Synthesis, Mode of Action, and Biological Activities of Rebeccamycin Bromo Derivatives

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Bromo analogues of the natural metabolite rebeccamycin with and without a methyl substituent on the imide nitrogen were synthesized. The effects of the drugs on protein kinase C, the binding to DNA, and the effect on topoisomerase I were determined. The drugs' uptake and their antiproliferative activities against P388 leukemia cells sensitive and resistant to camptothecin, their antimicrobial activity against a Gram-positive bacterium (*B. cereus*), and their anti-HIV-1 activity were measured and compared to those of the chlorinated and dechlorinated analogues. Dibrominated imide **5** shows a remarkable activity against topoisomerase I, affecting both the kinase and DNA cleavage activity of the enzyme. The marked cytotoxic potency of this compound depends essentially on its capacity to inhibit topoisomerase I.

Introduction

DNA topoisomerase I is a nuclear enzyme that controls the topological state of DNA and is implicated in various processes including replication, transcription, and recombination.[1,2] Topoisomerase I catalyzes unwinding of supercoiled DNA by nicking and rejoining a single strand of DNA.[3] Topoisomerase I-targeting drugs interact with the breakage−rejoining reaction between DNA and topoisomerase I referred to as a cleavable complex.[4,5] More recently, it has been shown that DNA topoisomerase I, isolated from different multicellular organisms, bears a protein kinase activity capable of phosphorylating splicing factors of the SR protein family.[6−9] This observation implies that DNA topoisomerase I is a dual enzyme with a DNA cleavage site juxtaposed to a functionally independent kinase site. In addition, DNA topoisomerase I is an important cellular target for clinically useful antitumor drugs such as derivatives of the plant alkaloid camptothecin: topotecan and irinotecan.[10,11] Indolocarbazole compounds including rebeccamycin (**1**) (Chart 1) and BE-13793C derivatives are a new family of topoisomerase I inhibitors.[12−15] Among them, the semisynthetic NB-506, ED-749, and NSC 655649 inhibit the growth of various human tumors and are now undergoing clinical trials.

In previous structure−activity relationship studies on rebeccamycin analogues, we observed that the functionality of the imide heterocycle can be modified (amide, anhydride) without abolishing the enzyme inhibitory potency.[16] Whereas a substitution on the second indole nitrogen is detrimental,[17] a substitution with a small group on the imide nitrogen does not affect significantly the activity of the drugs.[17−19] The sugar moiety linked to one of the indole nitrogens is a key element for both DNA binding and topoisomerase I inhibition.[17,20] Since the inhibition of topoisomerase I arises in part from the ability of the compounds to interact with DNA, various substituents were introduced in order to reinforce the interaction with DNA via a covalent bond. Chloro or bromoacetyl substituents, such as those fixed on the antibiotic distamycin to induce covalent binding to DNA,[21] were introduced either on the sugar moiety or on the imide nitrogen,[22] but these groups were not sufficiently reactive to induce covalent binding to DNA. A tight interaction with either DNA or topoisomerase I could be obtained by photochemical cross-linking. Aryl azides, aryl diazonium salts, diazirines leading to carbenes upon irradiation, and aryl halides have been largely used for their efficient photocoupling properties to proteins.[23,24] Rebeccamycin itself with its two chlorine atoms could be considered as a candidate for photochemical cross-linking. Dibromo derivatives in positions 3 and 9 on the indole moieties could also induce covalent bonds upon irradiation because of less bulkiness near these positions.

Moreover we have previously observed that the two chlorine atoms were detrimental to the interaction with DNA and to the topoisomerase I inhibition but not to the cytotoxicity. The chlorinated compounds were generally more active on cells than the dechlorinated analogues probably because of their better penetration in cells. These considerations prompted us to prepare derivatives of rebeccamycin brominated in positions 3

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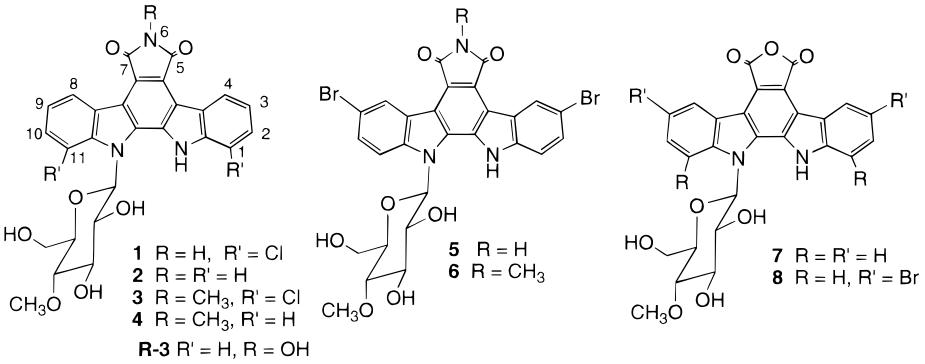
Chart 1



and 9 and to compare their biological activities to those of chlorinated and dechlorinated analogues (Chart 1).

Results and Discussion

Chemistry. The preparation of dechlorinated rebeccamycin **2** by catalytic hydrogenolysis of rebeccamycin on palladium on carbon was previously described.[16] Anhydride **7** was prepared from rebeccamycin by hydrogenolysis using Raney nickel in aqueous sodium hydroxide. Compound **8** was prepared from anhydride **7** by bromination with *N*-bromosuccinimide.[16] Dibromo imide **5** was obtained by treatment of anhydride **8** with ammonium acetate at 190 °C by application of the method used for the aglycones.[25] *N*-Methyl dibromo imide **6** was prepared from anhydride **8** by reaction with a solution of methylamine in THF at 70 °C in a sealed tube.

Protein Kinase C (PKC) Inhibition. Since structurally related antibiotics staurosporine and UCN-01 are well-known PKC inhibitors,[26,27] we investigated the PKC inhibitory properties of compounds **1**−**8**. The inhibitory properties toward PKC-α were tested using protamine sulfate as a substrate. The IC_{50} values for all the compounds described in Chart 1 were found to be >100 μM. As previously observed, the sugar unit linked to one of the indole nitrogens yields generally inactivity toward PKC.

Topoisomerase I/Kinase Inhibition. We have shown recently that an indolocarbazole derivative, the ***R*-3** compound (Chart 1), which is a close cousin of staurosporine but has no significant effect on PKC, can interfere with both kinase and relaxation activities of topoisomerase I. To seek for compounds that might turn out to be potent topoisomerase inhibitors endowed with interesting and possibly selective antitumor properties with a different pharmacological profile than camptothecins, we have compared the capacity of several other indolocarbazole derivatives to inhibit kinase, topoisomerase, or both activities. Kinase activity of DNA topoisomerase I was detected by its ability to phosphorylate bacterially expressed recombinant SF2/ASF, a member of the SR protein family,[28] with [γ-^{32}P]ATP. The results in Figure 1 show that the phosphorylation of SF2/ASF by topoisomerase I is markedly inhibited in the presence of dibrominated imide **5**. A concentration less than 1 μM suffices to reduce the kinase activity of topoisomerase I by 90%, and an equivalent drug concentration was shown to stabilize topoisomerase−DNA cleavable complexes (see below). Therefore the compound is equivalently potent at promoting the DNA cleavage and at inhibiting the kinase reactions of topoisomerase I. Inhibition of topo I/kinase activity was also observed with camptothecin[7] but required the presence of DNA. No inhibition was observed with camptothecin in the absence of DNA even when using a concentration as high as 500 μM. In sharp contrast, the strong inhibitory effect of compound **5** does not require DNA, implying that its action on the kinase activity is independent of topoisomerase I−DNA covalent complexes.

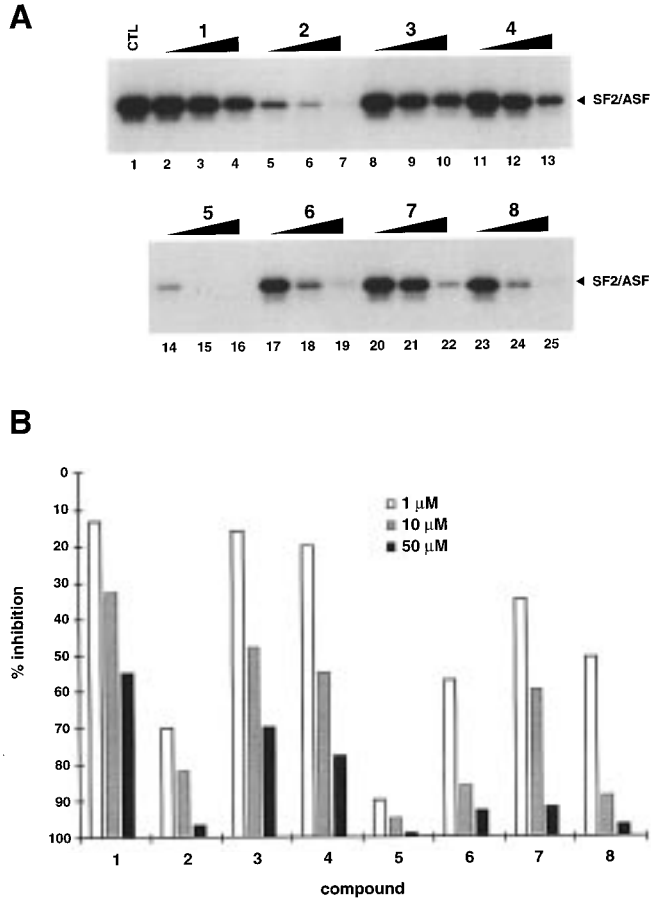


Figure 1. Inhibition of topoisomerase I kinase activity by derivatives of rebeccamycin. The kinase reactions were performed as described (Experimental Section) with 1, 10, or 50 μM of the indicated compounds. CTL (lane 1) refers to the control reaction with DMSO alone in place of rebeccamycin. The labeled SF2/ASF was analyzed on a 12% SDS−polyacrylamide gel and either revealed by autoradiography (A) or quantitated by phosphorimaging (B).

Neither rebeccamycin (**1**) nor compound **3** had significant inhibitory effect, implying that chlorine atoms

at positions 1 and 11 of the indole heterocycle are detrimental for the derivatives to act as powerful inhibitors of topo I/kinase. In agreement with this expectation, dechlorinated analogue **2** was as efficient as compound **5** to inhibit topo I/kinase. Methylation of the imide nitrogen also leads to compounds with less inhibitory potency, as demonstrated by compound **4** which has a slight inhibitory effect at a concentration of 50 μM, despite the fact that its differs from compound **2** just by the methyl group. Also compound **6** was 10 times less active than compound **5**. Additionally, compounds **7** and **8** with anhydride heterocycle have weak inhibitory potency compared to compounds **2** and **5** with imide nitrogen, respectively. Since compounds **1**−**8** are inactive toward protein kinase C, it can be concluded that the kinase site of topoisomerase I is different from the ATP-binding site of protein kinase C with which the indolocarbazole compounds usually interact.

Inhibition of Topoisomerase I-Mediated DNA Relaxation and Cleavage. Two complementary assays were employed to evaluate the topoisomerase I inhibitory properties of the drugs. First we ressorted to a relaxation assay. Negatively supercoiled plasmid pKMp27 was incubated with human topoisomerase I with and without the test drug (Figure 2). The DNA samples were treated with SDS and proteinase K to remove any covalently bound protein and resolved in a 1% agarose gel. The gel shown in Figure 2A indicates that the drugs at 20 μM do not unwind DNA. With compound **5**, the intensity of the band with the slowest migration in the gel (corresponding to nicked + fully relaxed DNA) is increased significantly. The relaxation experiments were repeated using ethidium-prestained agarose gels in order to differentiate the specific effect (e.g., stabilization of topoisomerase I−DNA cleavable complexes) and the nonspecific effect (e.g., the drug may prevent the enzyme from binding to DNA). In a recent study we showed that a nonspecific effect due to template binding can occur with indolocarbazoles.[29] In the presence of ethidium bromide nicked DNA and relaxed (close circular) DNA are easily separated (Figure 2B). Here again it can be seen very clearly that the level of nicked DNA molecules is considerably increased in the presence of compound **5**. Under identical conditions, the effect with **5** is more pronounced than with camptothecin. No effect was detected with compounds **6** and **8** (Figure 2C).

Second we studied DNA cleavage by calf thymus topoisomerase I 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 with and without the drugs at concentrations ranging from 0.01 to 10 μg/mL, and the resulting cleavage products were analyzed by agarose gel electrophoresis. The inhibitory potency (MIC values) of the tested compounds are reported in Table 2; the MIC value for camptothecin is given as a reference. Among all the rebeccamycin analogues we have synthesized thus far, dibrominated imide **5** has the strongest inhibitory potency toward topoisomerase I. Surprisingly, the analogue **6**, N-methylated on the imide nitrogen, was inactive, whereas compounds **2** and **4** were equally efficient against topoisomerase I. The inhibitory potencies of anhydride **7** and dibrominated anhydride **8** are in the same range.

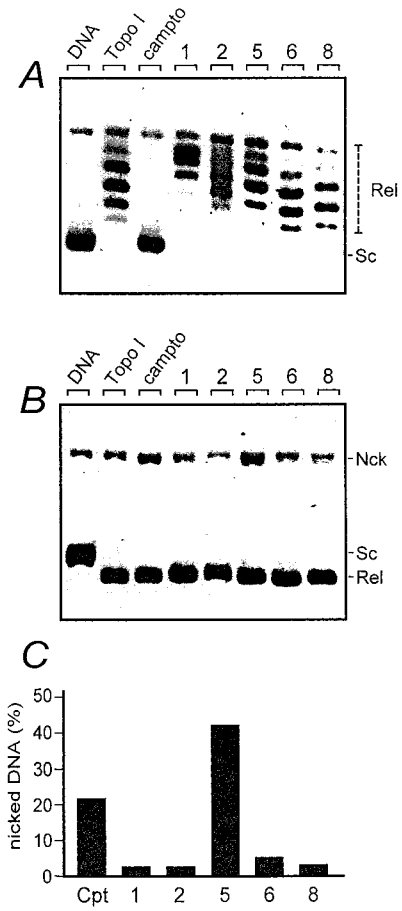


Figure 2. Effect of the drugs on the relaxation of plasmid DNA by topoisomerase I. Native supercoiled pKMp27 DNA (0.5 μg) (lane DNA) was incubated with 6 units of human topoisomerase I in the absence (lane Topo I) or presence of the indicated drug at 20 μM. Reactions were stopped with sodium dodecyl sulfate and treatment with proteinase K. DNA samples were separated by electrophoresis on an agarose gel (A) without ethidium bromide or (B) containing ethidium bromide. Gels were photographed under UV light. Nck, nicked; Rel, relaxed; Sc, supercoiled. (C) Comparison of the topoisomerase I-mediated cleavage efficiency of the different drugs. Data were compiled from quantitative analysis of three gels as the one shown in panel B and must be considered as a set of average values.

Table 1. Variation in Melting Temperature (ΔT_m, °C)[a]

	compound				
	1	**2**	**5**	**6**	**8**
poly(dA-dT)·(dA-dT)	0.1	6.1	3.8	0.4	0.1
calf thymus DNA	0.2	4.0	3.0	0.0	0.3

[a] 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 20 μM nucleic acids and 20 μM drug in 3-mL quartz cells at 260 nm with a heating rate of 1 °C/min. Each drug was tested in triplicate, and the results were averaged.

Binding to DNA. The ability of the drugs to alter the thermal denaturation profile of DNA was used as an indication of their propensity to bind to DNA. We measured the change of the absorbance at 260 nm as a function of the temperature for calf thymus DNA (which contains roughly equal proportions of A·T and G·C base pairs) and the synthetic polynucleotide poly(dA-dT)·(dA-dT) with and without the drugs (Figure 3). In all cases,

Table 2. Inhibitory Activities toward Topoisomerase I, Antiproliferative Activities in Vitro against Murine P388 Leukemia Cells and P388CPT5 Cells Resistant to Topoisomerase I Inhibitor Camptothecin, Antimicrobial Activities against *B. Cereus*, and Anti-HIV-1 Lai-Infected CEM-SS Cells[a]

	IC_{50} (μM)			*B. cereus*	HIV-1 Lai CEM-SS			topo I
compd	P388	P388CPT5	*R*	MIC (μM)	IC_{50} (μM)	CC_{50} (μM)	SI	MIC (μM)
CPT	0.032	2.8	87.5					0.086
1	1.22	10.5	8.5	10.9	0.52	1.05	2.0	1.75
2	0.69	>20	>28	>100	0.16	0.90	5.6	0.59
3	0.60			>85	4.9	8.0	1.6	>17
4	0.67	nd	nd	1.55	4.0	7.9	2.0	0.58
5	0.26	>15	>58	19	0.07	0.34	4.8	0.15
6	4.9	>15	>3.0	>74	1.17	1.49	1.3	>15
7	6.36	>20	>3.1	>99	>1.98		>1.98	1.99
8	0.91	7.58	8.3	>75	4.85	9.39	1.9	1.52

[a] *R*, IC_{50}(P388CPT5)/IC_{50}(P388); selectivity index (SI), CC_{50}/IC_{50}; CPT, camptothecin.

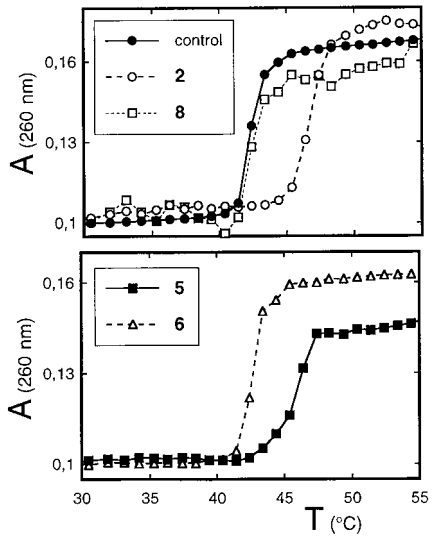


Figure 3. Representative thermal denaturation curves for poly(dA-dT)·(dA-dT) in the absence and presence of the drugs (2 μM each). The "melting" temperature T_m was taken as the midpoint of the hyperchromic transition determined from first-derivative plots.

monophasic melting curves were observed. The variations of the T_m (ΔT_m) of helix-to-coil transition of calf thymus DNA and the polynucleotide are collected in Table 1. The T_m of the nucleic acid is not significantly changed with compounds **6** and **8** as well as with rebeccamycin. The chlorine atoms are detrimental to the interaction with DNA.[20] Although the T_m shift is modest, interestingly compound **5** has a much greater effect on the T_m of both calf thymus DNA and poly(dA-dT)·(dA-dT) than analogues **6** and **8**. The effect of dibrominated imide **5** is comparable to that of dechlorinated rebeccamycin. The interaction of **2** and **5** with DNA is believed to represent a necessary event for the inhibition of topoisomerase I by these drugs.

In Vitro Antiproliferative Activity. The antiproliferative activities were tested in vitro against murine P388 leukemia cells. IC_{50} values are reported in Table 2. To have an insight into the involvement of topoisomerase I inhibition in the cytotoxicity, the toxicities of compounds **1**−**8** toward P388 CPT cells resistant to the topoisomerase I inhibitor camptothecin were also evaluated. The values for camptothecin are given as references. As expected from its strong inhibitory potency toward topoisomerase I, dibrominated imide **5** was the most cytotoxic compound against P388 leukemia cells. The large resistance index (>58) indicates a significant contribution of topoisomerase I inhibition to the cytotoxicity of this dibrominated analogue **5**.

Drug Binding to Cells. The intrinsic fluorescence of an indolocarbazole derivative is relatively weak in aqueous solution, but the fluorescence emission increases significantly when the drug is in a hydrophobic environment such as the cell membranes. Therefore, fluorescence spectroscopy provides a useful means to compare the interaction of the test drugs with living cells. In the presence of P388 leukemia cells, the fluorescence emission of rebeccamycin at 530 nm increases by about 30%. The fluorescence decreases when the drug-bound cell population is washed with a drug-free buffer, but the fluorescence of the cell pellet remains centered at 530 nm (Figure 4). The situation is markedly different with dechlorinated rebeccamycin. As indicated in Table 3, the fluorescence of **2** decreases by 26% in the presence of the cells and the maximum emission of the drug-bound whole cell population or the cell pellet obtained after washing the unbound ligand is shifted by 24 nm to a much lower wavelength (from 574 to 550 nm) compared to the spectrum of the free drug (Table 3). The behavior of the brominated imide analogues **5** and **6** is intermediate between that of rebeccamycin and its dechlorinated analogue **2**. With compound **5**, the fluorescence of the free drug increases considerably in the presence of P388 cells, as observed with rebeccamycin, but the emission maximum is shifted to the blue, as is the case with **2** (Figure 4). Similar spectra were recorded with **6**. The very weak fluorescence of anhydride **8** could not be accurately measured. The fluorescence data corroborate the idea that chlorine atoms of rebeccamycin facilitate the interaction of the antibiotic with the cell membranes. Recent experiments with a ^{3}H-labeled derivative of rebeccamycin confirm this view.[30] Moreover, the results suggest that in terms of cellular uptake, the newly introduced bromine atoms of compound **5** mimic the chlorine atoms of **1** facilitating the interaction of the drug with the cells. Although variations in fluorescence intensities must be analyzed with caution, it seems clear that compounds **5** and **6** have similar cell binding capacities. Therefore their very distinct biological activities cannot be explained in terms of different cellular uptake. The different biological activities likely arise from their distinct action on topoisomerase I.

Anti-HIV-1 Activity. Since topoisomerase I is known to activate HIV-1 reverse transcriptase activity, the anti-HIV-1 activity of compounds **1**−**8** was tested. The anti-HIV-1 activity in CEM-SS cells was measured by

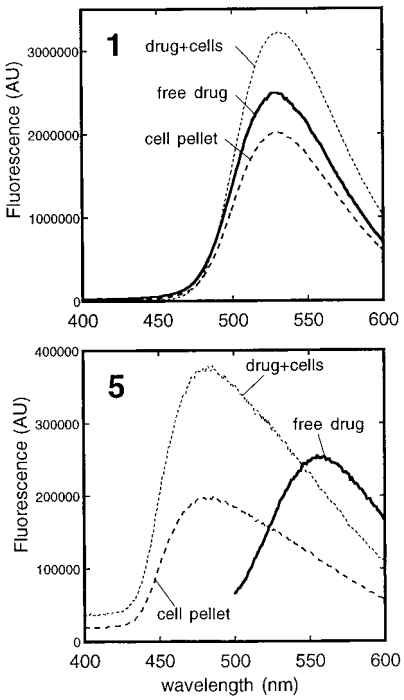


Figure 4. Fluorescence spectra of (top panel) rebeccamycin and (bottom panel) dibrominated imide **5** in the absence (solid line) and presence of P388 leukemia cells. Spectra were recorded with an excitation at 320 nm. The spectrum of the total cell population (dotted line) was obtained after incubating 1.5×10^6 cells with the drug at 100 μM for 30 min in 1 mL of Hepes buffer saline solution (HBSS). Cells were then pelleted by centrifugation and washed once with 5 mL of HBSS buffer prior to resuspending in 1 mL of drug-free buffer. The spectrum of the cell pellet (dashed line) was measured about 10 min after the spectrum corresponding to the whole cell population.

Table 3. Drug Binding to P388 Leukemia Cells

compd	free drug	drug-bound whole cells[b]	fraction change (%)	drug bound to cell pellet[c]
1	1[a] (530)[d]	1.29 (527)	+29	0.81
2	0.51 (574)	0.38 (550)	−26	0.33
5	0.12 (556)	0.15 (482)	+46	0.08
6	0.41 (570)	0.46 (490)	+13	0.24

[a] Relative fluorescence intensities are indicated with reference to that of free rebeccamycin with an excitation at 320 nm. The test drug was incubated with 1.5×10^6 P388 cells for 30 min at 37 °C prior to centrifugation. The fluorescence spectrum of the drug was recorded. [b] Before. [c] After centrifugation. [d] $\lambda_{em}{}^{max}$, in nm.

quantification of the reverse transcriptase activity associated with virus particles released from HIV-1-infected cells in the culture medium. The cytotoxicity of the drugs (CC_{50}) was evaluated in parallel to the IC_{50} values, and the selectivity index ($SI = CC_{50}/IC_{50}$) was calculated (Table 2). Compound **5** exhibited an interesting anti-HIV-1 activity; however the selectivity index is not sufficient to consider it as an anti-HIV-1 drug.

Antimicrobial Property. The antimicrobial activities against a Gram-positive bacterium, *Bacillus cereus*, were determined. The MIC values are reported in Table 2. Of the dibrominated compounds tested in this study, compound **5** was the most active against *B. cereus*.

Conclusion

Dibrominated imide **5** has very interesting effects on topoisomerase I. Not only does this compound act as a typical topoisomerase I poison like camptothecin, but in addition it strongly inhibits the kinase activity of the enzyme even in the absence of DNA. The effect is specific to topoisomerase I: there was absolutely no effect on PKC. The comparison of the cytotoxicity profiles with P388 leukemia cells sensitive and resistant to camptothecin leaves no room for doubt that topoisomerase I inhibition plays an important role for the cytotoxicity of this dibrominated analogue. We are now designing rebeccamycin analogues targeting selectively the kinase site without interfering with DNA relaxation activity of the enzyme. Such compounds might turn out to be potent topoisomerase inhibitors endowed with interesting and possibly selective antitumor properties with a different pharmacological profile than the camptothecins. The results reported here encourage us to believe that the indolocarbazole approach has the potential to yield important developments in the search for new and better anticancer drugs. Ongoing drug design will seek to explore this hypothesis.

Experimental Section

Chemistry. 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, abbreviations: singlet (s), doublet (d), doubled doublet (dd), multiplet (m), tertiary carbons (C tert), quaternary carbons (C quat)). The signals were assigned from $^1H-^1H$ COSY, $^{13}C-^1H$ correlations, exhange with D_2O, and inverse gate decoupling. Mass spectra (FAB+) were determined at CESAMO (Talence, France) on a high-resolution Fisons Autospec-Q spectrometer. Chromatographic purifications were performed by Kieselgel 60 (Merck) (0.063−0.200 mm) or Geduran SI 60 (Merck) (0.040−0.063 mm) column chromatography. For purity tests, TLC were performed on fluorescent silica gel plates (60 F_{254} from Merck).

3,9-Dibromo-12-(4-*O*-methyl-β-D-glucopyranosyl)-6,7,12,13-tetrahydroindolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7-dione (5). A mixture of anhydride **8** (243 mg, 0.37 mmol) and ammonium acetate (5.52 g, 72 mmol) was stirred at 190 °C for 3 h, then poured into water, and extracted with EtOAc. The organic phase was washed with water and then dried over $MgSO_4$. After removal of the solvent, the residue was purified by chromatography (eluent Et_2O:toluene:EtOAc, 1:1:8, for the first chromatography; eluent EtOAc for the second chromatography) to give **5** (163 mg, 0.248 mmol, 67% yield) as a yellow solid: mp 270 °C; IR (KBr) ν_{CO} 1710, 1750 cm^{-1}, $\nu_{NH,OH}$ 3100−3600 cm^{-1}; HRMS (FAB+) (M^+) calcd $C_{27}H_{21}N_3O_7Br_2$ 656.9746, found 656.9741; ^1H NMR (400 MHz, DMSO-d_6) 3.52 (1H, t, J = 8.4 Hz), 3.69 (1H, m), 3.72 (3H, s, OCH_3), 3.80 (2H, m), 3.98 (1H, d, J = 10.3 Hz), 4.05 (1H, m), 5.08 (1H, br s, OH), 5.38 (1H, br s, OH), 6.30 (1H, s, OH), 6.33 (1H, d, J = 9.3 Hz, $H_{1'}$), 7.69 (1H, dd, J_1 = 8.9 Hz, J_2 = 1.5 Hz), 7.73 (1H, d, J = 8.4 Hz), 7.78 (1H, dd, J_1 = 8.4 Hz, J_2 = 1.5 Hz), 7.97 (1H, d, J = 9.3 Hz), 9.26 (1H, d, J = 1.5 Hz), 9.30 (1H, d, J = 1.5 Hz), 11.30 (1H, s, N_{imide}-H), 11.80 (1H, s, N_{indole}-H); ^{13}C NMR (100 MHz, DMSO-d_6) 58.5 ($C_{6'}$), 60.1 (OCH_3), 73.2, 73.5, 76.1, 77.2, 84.3 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 112.5, 112.6, 116.1, 117.5, 120.0, 121.6, 122.7, 123.1, 128.5, 129.9, 139.4, 140.9 (C quat arom), 114.1, 114.3, 126.4, 126.5, 129.3, 129.6 (C tert arom), 170.8, 170.9 (C=O).

3,9-Dibromo-12-(4-*O*-methyl-β-D-glucopyranosyl)-6-methyl-6,7,12,13-tetrahydroindolo[2,3-*a*]pyrrolo[3,4-*c*]-carbazole-5,7-dione (6). A solution of 2 M methylamine in THF (3.4 mL) and anhydride **8** (103 mg, 0.16 mmol) was stirred at 70 °C in a sealed tube for 24 h. After cooling, the mixture was poured into water (260 mL). After extraction with

EtOAc, the organic phase was dried over $MgSO_4$, then the solvent was removed, and the residue was purified by chromatography (eluent THF:toluene, 70:30) to give **6** (58 mg, 0.086 mmol, 54% yield) as a yellow solid: mp 275 °C; IR (KBr) ν_{CO} 1700, 1750 cm^{-1}, $\nu_{NH,OH}$ 3100−3600 cm^{-1}; HRMS (FAB+) (M+) calcd for $C_{28}H_{23}N_3O_7Br_2$ 670.9903, found 670.9907; ^1H NMR (400 MHz, DMSO-d_6) 3.19 (3H, s, NCH_3), 3.50 (1H, m), 3.69 (3H, s, OCH_3), 3.77 (2H, m), 3.93 (1H, m), 4.03 (2H, m), 5.07 (1H, d, J = 5.4 Hz, OH), 5.36 (1H, d, J = 5.4 Hz, OH), 6.31 (1H, t, J = 3.9 Hz, OH), 6.35 (1H, d, J = 9.4 Hz, $H_{1'}$), 7.71 (1H, d, J = 9.4 Hz), 7.76 (1H, dd, J_1 = 8.9 Hz, J_2 = 2.0 Hz), 7.80 (1H, dd, J_1 = 8.9 Hz, J_2 = 2.0 Hz), 7.98 (1H, d, J = 9.4 Hz), 9.25 (1H, d, J = 2.0 Hz), 9.30 (1H, d, J = 2.0 Hz), 11.80 (1H, s, NH); ^{13}C NMR (100 MHz, DMSO-d_6) 23.7 (NCH_3), 58.5 ($C_{6'}$), 60.1 (OCH_3), 73.2, 76.1, 77.2 (2C), 84.3 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 112.5, 112.8, 116.1, 117.5, 118.9, 120.5, 122.5, 123.0, 128.3, 129.7, 139.5, 140.9 (C quat arom), 114.0, 114.2, 126.3, 126.4, 129.3, 129.7 (C tert arom), 169.3, 169.4 (C=O).

Protein Kinase C Inhibition. Protamine sulfate was from Merck (Darmstadt, Germany). Unless specified, chemicals were from Sigma (St. Louis, MO). [γ-^{33}P]ATP (1000−3000 Ci/mmol) was obtained from Amersham. Recombinant baculoviruses from protein kinase C subtypes were supplied by Dr. Silvia Stabel, Köln, Germany. Expression and partial purification of PKCs together with measurements of activities were carried out as previously described.[16] Data show IC_{50} values expressed in μM.

Topoisomerase I Kinase Assay. The reaction mixtures for protein kinase activity contained 100 ng of wild-type recombinant topoisomerase I protein, 300 ng of recombinant SF2/ASF protein, 1 μM ATP, 3 μCi [γ-^{32}P]ATP (3000 Ci/mmol), and 1 μL of compounds or DMSO in a final volume of 20 μL of buffer A (50 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 50 mM KCl, 0.5 mM DTT). The samples were incubated at 30 °C for 30 min, mixed with 6 μL of (4X) loading buffer, and appplied to a 12% SDS−polyacrylamide gel. Radioactivity incorporated into SF2/ASF on the dried gel was detected by autoradiography and by a Molecular Dynamics imaging analyzer. The relative amount of [γ-^{32}P]ATP incorporated in each assay was quantitated by densitometry scanning of the gel using ImageQuant software version 3.22.

DNA Relaxation Experiments. Supercoiled pKMp27 DNA (0.5 μg) was incubated with 6 units of human topoisomerase I (TopoGen Inc., Columbus, OH) at 37 °C for 45 min 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 electrophoresis dye mixture (3 μL) and electrophoresed in a 1% agarose gel at room temperature for 3 h. Gels were stained with ethidium bromide (1 mg/mL), washed, and photographed under UV light. Similar experiments were performed using ethidium-containing agarose gels.

Topoisomerase I-Mediated Cleavage of DNA. The procedure previously described was followed.[16] pBR322 DNA (Boehringer 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 calf thymus topoisomerase I (40 units in a 20-μL 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 μL, followed by incubation for 30 min at 50 °C. Samples were denatured by the addition of 10 μL of denaturating loading buffer consisting of 0.45 M NaOH, 30 mM EDTA, 15% (w/v) sucrose, 0.1% bromocresol green prior to loading onto a 1% agarose gel in TBE buffer containing 0.1% SDS. Electrophoresis was conducted at 2 V/cm for 18 h. The MIC values correspond to the lowest concentration (μM) producing a detectable DNA cleavage.

DNA 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 cells (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 cell was measured with a platinum probe; it was increased over the range 20−100 °C with a heating rate of 1 °C/min.

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 previously described.[20] The concentrations of drugs giving 50% of growth inhibition (IC_{50}) were determined.

Drug Binding to Cells. Exponentially growing P388 leukemia cells were incubated with the test drug (usually at 100 μM) in 1 mL of culture medium (1.5 × 10^6 cells). After 30 min of incubation in a CO_2 incubator at 37 °C, cells were pelleted, rinced once with 3 mL of Hepes buffer saline solution (HBSS from Life Science, Cergy-Pontoise, France), and resuspended at an appropriate density for fluorescence measurements. The indolocarbazole chromophore was excited at 320 nm, whereas the emission was measured between 400 and 600 nm (8-nm band-pass filter) using a SPEX fluorolog spectrofluorimeter.

MIC Determination. MIC values of **1**−**8** were determined classically on *B. cereus* ATCC 14579 in Mueller-Hilton broth, pH 7.4 (Difco), after a 24-h incubation at 27 °C. The compounds diluted in DMSO were added to 12 tubes; the concentration range was from 100 to 0.05 μg/mL.

Antiviral HIV-1 Activity. The cultures of CEM-SS cells were maintained at 37 °C in 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% decomplemented fetal bovine serum (FBS). The antiviral HIV-1 activities in CEM-SS cells (IC_{50} and CC_{50}) were measured as previously described.[16]

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