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Semi-Synthesis, Topoisomerase I and Kinases Inhibitory Properties, and Antiproliferative Activities of New Rebeccamycin Derivatives

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Abstract—In the course of structure–activity relationship studies, new rebeccamycin derivatives substituted in 3,9-positions on the indolocarbazole framework, and a 2′,3′-anhydro derivative were prepared by semi-synthesis from rebeccamycin. The antiproliferative activities against nine tumor cell lines were determined and the effect on the cell cycle of murine leukemia L1210 cells was examined. Their DNA binding properties and inhibitory properties toward topoisomerase I and three kinases PKCζ, CDK1/cyclin B, CDK5/p25 and a phosphatase cdc25A were evaluated. The 3,9-dihydroxy derivative is the most efficient compound of this series toward CDK1/cyclin B and CDK5/p25. It is also characterized as a DNA binding topoisomerase I poison. Its broad spectrum of molecular activities likely accounts for its cytotoxic potential. This compound which displays a tumor cell line-selectivity may represent a new lead for subsequent drug design in this series of glycosylated indolocarbazoles.

Introduction

Rebeccamycin is a microbial metabolite isolated from cultures of *Saccharothrix aerocolonigenes*. Its antiproliferative activity is linked, at least in part, to topoisomerase I inhibition.[1,2] Like camptothecin, rebeccamycin is a topoisomerase I poison which stabilizes the topoisomerase I-DNA cleavable complex and prevents the religation of the DNA strand cleaved by topoisomerase I.[3–5] Structure–activity relationship studies has been performed and several families of rebeccamycin analogues have been prepared either by semi-synthesis from rebeccamycin or by total synthesis, and their biological properties have been examined.[2,6–11] Various modifications were achieved by semi-synthesis: substitutions and modifications of the functionalities on the upper heterocycle and on the sugar unit, coupling of the sugar moiety to the second indole nitrogen. Other analogues were obtained by total synthesis: introduction of various carbohydrate moieties or introduction of an amino function in the 2′ position of the sugar part or replacement of one or both indole moieties by 7-azaindole units.[12–17] A water soluble derivative of rebeccamycin, NCS 655649, is currently undergoing clinical trials.[18]

In this paper, semi-syntheses from rebeccamycin of new compounds bearing substituents at 3,9-positions of the indolocarbazole framework are described as well as a newly obtained 2′,3′-epoxide. The antiproliferative activities of the new compounds against nine tumor cell lines and the effect on the cell cycle of murine leukemia L1210 cells were determined. We previously observed that, if topoisomerase I is the main target for most of rebeccamycin analogues, the absence of a strict correlation between topoisomerase I inhibitory properties and antiproliferative activities suggests other targets for these molecules. Since rebeccamycin is structurally

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related to staurosporine (Fig. 1), a non selective kinase inhibitor,[19,20] the inhibitory activities of the new analogues were examined toward three kinases: PKCζ a protein kinase C isoform, CDK1/cyclin B a cyclin-dependent kinase, CDK5/p25 a kinase involved in the phosphorylation of neuron cytoskeletons, and cdc25A a phosphatase involved in the progression of the cell cycle.

Results and Discussion

Chemistry

Rebeccamycin **1** and demethylated rebeccamycin **2** (Fig. 1) were obtained from cultures of *S. aerocolonigenes*. 3,9-Diformylated compound **4** was prepared from dechlorinated rebeccamycin **3** after peracetylation using acetic anhydride in pyridine. This step was necessary because, in the absence of protective groups on the sugar moiety, substitution may occur on the carbohydrate part.[6] Crude 2′,3′,6′-triacetylated compound **3′** was formylated using dichloromethylmethylether in the presence of titanium chloride in methylene chloride according to a method used for the formylation of staurosporine.[21] Deprotection of the hydroxyl groups of the sugar moiety led to compound **4** from which 3,9-dihydroxymethyl derivative **5** was obtained by hydrogenation over Raney Nickel (Scheme 1). To prepare 3,9-dinitro compound **6**, a similar sequence of reactions as for **4** was carried out: protection of the hydroxyl groups of the sugar part, nitration using fuming HNO_3, then hydrolysis of the acetate groups. The nitro groups of compound **6** have been reduced to amino groups with stannous chloride[22] to give the diamino derivative **7**. Baeyer–Villiger oxidation[23] of diformylated analogue **4′**, bearing protective groups on the hydroxyl functions of the sugar part, using H_2O_2/H_2SO_4 in methanol gave either the dihydroxy derivative **8** or the diester **9** according to the experimental conditions. From **4′** (1 mmol), using H_2O_2 (7.7 mmol) and H_2SO_4 (0.08 mL), dihydroxy **8** was isolated, whereas with 10-fold more equivalents of H_2O_2/H_2SO_4, diester **9** was obtained together with degradation products but without any amounts of **8**.

Tosylate **10** (Scheme 2), obtained from rebeccamycin by selective tosylation, was an intermediate for the preparation of a new series of rebeccamycin analogues in which the sugar moiety is linked to both indole nitrogens.[12] In a basic medium (NaH), tosylate **10** was transformed to epoxide **11**.

Antiproliferative activities

The in vitro antiproliferative activities of **1**–**11** were evaluated against eight human tumor cell lines: ovarian carcinoma IgROV, neuroblastoma SK-N-MC, colon carcinoma HT29, non-small cell lung carcinoma A549, small cell lung carcinoma NCl-H69, epidermoïd carcinoma A431 and KB-3-1, human leukemia K-562). A ninth cell line from murine origin, the L1210 leukemia cell line, was used for both cytotoxicity and cell cycle assays (Table 1). The percentage of cells recovered in the G2 + M phases at a given drug concentration was determined. There is no marked differences between the cytotoxicities of rebeccamycin **1** and the minor product of fermentation **2** except toward IgROV and A549 cells against which rebeccamycin is ten times more potent than its demethylated analogue **2**. Compound **2** and dechlorinated rebeccamycin **3** exhibit similar profiles of cytotoxicity. Dimethoxycarbonyl **9** is only weakly active against L1210 cells. Dinitro compound **6** is strongly cytotoxic without selectivity. Compounds **11** is also non selective. In contrast, 3,9-dihydroxy compound **8** exhibits strong antiproliferative activities toward L1210, SK-N-MC, NCI-H69 and K-562 cells but is inactive against IgROV and A431 cells. Diformyl **4** is selective toward SK-N-MC, NCI-H69 and K-562 cells. Diamino **7** exhibits selectivity toward NCI-H69 and SK-N-MC cells. Dihydroxymethyl **5** is only weakly active against SK-N-MC and NCI-H69. The most sensitive tumor cells lines toward the rebeccamycin analogues of this series are neuroblastoma SK-N-MC and small cell lung carcinoma NCI-H69. Most of the compounds induce an accumulation of the cells in the G2 + M phases which could be linked to the inhibition of cyclin-dependent kinases and especially CDK1/cyclin B which activates the G2-M progression.[24,25]

Kinases inhibition

Various kinases are involved in the progression of the cell cycle as well as in essential cell functions through kinases cascades. The inhibitory properties of compounds **1**–**11** toward PKCζ, a cyclin-dependent kinase CDK1/cyclin B, a kinase important in phosphorylation

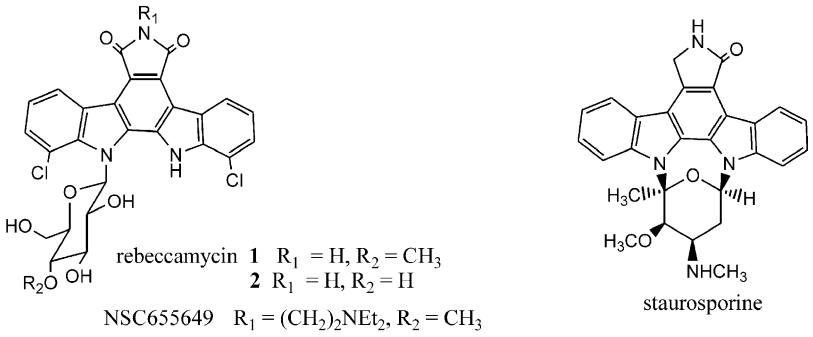
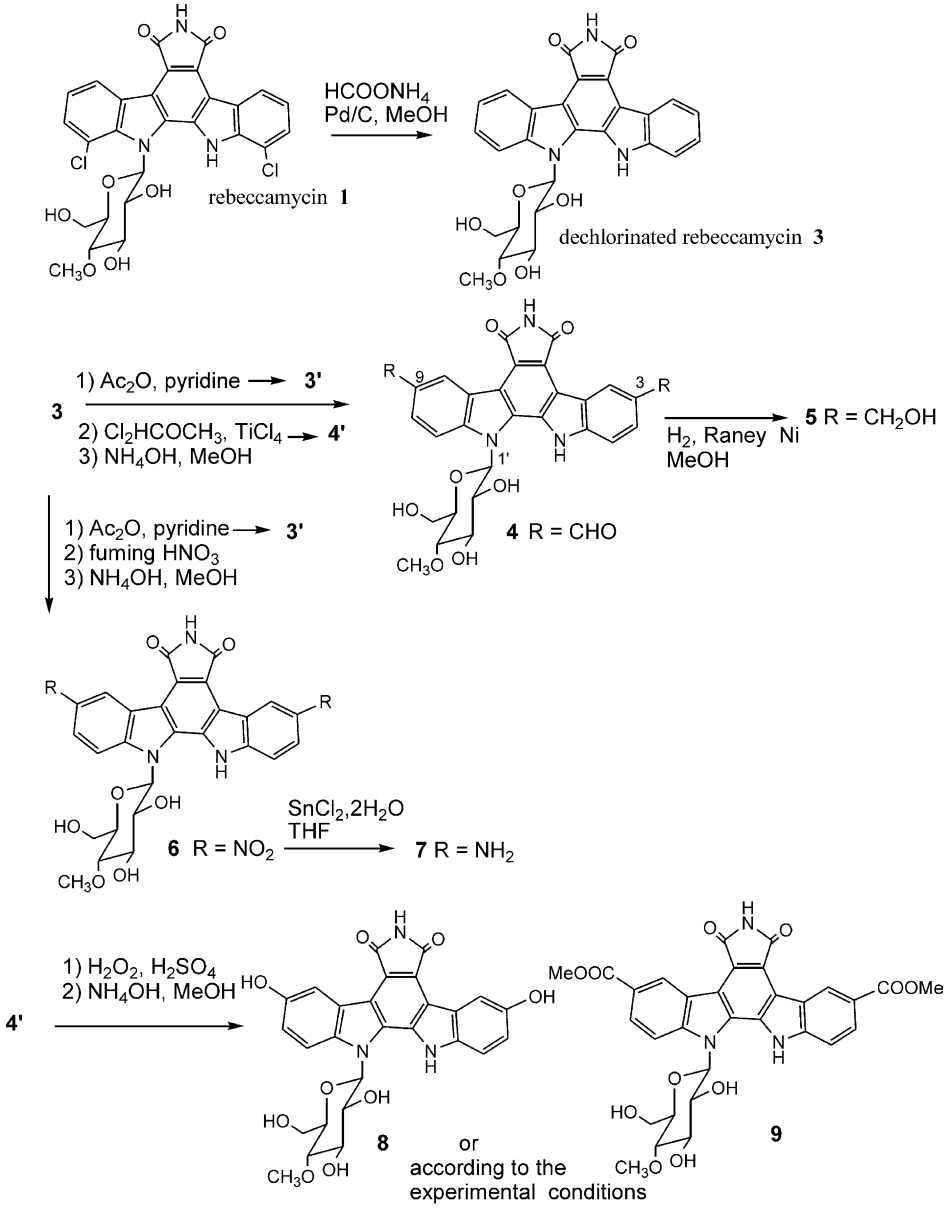


Figure 1.



Scheme 1.

Table 1. In vitro antiproliferative activities (IC_{50} μM) and cell cycle effects

Compd	L1210	% of L1210 cells in the G2 + M phase[a]	IgROV	SK-N-MC	HT29	A549	A431	NCl-H69	K-562	KB-3-1
1	0.14	69% (1 μM)	0.25	<0.1	0.3	0.3	0.25	<0.1	0.2	0.3
2	0.16	68% (2 μM)	3	<0.1	1	3	1	<0.1	0.1	0.4
3	0.11	71% (1 μM)	2.6	<0.1	2.5	2	3.8	<0.1	<0.1	0.28
4	1.1	79% (0.5 μM)	>10	<0.1	>10	>10	>10	0.3	0.5	>10
5	11.3	NE	>10	4.5	>10	>10	>10	6	9	>10
6	0.29	74% (25 μM)	1	<0.1	0.4	2	0.1	<0.1	<0.1	0.2
7	1.1	69% (5 μM)	>10	0.25	>10	>10	>10	0.1	0.7	10
8	0.11	78% (0.25 μM)	>10	<0.1	7	2	>10	<0.1	0.1	1.5
9	4.9	34% (10 μM)	>10	~10	>10	>10	10	~10	>10	>10
11	12	NS, toxic at 50μM	9	5	8	9.5	2.5	5	6.5	8.5

Murine leukemia L1210, ovarian carcinoma IgROV, neuroblastoma SK-N-MC, colon carcinoma HT29, non-small cell lung carcinoma A549, small cell lung carcinoma NCl-H69, epidermoïd carcinoma A431 and KB-3-1, human leukemia K-562. Percentage of L1210 cells in G2 + M phase (at the indicated drug concentration).

[a]24% of untreated control cells were in the G2 + M phase of the cell cycle; NE, not evaluated; NS, not specific.

Table 2. Effects on kinases and phosphatase (IC_{50} μM)

Compd	CDK1/cyclin B	CDK5/p25	PKCζ	cdc25A
1	>5	>5	>5	>5
2	>5	>5	>5	>5
3	>10	>5	>5	>5
4	>5	>5	>5	>5
5	>5	>5	>5	>5
6	>5	>5	>5	>5
7	10	>5	>5	>5
8	0.08	0.04	<5	>5
9	>5	>5	>5	>5
11	>5	>5	>5	>5

PKCζ a protein kinase C isoform, CDK1/cyclin B a cyclin-dependent kinase, CDK5/p25 a kinase involved in phosphorylation of neuron cytoskeletons, and a phosphatase involved in the progression of the cell cycle cdc25A.

of neuron cytoskeletons CDK5/p25 and toward cdc25A, a phosphatase involved in the cell cycle (Table 2). None of the compounds inhibits cdc25A and PKCζ. Only one compound, dihydroxy **8** inhibits markedly the cyclin-dependent kinase CDK1/cyclinB and CDK5/p25.

DNA binding and topoisomerase I inhibition

Rebeccamycin derivatives usually behave as DNA intercalating agents.[3] This property manifests by the capacity of the drugs to unwind supercoiled DNA and this can be monitored by a DNA relaxation assay using topoisomerase I.[26] In the absence of ethidium bromide in the agarose gel during the electrophoresis, the relaxation of supercoiled DNA by topoisomerase I gives a population of topoisomers and the presence of an

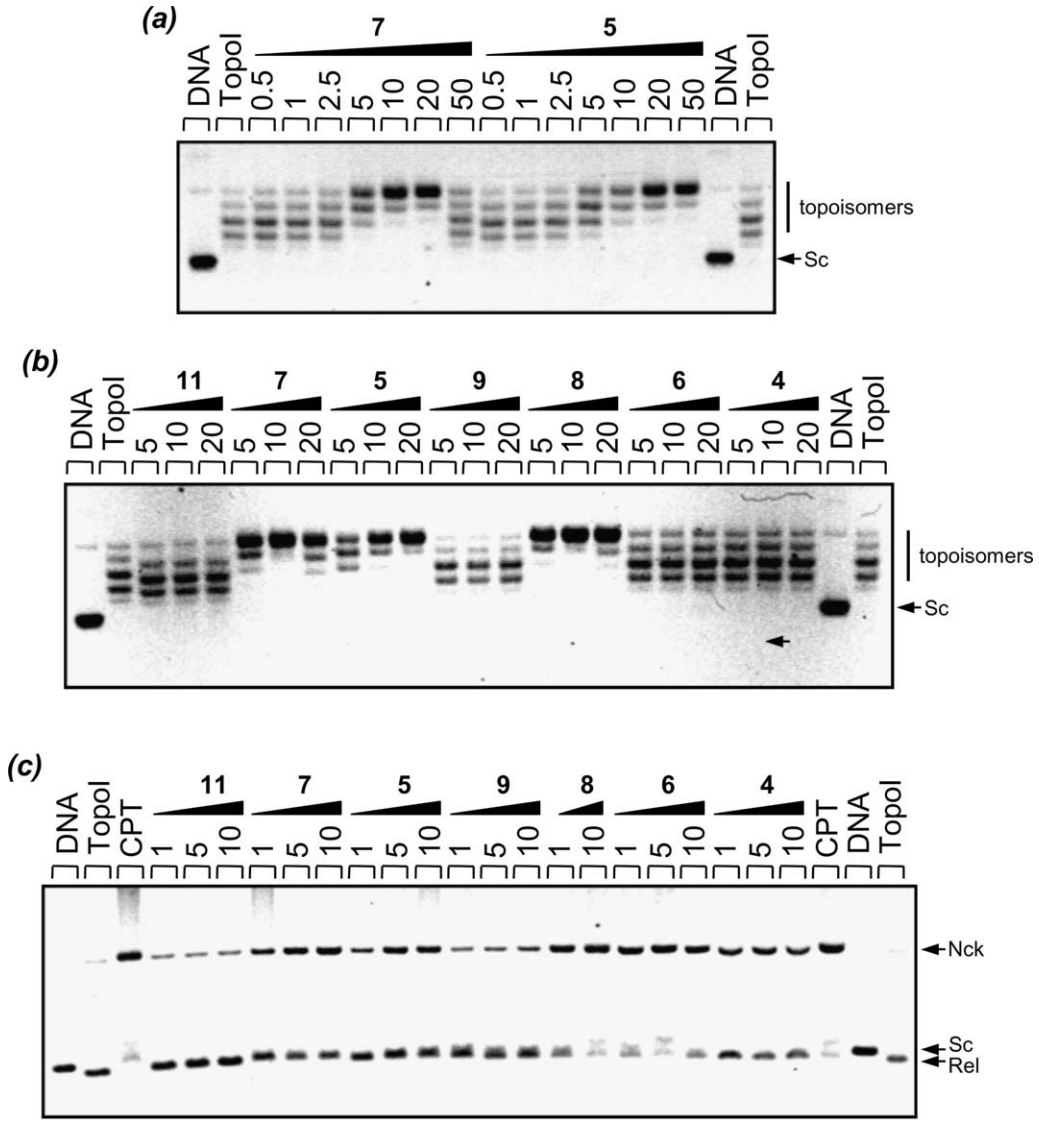
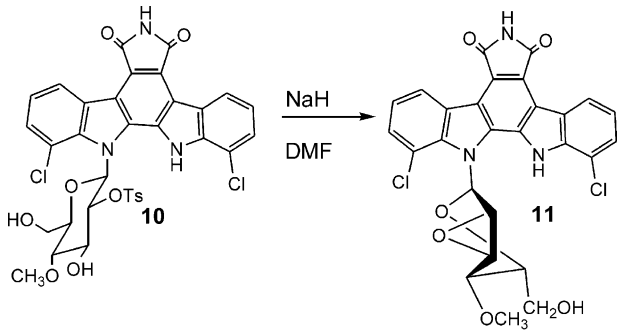


Figure 2. Topoisomerase I inhibition. Effect of the compounds on the relaxation of plasmid DNA by human topoisomerase I, (a, b) in the absence or (c) in the presence of ethidium bromide during the electrophoresis. Native supercoiled pKMp27 DNA (0.25 μg) (lane DNA) was incubated with 4 units topoisomerase I in the absence (lane TopoI) or presence of drug at the indicated concentration (μM). Reactions were stopped with sodium dodecylsulfate and treatment with proteinase K. DNA samples were separated by electrophoresis on 1% agarose gels. The gel was stained with ethidium bromide after the electrophoresis (a, b) or contained ethidium (1 μg/mL) prior to the electrophoresis (c). Nck, nicked; Rel, relaxed; Sc, supercoiled.



Scheme 2.

intercalating agent affects the distribution of the topoisomers population due to an unwinding effect. The typical gels presented in Figure 2a and b show that compounds **5**, **7** and **8** produce dose-dependent alterations in plasmid linking number which are characteristic of an intercalating agent. As expected, the unwinding effect is observed with the amino derivative **7** but not with the corresponding nitro analogue **6**. Similarly, the hydroxy compounds **5** and **8** unwind supercoiled DNA whereas the related diformyl **4** and dimethoxycarbonyl **9** derivatives do not affect the electrophoretic mobility of DNA in the presence of the enzyme. Similar results were obtained when evaluating DNA interaction by absorption spectroscopy. Compounds **5**, **7** and **8** slightly stabilized duplex DNA against heat denaturation (ΔT_m values $<5\,^{\circ}C$) and hypochromic and bathochromic shifts were observed upon binding to DNA (data not shown).

The three DNA binding agents **5**, **7** and **8** also function as topoisomerase I poisons. This is revealed by the DNA relaxation experiments performed using ethidium bromide-containing agarose gels in order to visualize the activity of the test compounds on topoisomerase I-mediated DNA cleavage. Compounds **5**, **7** and **8** promote the formation of nicked DNA molecules, as observed with the reference drug camptothecin (Fig. 2c). This topoisomerase I poisoning activity was also observed when using a 117-bp radiolabeled DNA substrate. Three cleavage sites, at T-G dinucleotide steps, were detected with **5**, **7** and **8** (data not shown). DNA intercalation is not required for this poisoning effect because a marked increase of the intensity of the nicked DNA content was also detected with compounds **6** and **4** which have no effect on DNA unwinding. In the indolocarbazole series, it is known that DNA intercalation and topoisomerase I are not necessarily correlated and can both contribute to the cytotoxic action.[27] In fact, tight binding to DNA can be detrimental for trapping the covalent DNA-topoisomerase I complexes.[28] Compounds **11** and **9** have no effect on topoisomerase I-mediated DNA cleavage and they are also the least cytotoxic compounds in the series. However, no direct correlation can be established between cytotoxicity and topoisomerase I inhibition. For example, compound **5** binds to DNA and inhibits topoisomerase I but it is not particularly toxic to cells.

Conclusion

Depending on to the structural modifications, the new rebeccamycin derivatives exhibit or not selectivity toward the various tumor cell lines tested. 3,9-Substituents can enhance or abolish the cytotoxicity, but they can also induce selectivity. 3,9-Dihydroxymethyl compound **5** and 3,9-dimethoxycarbonyl **9** are almost inactive. In contrast to rebeccamycin, dechlorinated rebeccamycin and compound **2**, which are non selective toward the various tumor cell lines tested, 3,9-diformyl compound **4**, 3,9-diamino **7** and diphenol **8** are highly selective. 2′-3′-Epoxy **11** is weakly active and not selective, however this compound is especially interesting because it can be used for the introduction of various functionalities in the positions 2′ and 3′ of the sugar moiety. Small cell lung carcinoma NCI-H69 and neuroblastoma SK-N-MC are the most sensitive tumor cells lines to the rebeccamycin derivatives reported in this paper. The new compounds do not inhibit efficiently the kinases PKCζ, CDK1/cyclin B and CDK5/p25 and the phosphatase cdc25A except diphenol **8** which inhibits markedly CDK1/cyclin B and CDK5/p25. The efficiency of **8** toward CDK5/p25 is especially interesting since this kinase, activated by p25, is thought to play an important role in the phosphorylation of tau protein involved in Alzheimer′s disease.[20] The activation of CDK1/cyclin B is required in G2 + M phases, therefore the inhibitory activity of compound **8** toward CDK1/cyclin B seems to be correlated to the observed accumulation of L1210 cells in the G2 + M phase. No direct correlation can be observed between the topoisomerase I inhibitory activities and the cytotoxicities of the newly synthesized compounds. The selectivity toward the tumor cell lines tested could be explained by the possible action of the compounds on other cellular targets such as the multiple kinases not examined in this study and well known to be expressed at different levels in the various tumor cell lines.[29,30] The levels of topoisomerase I also vary in the different tumor cell lines.[31] Diphenol derivative **8** exhibits a large spectrum of molecular activities because, in addition to its action at the CDK1 and CDK5 levels, it is also a DNA binder and a topoisomerase I poison. In vivo studies have now been initiated with this multitarget inhibitor.

Experimental

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, the following abbreviations are used: singlet (s), broad singlet (br s), doublet (d), triplet (t), pseudo-triplet (pt), multiplet (m), tertiary carbons (C tert), quaternary carbons (C quat). The signals were assigned from ^1H-^1H COSY and ^{13}C-^1H correlations. Mass spectra (FAB+) were determined CESAMO (Talence, France) on a high resolution Fisons Autospec-Q spectrometer. Chromatographic purifications were performed by flash silicagel Geduran SI 60 (Merck)

0.040–0.063 mm or Kieselgel 60 (Merck) 0.063–0.200 mm column chromatography. For purity tests, TLC were performed on fluorescent silica gel plates (60 F_{254} from Merck). Rebeccamycin was from our laboratory stock sample.

3,9-Diformyl-12-(4-*O*-methyl-β-D-glucopyranosyl)-6-methyl-6,7,12,13-tetrahydro-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7-dione (4). To dechlorinated rebeccamycin **3** (68 mg, 0.136 mmol) at 0 °C are successively added acetic anhydride (0.130 mL, 1.37 mmol) and pyridine (0.25 mL, 3 mmol). The mixture was stirred at room temperature for 19 h, poured into water and extracted with EtOAc. The organic phase was washed with Na_2CO_3 and brine and dried over $MgSO_4$. The solvent was removed and the residue purified by chromatography (eluent, EtOAc) to give conformers of triacetylated dechlorinated rebeccamycin (75 mg, 0.119 mmol, 88% yield) as an orange solid. To the mixture of conformers in dichloromethane (2 mL) was added α,α-dichloromethyl methyl ether (0.22 mL, 2.4 mmol). The mixture was cooled to 0 °C before addition of 1 M $TiCl_4$ in CH_2Cl_2 (2.4 mL, 2.4 mmol) then stirred at room temperature for 24 h. The mixture was poured into water and stirred at room temperature for 30 min. After extraction with CH_2Cl_2, the organic phase was washed with brine and dried over $MgSO_4$. After removal of the solvent, the residue was dissolved in methanol (13 mL) before addition of 30% aqueous NH_4OH (6 mL). After stirring for 24 h at room temperature, the mixture was evaporated to dryness. The residue was dissolved in a mixture of EtOAc–THF, acidified with 1 N HCl and extracted with EtOAc. The organic phase was dried over $MgSO_4$ and the solvent was removed. The residue was purified by chromatography (eluent cyclohexane–acetone 20:80 to give **4** (30 mg, 0.054 mmol, 45% yield) as a yellow solid. Mp >300 °C. IR (KBr) ν_{CO} 1680, 1720, 1750 cm^{-1}, $\nu_{NH,OH}$ 3100–3600 cm^{-1} HRMS (FAB+) $(M+H)^+$: calcd for $C_{29}H_{24}N_3O_9$, 558.1512; found, 558.1514. ^1H NMR (400 MHz, DMSO-d_6): 3.55 (1H, m), 3.70 (3H, s), 3.81 (2H, m), 3.95 (1H, m), 4.03 (1H, dd, $J_1=12.3$ Hz, $J_2=4.4$ Hz), 4.18 (1H, m), 5.14 (1H, d, $J=5.5$ Hz, OH), 5.39 (1H, d, $J=5.4$ Hz, OH), 6.39 (1H, br s, OH), 6.49 (1H, d, $J=8.9$ Hz), 7.91 (1H, d, $J=8.4$ Hz), 8.15 (1H, d, $J=9.8$ Hz), 8.18 (1H, d, $J=9.8$ Hz), 8.22 (1H, d, $J=9.4$ Hz), 9.65 (1H, s), 9.72 (1H, s), 10.16 (1H, s), 10.19 (1H, s), 11.40 (1H, s, N–H), 12.00 (1H, s, N–H). ^{13}C NMR (100 MHz, DMSO-d_6): 58.5 ($C_{6'}$), 60.1 (OCH_3), 73.1, 76.1, 77.2, 77.3, 84.5 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 112.6, 112.8, 127.4, 127.6, 128.1, 128.5 (C *tert* arom), 117.5, 119.0, 120.5, 121.0, 121.3, 122.0, 128.9, 129.8, 129.9, 130.1, 144.2, 145.4 (C quat arom), 170.5, 170.6 (C=O quat), 191.9, 192.1 (CHO).

3,9-Dihydroxymethyl-12-(4-*O*-methyl-β-D-glucopyranosyl)-6-methyl-6,7,12,13-tetrahydro-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7-dione (5). To a solution of **4** (50 mg, 0.090 mmol) in methanol (28 mL) was added Raney Nickel (50% w/w in water, 20 mg). The mixture was stirred for 3 days at room temperature under hydrogen (1 bar). After filtration over Celite and washing the solid with methanol, THF and acetone, the solvents were removed and the residue purified by flash chromatography (eluent/cyclohexane–acetone 1:1) to give **5** (10.4 mg, 0.019 mmol, 21% yield) as a yellow solid. Mp >300 °C. IR (KBr) ν_{CO} 1720, 1740 cm^{-1}, $\nu_{NH,OH}$ 3100–3600 cm^{-1}. HRMS (FAB+) $(M+H)^+$: calcd for $C_{29}H_{25}N_3O_8$, 543.1641; found, 543.1654. ^1H NMR (400 MHz, DMSO-d_6): 3.68 (3H, s, OCH_3), 3.44–4.10 (6H, m), 4.71 (2H, d, $J=5.0$ Hz), 4.74 (2H, d, $J=4.9$ Hz), 5.02 (1H, d, $J=5.6$ Hz, OH), 5.25 (1H, t, $J=5.5$ Hz, OH), 5.27 (1H, t, $J=5.7$ Hz, OH), 5.36 (1H, d, $J=5.6$ Hz, OH), 6.20 (1H, t, $J=3.9$ Hz, OH), 6.35 (1H, d, $J=9.0$ Hz, $H_{1'}$), 7.57 (1H, d, $J=8.3$ Hz), 7.58 (1H, d, $J=8.3$ Hz), 7.69 (1H, d, $J=8.4$ Hz), 7.93 (1H, d, $J=8.7$ Hz), 9.07 (1H, s), 9.13 (1H, s), 11.15 (1H, s, NH), 11.58 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 58.5 ($C_{6'}$), 60.1 (OCH_3), 63.6, 63.7 (CH_2OH), 73.1, 76.3, 77.1, 77.3, 84.2 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 111.3, 111.8, 122.7, 122.8, 126.3, 126.5 (C *tert* arom), 116.9, 118.4, 119.3, 120.9, 121.0, 121.3, 128.6, 130.0, 134.6, 134.9, 140.0, 141.4 (C quat arom), 170.9, 171.0 (C=O).

3,9-Dinitro-12-(4-*O*-methyl-β-D-glucopyranosyl)-6-methyl-6,7,12,13-tetrahydro-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7-dione (6). To peracetylated dechlorinated rebeccamycin **3′** (200 mg, 0.32 mmol) was added Mallinckrodt acid solution (7.7 mL, prepared at 0 °C by addition of 7 mL fuming nitric acid to 50 mL of Et_2O). The mixture was stirred at room temperature for 27 h, then poured into water and extracted with EtOAc. The organic phase was washed with water, dried over $MgSO_4$. After removal of the solvent, the residue was dissolved in methanol (78 mL) before addition of 30% aqueous NH_4OH (35 mL). The mixture was stirred at room temperature for 19 h, evaporated to dryness and dissolved in EtOAc. The organic solution was washed with brine, dried over $MgSO_4$. The solvent was removed and the residue purified by silicagel chromatography column (eluent toluene–THF, 60:40), then silicagel chromatography plate (eluent toluene–THF, 40:60) to give **6** as a yellow solid (27 mg, 0.045 mmol, 14% yield). Mp >297 °C. IR (KBr) ν_{CO} 1720, 1760 cm^{-1}, $\nu_{NH,OH}$ 3140-3660 cm^{-1}. HRMS (FAB+) $(M+H)^+$: calcd for $C_{27}H_{22}N_5O_{11}$, 592.1316; found, 592.1327. ^1H NMR (400 MHz, DMSO-d_6): 3.27 (1H, m), 3.69 (3H, s, OCH_3), 3.65–3.81 (2H, m), 4.09 (2H, m), 4.19 (1H, d, $J=9.8$ Hz), 5.00 (1H, d, $J=5.4$ Hz, OH), 5.33 (1H, d, $J=5.9$ Hz, OH), 6.32 (1H, d, $J=9.3$ Hz, $H_{1'}$), 6.43 (1H, br s, OH), 7.76 (1H, d, $J=8.9$ Hz), 7.97 (1H, d, $J=9.4$ Hz), 8.15 (1H, dd, $J_1=9.4$ Hz, $J_2=2.0$ Hz), 8.41 (1H, dd, $J_1=8.9$ Hz, $J_2=2.0$ Hz), 9.71 (1H, d, $J=2.5$ Hz), 9.75 (1H, d, $J=2.5$ Hz), 11.39 (1H, s, NH), 11.94 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 58.6 ($C_{6'}$), 60.1 (OCH_3), 73.2, 75.8, 77.2, 77.5, 84.7 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 112.5, 112.6, 121.1, 121.7, 122.1, 122.4 (C *tert* arom), 117.3, 118.9, 120.1, 120.4, 120.5, 120.6, 129.0, 130.3, 140.9, 141.0, 143.6, 144.8 (C quat arom), 170.1, 170.7 (C=O).

3,9-Diamino-12-(4-*O*-methyl-β-D-glucopyranosyl)-6-methyl-6,7,12,13-tetrahydro-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7-dione (7). To a solution of **6** (71 mg, 0.12 mmol) in THF (14 mL), $SnCl_2$, $2H_2O$ (228 mg, 1.2 mmol) was added. The reaction mixture was stirred at 60 °C for 48 h before another addition of $SnCl_2$, $2H_2O$

(200 mg, 1.06 mmol). The reaction was then stirred at 60 °C for 24 h. After cooling, addition of water allowed the precipitation of a white solid which was filtered off. The filtrate was extracted with EtOAc. The resulting aqueous phase was then basified with a saturated aqueous solution of $NaHCO_3$ and extracted with EtOAc. The organic phase was dried over $MgSO_4$. The solvent was removed to give **7** as dark red crystals (52 mg, 0.10 mmol, 82%). Mp >300 °C. IR (KBr): ν_{CO} 1710, 1740 cm^{-1}, $\nu_{NH,\ OH,\ NH_2}$ 3000–3640 cm^{-1}. HRMS (FAB+) $(M+H)^+$: calcd for $C_{27}H_{26}N_5O_7$ 532.1832 found, 532.1814. ^1H NMR (400 MHz, DMSO-d_6): 3.69 (3H, s, OCH_3), 3.53–3.99 (6H, m), 4.95 (1H, d, $J=5.4$ Hz, OH), 5.04 (4H, br s, $2NH_2$), 5.32 (1H, d, $J=5.4$ Hz, OH), 6.03 (1H, br s, OH), 6.08 (1H, d, $J=9.3$ Hz, $H_{1'}$), 6.93 (1H, dd, $J_1=7.9$ Hz, $J_2=2.5$ Hz), 6.95 (1H, dd, $J_1=9.3$ Hz, $J_2=2.0$ Hz), 7.42 (1H, d, $J=8.4$ Hz), 7.62 (1H, d, $J=8.9$ Hz), 8.30 (1H, d, $J=2.0$ Hz), 8.36 (1H, d, $J=2.0$ Hz), 10.90 (1H, s, NH), 11.16 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 58.6 ($C_{6'}$), 60.0, 73.1, 76.5, 77.0, 77.3, 84.1 (OCH_3, $C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 107.6 (2C), 111.6, 112.2, 116.0, 116.5 (C *tert* arom), 116.3, 117.7, 118.6, 120.4, 122.1, 122.5, 128.5, 130.1, 133.9, 135.2, 142.5, 142.7 (C quat arom), 171.0, 171.1 (C=O).

3,9-Dihydroxy-12-(4-*O*-methyl-β-D-glucopyranosyl)-6,7,12,13-tetrahydro(5*H*)-indolo[2,3-a]-pyrrolo[3,4-*c*]-carbazole-5,7-dione (8). To a solution of compound **4′** (374 mg, 0.55 mmol) in MeOH (22 mL) were added a solution of H_2O_2 (50% in water, 0.24 mL, 4.24 mmol) and concentrated H_2SO_4 (44 μL). The reaction mixture was stirred at room temperature for 72 h before addition of water and then stirred for 30 min at room temperature before extraction with EtOAc. The organic phase was dried over $MgSO_4$ and the solvent removed to give a yellow solid which was used directly in the next step. The yellow solid was dissolved in MeOH (210 mL) before addition of NH_4OH (28% in water, 94 mL). The mixture was stirred at room temperature for 24 h. After evaporation of the mixture, EtOAc was added. The organic phase was washed with a saturated aqueous solution of NaCl and dried over $MgSO_4$. The solvent was removed and the residue purified by chromatography (first column: eluent, EtOAc; second purification: PLC plate: eluent MeOH–EtOAc, 3:97) to yield **8** (33 mg, 0.06 mmol, 7% yield for three steps from acetylated rebeccamycin) as orange crystals. Mp >300 °C. IR (KBr): $\nu_{C=O}$ 1700, 1750 cm^{-1}, $\nu_{NH,\ OH}$ 3100–3700 cm^{-1}. HRMS (FAB+) (M^+) calcd for $C_{27}H_{23}N_3O_9$, 533.1434, found, 533.1431. ^1H NMR (400 MHz, DMSO-d_6): 3.50–3.60 (2H, m), 3.68 (3H, s, OCH_3), 3.78 (1H, t, $J=9.3$ Hz), 3.87 (1H, m), 3.98 (2H, m), 4.98 (1H, d, $J=5.4$ Hz, OH), 5.31 (1H, d, $J=5.9$ Hz, OH), 6.09 (1H, t, $J=3.4$ Hz), 6.16 (1H, d, $J=10.9$ Hz, $H_{1'}$), 7.07 (1H, dd, $J_1=6.4$ Hz, $J_2=2.4$ Hz), 7.10 (1H, dd, $J_1=6.4$ Hz, $J_2=2.4$ Hz), 7.53 (1H, d, $J=8.8$ Hz), 7.76 (1H, d, $J=8.8$ Hz), 8.52 (1H, d, $J=2.0$ Hz), 8.59 (1H, d, $J=2.0$ Hz), 9.26 (1H, s, OH), 9.31 (1H, s, OH), 11.02 (1H, s, NH), 11.34 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 58.5 ($C_{6'}$), 60.0 (OCH_3), 73.2, 76.4, 77.1, 77.3, 84.2 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 108.9 (2C), 112.0, 112.5, 116.2, 116.6 (C *tert* arom), 116.5, 117.9, 118.9, 120.6, 121.8, 122.3, 128.7, 130.3, 134.8, 136.2, 151.5, 151.6 (C quat arom), 171.0, 171.1 (C=O).

3,9-Dimethoxycarbonyl-12-(4-*O*-methyl-β-D-glucopyranosyl) - 6,7,12,13 - tetrahydro(5*H*) - indolo[2,3 - *a*] - pyrrolo[3,4-*c*]-carbazole-5,7-dione (9). To a solution of **4′** (63 mg, 0.09 mmol) in MeOH (6 mL) were added H_2O_2 (50% in water, 0.4 mL, 7.07 mmol) and concentrated H_2SO_4 (1.6 mL). The mixture was stirred at room temperature for 72 h before addition of water and then stirred at room temperature for 20 min before extraction with EtOAc. The organic phase was dried over $MgSO_4$ and the solvent removed to give a yellow solid which was dissolved in MeOH (22 mL) before addition of NH_4OH (28% in water, 9.7 mL). The mixture was stirred at room temperature for 24 h. After evaporation of the mixture, EtOAc was added. The organic phase was washed with a saturated aqueous solution of NaCl and dried over $MgSO_4$. The solvent was removed and the residue purified by column chromatography (eluent EtOAc–cyclohexane, 70:30) to yield **9** (8 mg, 0.013 mmol, 14% yield) as a yellow solid. Mp >300 °C. IR (KBr): $\nu_{C=O}$ 1700, 1710, 1750 cm^{-1}, $\nu_{NH,OH}$ 3200–3700 cm^{-1}. HRMS (FAB+) $(M+H)^+$ calcd for $C_{31}H_{28}N_3O_{11}$ 618.1724, found, 618.1736. ^1H NMR (400 MHz, DMSO-d_6): 3.55 (1H, m), 3.70 (3H, s), 3.75–3.85 (2H, m), 3.93 (1H, m), 3.97 (3H, s), 3.98 (3H, s), 4.00 (1H, m), 4.09 (1H, m), 5.12 (1H, d, $J=5.4$ Hz, OH), 5.36 (1H, d, $J=5.4$ Hz, OH), 6.34 (1H, t, $J=3.9$ Hz, OH), 6.45 (1H, d, $J=8.9$ Hz, $H_{1'}$), 7.83 (1H, d, $J=8.9$ Hz), 8.12 (1H, d, $J=9.4$ Hz), 8.22 (1H, dd, $J_1=9.3$ Hz, $J_2=1.5$ Hz), 8.24 (1H, dd, $J_1=8.4$ Hz, $J_2=1.5$ Hz), 9.83 (1H, d, $J=1.5$ Hz), 9.90 (1H, d, $J=2.0$ Hz), 11.38 (1H, s, NH), 11.96 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 52.1 (2C, COO<u>Me</u>), 58.5 ($C_{6'}$), 60.1 (OCH_3), 73.1, 76.2, 77.3 (2C), 84.4 ($C_{1'}$, $C_{2'}$, $C_{3'}$, $C_{4'}$, $C_{5'}$), 112.0, 112.2, 126.9, 127.1, 127.7, 128.0 (C *tert* arom), 117.5, 118.9, 120.4, 120.8, 121.1, 121.9, 122.0, 122.3, 128.9, 130.2, 143.4, 144.6 (C quat arom), 166.6, 166.8, 170.6, 171.0 (C=O).

1,11-Dichloro-12-(2,3-anhydro-4-*O*-methyl-β-D-glucopyranosyl) - 6,7,12,13 - tetrahydro(5*H*) - indolo[2,3 - *a*] - pyrrolo[3,4-*c*]-carbazole-5,7-dione (11). To a solution of compound **10** (50 mg, 0.07 mmol) in DMF (1 mL) was added sodium hydride (60% in mineral oil, 27.6 mg, 0.70 mmol). The mixture was stirred at 60 °C for 4 h. Water was added and the mixture was extracted with EtOAc. The organic phase was washed with brine, the solvent was removed and the residue purified by chromatography (neutral alumina 507C STD grade, 150 mesh, 58 Å) (eluent EtOAc–CH_2Cl_2, 60:40) to give epoxide **11** (22 mg, 0.04 mmol, 57% yield) as a yellow solid Mp >300 °C. IR (KBr), $\nu_{C=O}$ 1710, 1760 cm^{-1}; $\nu_{NH,\ OH}$ 3100–3600 cm^{-1}. HRMS (FAB$^+$) (M^+) calcd for $C_{27}H_{19}N_3O_6Cl_2$ 551.0651, found, 551.0654. ^1H NMR (400 MHz, DMSO-d_6) δ 3.48 (1H, m), 3.57 (1H, m), 3.61 (3H, s, OCH_3), 3.77 (1H, m), 3.85 (1H, d, $J=9.2$ Hz), 3.95 (1H, d, $J=4.1$ Hz), 4.08 (1H, d, $J=3.9$ Hz), 4.85 (1H, t, $J=5.2$ Hz, OH), 7.34 (1H, t, $J=7.9$ Hz), 7.38 (1H, t, $J=7.9$ Hz), 7.41 (1H, s), 7.60 (2H, pt, $J=7.0$ Hz), 8.95 (1H, d, $J=7.9$ Hz), 9.20 (1H, dd, $J_1=7.9$ Hz, $J_2=0.7$ Hz), 11.25 (1H, s, NH), 11.36 (1H,

s, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 52.0, 54.0 ($C_{2'}$, $C_{3'}$), 57.8 (OCH_3), 60.9 ($C_{6'}$), 69.4, 81.4, 82.1 ($C_{1'}$, $C_{4'}$, $C_{5'}$), 115.5, 116.3, 116.8, 118.5, 119.4, 122.1, 122.6, 125.5, 128.9, 129.5, 136.5, 137.2 (C quat arom), 121.0, 122.9, 123.3, 123.9, 126.2, 129.4 (C *tert* arom), 170.2, 170.4 (C=O).

Growth inhibition assays

Tumor cells were provided by American Type Culture Collection (Frederik, MD, USA). They were cultivated in RPMI 1640 medium (Life Science technologies, Cergy-Pontoise, France) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10 mM Hepes buffer (pH = 7.4). Cytotoxicity was measured by the microculture tetrazolium assay as described.[32] Cells were continuously exposed to graded concentrations of the compounds for four doubling times, then 15 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were added to each well and the plates were incubated for 4 h at 37 °C. The medium was then aspirated and the formazan solubilized by 100 μL of DMSO. Results are expressed as IC_{50}, concentration which reduced by 50% the optical density of treated cells with respect to untreated controls.

Cell cycle analysis

For the cell cycle analysis, L1210 cells (2.5×10^5 cells/mL) were incubated for 21 h with various concentrations of the compounds, then fixed by 70% ethanol (v/v), washed and incubated in PBS containing 100 μg/mL RNAse and 25 μg/mL propidium iodide for 30 min at 20 °C. For each sample, 10^4 cells were analyzed on a XL/MCL flow cytometer (Beckman Coulter). The fluorescence of propidium iodide was collected through a 615-nm long-pass filter.

Kinases inhibition

Biochemical reagents. Sodium ortho-vanadate, EGTA, EDTA, RNAse A, Mops, β-glycerophosphate, phenylphosphate, sodium fluoride, glutathione-agarose, dithiothreitol (DTT), bovine serum albumin (BSA), nitrophenylphosphate (p-NPP), leupeptin, aprotinin, pepstatin, soybean trypsin inhibitor, benzamidine, histone H1 (type III-S) were obtained from Sigma Chemicals. [γ-^{32}P]-ATP (PB 168) was obtained from Amersham.

Buffers. Homogenization buffer. 60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 25 mM Mops (pH 7.2), 15 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 1 mM sodium vanadate, 1 mM NaF, 1 mM phenylphosphate, 10 μg leupeptin/mL, 10 μg aprotinin/mL, 10 μg soybean trypsin inhibitor/mL and 100 μM benzamidine.

Buffer A. 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 25 mM Tris–HCl pH 7.5, 50 μg heparin/mL.

Buffer C. Homogenization buffer but 5 mM EGTA, no NaF and no protease inhibitors.

Kinases preparations and assays

Kinases activities were assayed in buffer A or C, at 30 °C, at a final ATP concentration of 15 μM. Blank values were substracted and activities calculated as pmoles of phosphate incorporated for a 10-min incubation. The activities are usually expressed in% of the maximal activity, that is in the absence of inhibitors. Controls were performed with appropriate dilutions of dimethylsulfoxide.

CDK1/cyclinB and CDK5/p25. Their activities were assayed in buffer C as previously described.[33] The same method in buffer C was used with PKCζ.

cdc25A. Its activity was assayed in buffer A as previously described.[34]

DNA relaxation and topoisomerase I inhibition

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

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