bleomycin, and anthracycline, antibiotics bearing di- or trisaccharide side chains (e.g., betaclamycin A and ditrisarubicin B), we have initiated the synthesis of novel rebeccamycin analogs equipped with aminooligosaccharide side chain.

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