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Rebeccamycin Derivatives as Dual DNA-Damaging Agents and Potent Checkpoint Kinase 1 Inhibitors

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ABSTRACT

Rebeccamycin is an indolocarbazole class inhibitor of topoisomerase I. In the course of structure-activity relationship studies on rebeccamycin derivatives, we have synthesized analogs with the sugar moiety attached to either one or both indole nitrogens. Some analogs, especially those with substitutions at the 6′ position of the carbohydrate moiety, exhibit potent inhibitory activity toward checkpoint kinase 1 (Chk1), a kinase that has a major role in the G_2/M checkpoint in response to DNA damage. Some of these compounds retained a genotoxic activity either through intercalation into the DNA and/or by topoisomerase I-mediated DNA cleavage. We explored the structure-activity relationship between these compounds and their multiple targets. These rebeccamycin derivatives represent a novel class of potential antitumor agents that have a dual effect and might selectively induce the death of cancer cells.

Indolocarbazole structures are found in many bacterial metabolites (Prudhomme, 1997). One of these, rebeccamycin, is a topoisomerase I inhibitor isolated from cultures of *Saccharothrix aerocolonigenes* (Bush et al., 1987). Other derivatives, such as staurosporine and UCN-01, which have been isolated from cultures of *Streptomyces* spp., are nonselective protein kinase inhibitors devoid of effects on topoisomerase I (Tamaoki et al., 1986; Takahashi et al., 1987) (Fig. 1).

Rebeccamycin behaves as a conventional topoisomerase I poison capable of stabilizing DNA-topoisomerase I covalent complexes, thereby promoting secondary DNA double strand breaks as a result of the collision of the replication fork with the cleavage complex (Meng et al., 2003). The structurally related compounds, staurosporine and UCN-01, do not inhibit topoisomerase I, but they are potent inhibitors of many protein kinase, including checkpoint kinase 1 (Chk1) (Davies et al., 2000; Graves et al., 2000; Jackson et al., 2000). Analysis of the Chk1 and inhibitor cocrystals by X-ray crystallography showed that these indolocarbazoles bind to the ATP pocket of the enzyme (Zhao et al., 2002).

Over the past decade, much interest has been devoted to cell cycle checkpoints in the development of antitumor agents (Prudhomme, 2006; Tao and Lin, 2006; Garbaccio et al., 2007; Tao et al., 2007).

Cell cycle checkpoints are activated in response to DNA damage. Once activated, they block the cell cycle to allow time for DNA repair, or if the damage is too important, they engage cell death. The G_1/S phase checkpoint depends upon p53 protein, which is either absent or mutated in more than 50% of human tumors. When the G_1/S phase checkpoint is not functional, tumors are more dependent upon the G_2/M phase checkpoint. Among the regulators of the G_2/M checkpoint, Chk1 plays a major role. Based upon this model, it is

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ABBREVIATIONS: Chk1, Checkpoint kinase 1; CPT, camptothecin; Nck, nicked; Rel, relaxed; Sc, supercoiled; NB506, 6*N*-formylamido-12,13-dihydro-1,11-dihydroxy-13-(β-D-glucopyranosyl)-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]-5,7(6*H*)-dione.

proposed that the majority of cancer cells, when treated with a genotoxic cancer drug, would be selectively sensitive to cotreatment with a Chk1 inhibitor (Levesque et al., 2008). This cotreatment should force cancer cells to bypass the G_2/M checkpoint and enter a premature and lethal mitosis, also known as mitotic catastrophe (Prudhomme, 2004b; Zhou and Bartek, 2004). Chk1 is a relevant target for new antitumor agents because it has a major role in the regulation of the DNA damage checkpoint (Meijer, 2003; Xiao et al., 2003; Kawabe, 2004). Phase I and II clinical trials have been reported using a combination of UCN-01 with cisplatin, carboplatin, and other DNA-damaging agents (Perez et al., 2006; Edelman et al., 2007; Welch et al., 2007). However, this chemotherapeutic approach requires the combination of two drugs. Otherwise, it might be advantageous to use a single drug simultaneously able to induce DNA lesions and to abrogate the G_2/M checkpoint.

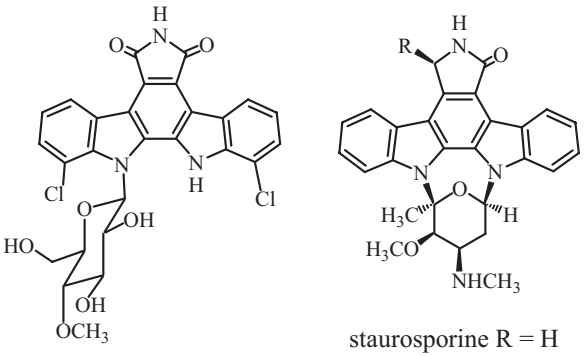
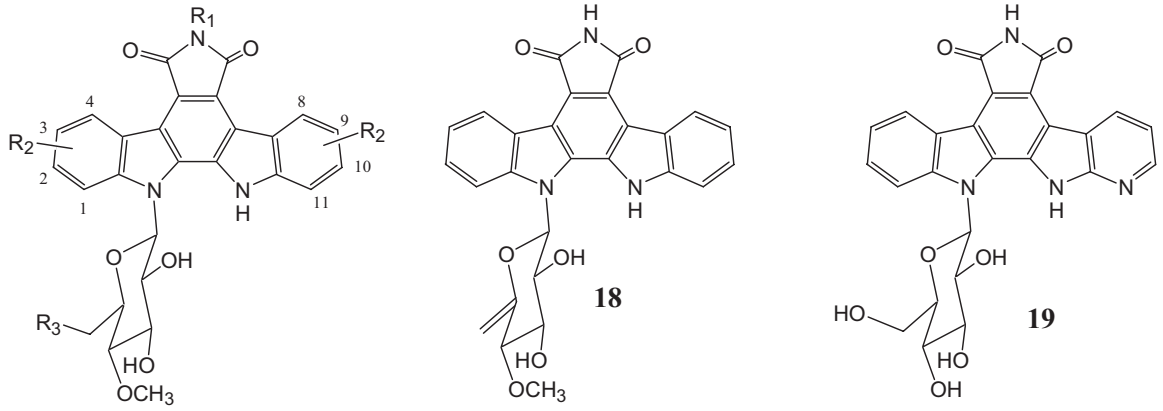
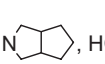


Fig. 1. Chemical structures of rebeccamycin, staurosporine, and UCN-01.

The similarities between the structures of the indocarbazole synthon that inhibits either protein kinases or topoisomerases led us to explore in detail whether derivatives of indolocarbazoles might retain both activities (Prudhomme, 2004a). We have synthesized a large number of rebeccamycin derivatives, substituted on the imide nitrogen or at 3,9 positions of the indole moieties, or on the sugar moiety (Fig. 2) (Rodrigues Pereira et al., 1996; Anizon et al., 1997, 2003; Moreau et al., 1998, 1999a,b, 2003). We have also synthesized staurosporine analogs from rebeccamycin in which the sugar unit was linked to both indole nitrogens (Fig. 3) (Facompré et al., 2002; Marminon et al., 2002). Finally, because replacing an indole moiety by a 7-azaindole could modify the interactions with the biological targets, we have synthesized aza-rebeccamycins in which one or both indole moieties were replaced by 7-azaindoles (e.g., compound **19**) (Fig. 2) (Marminon et al., 2003a,b; Messaoudi et al., 2004, 2005a,b). The



1 Rebeccamycin $R_1 = R_3 = H$, $R_2 = 1, 11\text{-Cl}$
2 $R_1 = H$, $R_2 = 1, 11\text{-Cl}$, $R_3 = Cl$
3 $R_1 = R_3 = R_2 = H$
4 $R_1 = R_2 = H$, $R_3 = Cl$
5 $R_1 = R_2 = H$, $R_3 = I$
6 $R_1 = R_2 = H$, $R_3 = N_3$
7 $R_1 = R_2 = H$, $R_3 = NH_2$, HCl
8 $R_1 = R_2 = H$, $R_3 = NHCH_3$, HCl
9 $R_1 = R_2 = H$, $R_3 = N(CH_3)_2$, HCl

10 $R_1 = R_2 = H$, $R_3 =$  , HCl

11 $R_1 = R_3 = H$, $R_2 = 3,9\text{-di}NO_2$
12 $R_1 = R_3 = H$, $R_2 = 3,9\text{-di}NH_2$
13 $R_1 = R_3 = H$, $R_2 = 3,9\text{-di}OH$
14 $R_1 = R_3 = H$, $R_2 = 3,9\text{-di}CHO$
15 $R_1 = R_3 = H$, $R_2 = 3,9\text{-di}COOCH_3$
16 $R_1 = R_3 = H$, $R_2 = 3,9\text{-di}Br$
17 $R_1 = R_3 = H$, $R_2 = 3,9\text{-di}CH_2OH$

Fig. 2. Rebeccamycin analogs.

activities of these derivatives were tested for tumor cell proliferation, topoisomerase I inhibition and DNA binding. We identified several compounds that have all three activities.

Materials and Methods

Chemicals

Camptothecin was purchased from Sigma (St. Louis, MO), UCN-01 was purchased from Merck (Darmstadt, Germany). Rebeccamycin was isolated from cultures of *S. aerocolonigenes* in our laboratory. Compounds **2** to **31** have been synthesized in our laboratory (Moreau et al., 1999b, 2003; Marminon et al., 2002, 2003a; Anizon et al., 2003).

Kinase Inhibition Assays

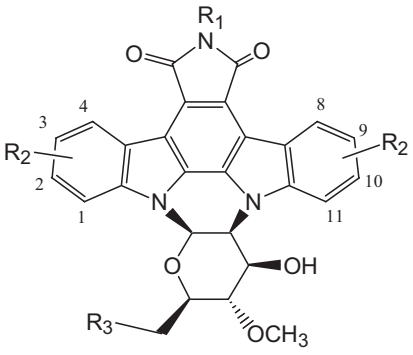
Chk1 Inhibition Assays. Human Chk1 full-length enzyme with an N-terminal GST sequence was purchased from Millipore (Billerica, MA). Assays for compound testing were based upon the method described by Davies et al. (2000) except that the final ATP concentration was 15 μM. Compounds (final concentration, 10 μM) were tested in duplicate, and the average inhibition was calculated as a percentage relative to samples without compound. The reproducibility of assays was monitored by testing a control compound in every experiment.

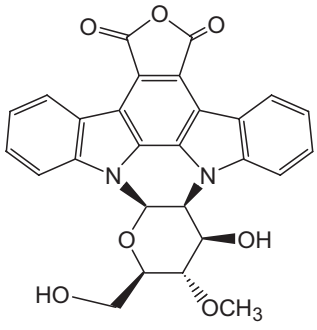
Src Inhibition Assays. Src kinase was purchased from Invitrogen. Inhibitors were diluted with a Tecan Evo150 robot. The kinase assay was performed with 4 μl of inhibitor (10% DMSO), 10 μl of 4× kinase assay buffer (80 mM $MgCl_2$, 200 mM HEPES, 0.4 mM EDTA, and 2 mM dithiothreitol), 10 μl of substrate peptide (KVEKIGEGYYGVVYK, 370 nM), and 6 μl of Src kinase (stock GTP purified and diluted with 1× kinase assay buffer to 200 nM). Ten microliters of cosubstrate (40 μM ATP with 0.2 μCi of -γ-^{33}P-ATP) was added with a Precision 2000 robot (BioTek, Winooski, VT). The incubation was run for 20 min at 30°C then stopped by addition of 200 μl of 0.85% orthophosphoric acid, and the mix was then transferred to a phosphocellulose filter microplate (Whatman - P81). The plate was washed 3 times with 200 μl of 0.85% orthophosphoric acid and dried with 200 μl of acetone. The bound ^{33}P-labeled substrate was measured in a TopCount liquid scintillation counter with 25 μl of Ultima Gold scintillation solution (both from PerkinElmer Life and Analytical Sciences, Waltham, MA). The tests were performed in duplicate. Furthermore, the assays were validated with a reference compound to ensure reproducibility of the assays.

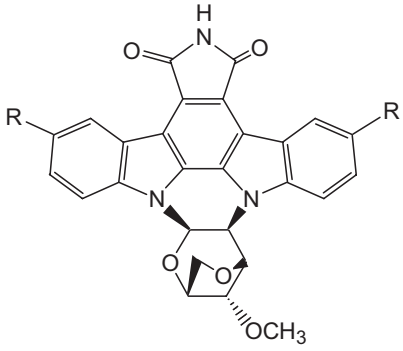
Inhibition assays toward other kinases were performed by Millipore's kinase profiler screening service. Inhibition assays toward the six kinases (CDK1/cyclin B, CDK5/p25, PIM1, PKCζ, ERK1, GSK3) and the cdc25A phosphatase shown in Table 3 were performed as described previously (Leclerc et al., 2001).

DNA Binding Assays. UV/visible absorption spectrometry was performed using an Uvikon 943 spectrophotometer (Kontron, Zurich, Switzerland). The spectrum of a solution of 20 μM concentrations of the various drugs prepared in 1 ml of BPE buffer [6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA, pH 7.1] was recorded from 230 to 500 nm in the presence or absence of calf thymus DNA (20 μM) and were referenced against a quartz cuvette containing the same DNA concentration in the same buffer.

Topoisomerase I-Mediated DNA Relaxation and Cleavage Assays. Increasing concentrations of the various rebeccamycin derivatives, as indicated in the figures, or camptothecin (CPT, 20 μM) were incubated with supercoiled pUC19 plasmid DNA (120 ng) or a 117-base pair 3′-end-labeled DNA fragment as described previously (Messaoudi et al., 2006) in 20 μl of relaxation buffer (50 mM tris(hydroxymethyl) aminomethane, pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, and 1 mM ATP) for 15 min at 37°C to ensure binding equilibrium before the addition of human recombinant topoisomerase I enzyme (4 units; TopoGEN, Inc., Port Orange, FL) for a further 30-min incubation at 37°C. The cleavage reactions were then stopped through the inhibition of topoisomerase I by addition of SDS for a final concentration of 0.25% and proteinase K to a final concentration of 250 μg/ml and a 30-min incubation at 50°C. The DNA samples were then differentially separated depending on the aim of the experiment (DNA relaxation or cleavage potency) and on the size of the various DNA substrates. For topoisomerase I-induced DNA relaxation profiling, the plasmid DNA samples supplemented with 3 μl of the loading buffer were loaded onto a 1% agarose gel lacking ethidium bromide at room temperature for 2 h at 120 V in Tris-borate/EDTA buffer (89 mM Tris base, 89 mM boric acid, and 2.5







20 $R_1 = R_2 = H$, $R_3 = NH_2$, HCl
21 $R_1 = CH_2\text{-}CH_2\text{-}N(C_2H_5)_2$, HCl, $R_2 = R_3 = H$
22 $R_1 = R_3 = H$, R_2 = 3,9-diCHO
23 $R_1 = R_3 = H$, R_2 = 3,9-diCH_2OH
24 $R_1 = R_3 = H$, R_2 = 3,9-diNH_2
25 $R_1 = R_3 = H$, R_2 = 3,9-diBr
26 $R_1 = R_3 = H$, R_2 = 3,9-diNO_2
27 $R_1 = R_3 = H$, R_2 = 3 or 9-NO_2
28 $R_1 = R_3 = H$, R_2 = 3,9-diOH

29

30 R = H
31 R = NO_2

Fig. 3. Bridged rebeccamycin analogs with the sugar moiety linked to both indole nitrogens.

mM Na_2EDTA, pH 8.3). Gels were stained after migration using ethidium bromide. By contrast, for the topoisomerase I cleavable complex formation assay using plasmid as a substrate, the DNA samples were run on an ethidium bromide-containing agarose gel (1%) in the same condition as above. Both gels were washed and finally photographed under UV light. To identify the sequence selectivity of the cleavage sites induced by topoisomerase I poisoning effect, the radiolabeled 117-bp DNA samples treated as previously were precipitated to be then diluted in 5 μl of denaturing loading buffer containing tracking dyes. The DNA fragments were denatured by heating the samples at 90°C for 3 min and subsequent cooling on ice and resolved by electrophoresis on 8% denaturing polyacrylamide gels for 90 min at 65 W in Tris-borate/EDTA buffer. The cleaved bands were revealed using the 445SI PhosphorImager (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The identity of the bases on the DNA fragment was established from comparison with the position of guanines within the known sequence in the G-track lane obtained from the Maxam and Gilbert (1980) procedure.

In Vitro Antiproliferative Assays. The cytotoxicities toward murine leukemia L1210 and human A549 non–small-cell lung carcinoma and HT29 colon carcinoma (micromolar IC_{50} values) were evaluated as previously reported (Moreau et al., 1999b, 2003; Marminon et al., 2003a). The cytotoxicity of UCN-01 toward these three cancer cells was evaluated using the same methods.

Results

The percentages of Chk1 inhibition were evaluated at a drug concentration of 10 μM. IC_{50} values were determined for the most efficient compounds and for UCN-01 as reference

Topoisomerase I-mediated DNA cleavage and binding to DNA were evaluated by comparison with camptothecin and NB506 (Table 1). Topoisomerase I-mediated DNA cleavage by UCN-01 was also studied under the same conditions. The results concerning topoisomerase I-mediated DNA cleavage, binding to DNA, and antiproliferative activities by compounds **1** to **10** and **18** have been reported previously (Anizon et al., 2003; Prudhomme, 2004a), as well as those for compounds **11** to **15** and **17** (Moreau et al., 2003), **16** (Moreau et al., 1999b), **19** (Marminon et al., 2003a), **20** and **21** (Facompré et al., 2002). The antiproliferative activities of compounds **20** to **30** have been reported previously (Marminon et al., 2002).

UV/visible absorbance spectroscopy was performed to get an insight into the DNA binding efficiency of compounds **22** to **31**. Only small changes were observed, suggesting poor DNA binding potency of either bridged derivatives (Fig. 4). Melting temperatures studies failed to give information on

TABLE 1

Rebeccamycin analogs

Percentage of Chk1 inhibition at a drug concentration of 10 μM, IC_{50} values toward Chk1, topoisomerase I-mediated DNA cleavage, DNA binding, and in vitro antiproliferative activities against three tumor cell lines: murine leukemia L1210, and human A549 non-small cell lung carcinoma and HT29 colon carcinoma (IC_{50} μM).

Compound	Inhibition Chk1 at 10 mM	IC_{50} Chk1	Topo I	DNA	L1210	A549	HT29
	%	μM					
UCN-01		7.7	−	N.E.	0.271	N.E.	0.253
1	61.8	>10	−	−	0.14	0.3	0.3
2	5.29	N.D.	+/−	−	3.3	<10	<10
3	89.2	0.160	+++	++	0.11	2.0	2.5
4	96	0.897	+/−	++	1.1	<10	<10
5	92.3	N.D.	+	−	1.5	<10	<10
6	89.1	0.091	+/−	−	0.68	<10	<10
7	97.57	0.116	−	+++	0.023	<1	0.1
8	92.4	0.083	−	+++	0.025	<0.1	<0.1
9	96.97	0.273	−	+++	0.035	<0.1	<0.1
10	100	0.158	+/−	+++	0.35	1	1
11	6.86	N.D.	+++	−	0.29	1	<1
12	38.53	N.D.	++ (104%)	+	1.1	>10	>10
13	100	0.010	+++	+	0.11	<10	10
14	49	N.D.	++	−	1.1	>10	>10
15	11.47	N.D.	+/−	−	4.9	>10	>10
16	55.8	N.D.	+++	−	0.11	0.37	0.14
17	87	1.0	++	+	11.3	>10	>10
18	93.1	0.127	+	+	0.31	<1	1
19	88.6	0.327	++[a]	+	0.066	5.3	4.8
20	94	0.0028	−	+++	0.22	0.36	0.16
21	Inactive		−	+++	0.38	1.2	0.9
22	39	N.D.	+	−	0.17	>10	>10
23	26.55	N.D.	+	−	18.1	>10	>10
24	88.9	0.017	+	−	19.3	>10	>10
25	91.25	0.165	+	−	0.16	0.40	0.21
26	8.76	N.D.	+	−	0.13	>10	>10
27	39.73	N.D.	++	−	0.081	0.3	0.3
28	90.9	0.013	+++	−	0.68	10	>10
29	3.83	N.D.	+/−	−	70.3	>10	10
30	81	1.0	+/−	−	2.9	>10	>10
31	12.44	N.D.	+/−	−	0.26	0.1	<0.1

N.D., not determined; N.E., not evaluated.

<10, IC_{50} = 1–10; <1, IC_{50} = 0.1–1.

Topoisomerase I inhibition quantified as the percentage of DNA cleavage with the drug/percentage of DNA cleavage with camptothecin: −, <20%/CPT; +/−, 20–34%/CPT; +, 35–49%/CPT; ++, 50–74%/CPT; +++, >75%/CPT

Binding to DNA evaluated by thermal denaturation profile of the synthetic polynucleotide poly(dA-dT)·(dA-dT) with and without the drugs. Variations in melting temperature ΔT_m ($T_m^{\text{drug-DNA complex}} - T_m^{\text{DNA alone}}$) in °C: −, $-\Delta T_m \leq 5$°C; +, 5°C < ΔT_m< 20°C; ++, 20°C ≤ $\Delta T_m \leq$ 30°C; +++, ΔT_m > 30°C

[a] DNA cleavage by topoisomerase I in the presence of the drug determined using sequencing gels and compared with DNA cleavage in the presence of camptothecin (Marminon, 2003).

the relative DNA binding affinity of the various derivatives because of the strong change in the absorbance at 260 nm of the compounds in the course of heat denaturation of the DNA helix. The mode of binding to the DNA could not have been evaluated using circular dichroism measurement because of intrinsic circular dichroism of the bridged compounds. Therefore, we performed topoisomerase I-mediated relaxation of plasmid DNA in the presence of either derivative to evidence potential intercalative effects (Fig. 5). Incubation of increasing concentrations of each molecule reveals no DNA intercalative profile but an increase in the comigrating relaxed and nicked DNA forms. Separation of the open circular DNA (Nck) from the relaxed form of plasmid DNA was then obtained from migration of the samples on an ethidium bromide containing gel. By this assay, compounds such as **22**, **23**, **26**, **27**, and **28** displayed topoisomerase I poisoning effects, which are weaker than that obtained using 20 μM CPT (Fig. 6, A and B). Quantification of the covalent complex formation showed that, although compounds **23** and **28** were less active than CPT, the curve obtained using compound **28** nearly reached the reference point for CPT (20 μM, ▲, Fig. 6C). Figure 7 shows the lack or the absence of topoisomerase I poisoning effect of UCN-01 compared with camptothecin at various drug concentrations.

Localization of the cleavage site was determined by analysis on denaturing gels from comparison of either CPT or NB506 as an indolocarbazole derivative bearing strong topoisomerase I poisoning effect at sites different from that obtained with the standard poison CPT (Fig. 8). Comparison of the various sequences directly surrounding the cleavage sites reveals a strong selectivity for the 5′-TG dinucleotide sequence as for CPT (short arrows), but no poisoning effect was observed at the 5′-CG dimer, which is recognized in the same manner as the 5′-TG site by the indolocarbazole derivative NB506 (long arrows). This experiment also comforted previous analysis using agarose gels (Fig. 6) in validating the potency of compounds **22**, **23**, **24**, **26**, **27**, and **28** to act as topoisomerase I poisoning agents, whereas compounds **29** and **31** displayed much weaker inhibitory effects, and compound **30** totally failed to inhibit topoisomerase I.

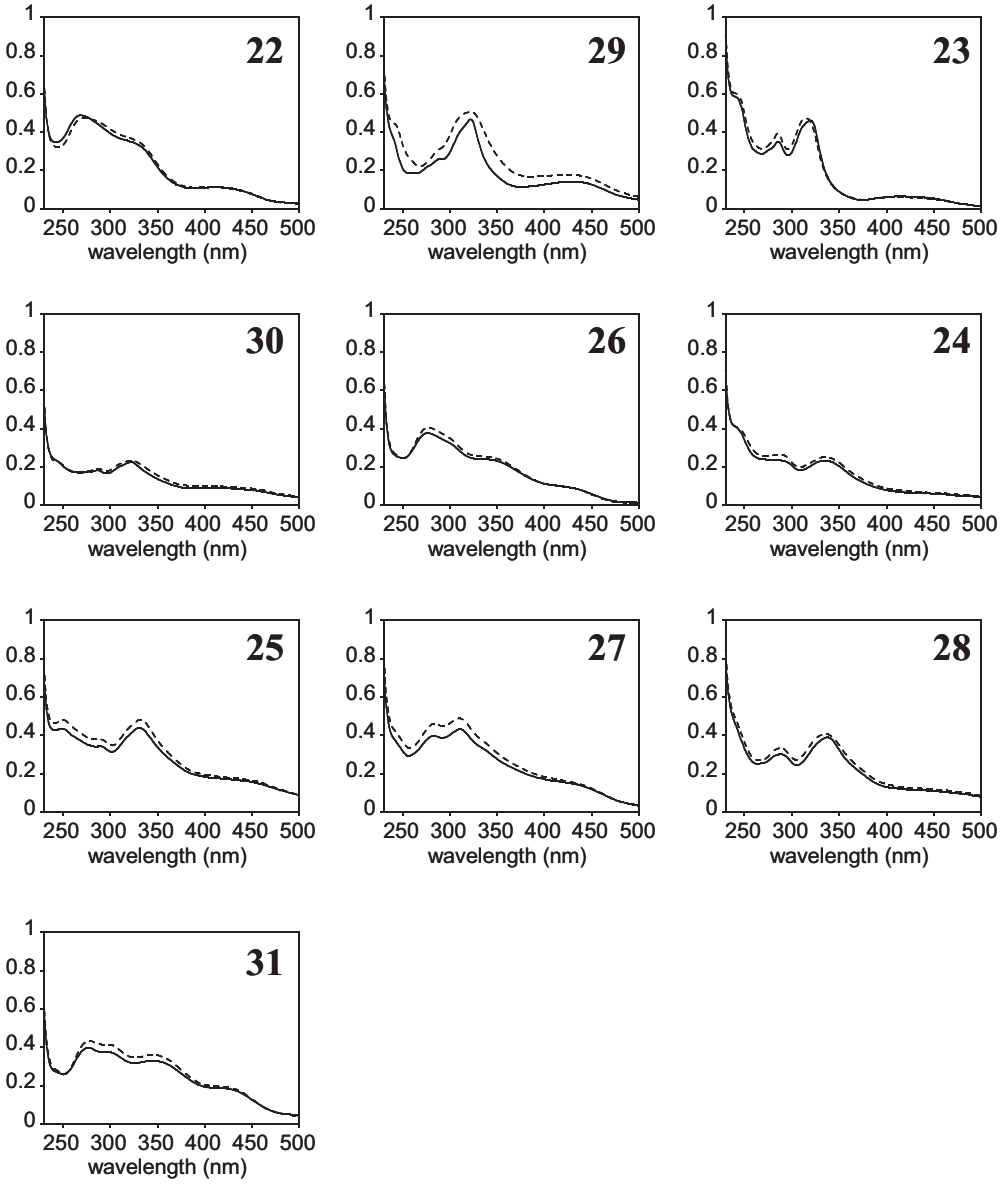


Fig. 4. DNA binding. UV/Visible absorption spectra were recorded from 230 to 550 nm using compounds **22** to **31** (20 μM) in the absence (dashed curve) or presence (plain curve) of calf thymus DNA at a phosphate/drug ratio of 20 in BPE buffer.

In the non-"aza" series, all the rebeccamycin analogs presented in Table 1 were Chk1 inhibitors except compound **21**. Because compound **21** did not induce topoisomerase I-mediated DNA cleavage, its cytotoxicity seems, at first glance, to be linked to its capacity to strongly bind to the DNA, as observed with almost all the other charged derivatives (**7**, **8**, **9**, **10**, **20**). The chlorine atoms at positions 1 and 11 that were previously found to be detrimental to DNA binding and to topoisomerase I inhibition (Bailly et al., 1997) are also detrimental to Chk1 inhibition (compare compounds **1** and **3**). In the nonbridged series, compounds **11**, **12**, **14**, **15**, and **16** substituted at positions 3 and 9 of the indole heterocycles with nitro, amino, formyl, methyl carboxylate, and bromo substituents, respectively, were poor Chk1 inhibitors. In contrast, compound **17**, bearing hydroxymethyl, and, above all, compound **13**, bearing hydroxy substituents at positions 3 and 9, were potent Chk1 inhibitors. Substitution on the imide nitrogen was detrimental to Chk1 inhibition (compound **21**). This is not surprising because in the crystal structures of staurosporine and UCN-01 in complex with Chk1, the NH of the upper heterocycle makes a fundamental hydrogen bond to the backbone carbonyl oxygen of the Glu^{85} residue.

Discussion

Among the best Chk1 inhibitors were compounds **6**, **8, 13**, **20**, **24**, and **28**. The IC_{50} values toward Chk1 of the bridged compounds **20**, **24**, **28** and that of the nonbridged compound **13** were in the nanomolar range. It can be observed that, for the cationic compounds **7** to **10**, **20**, and **21**, the strong interaction with DNA is at the expense of topoisomerase I inhibition because the cationic drugs have lost their capacity to stimulate topoisomerase I-mediated DNA cleavage. Nevertheless, compounds **7** to **10**, and especially compound **20**, were remarkable as strong Chk1 inhibitors and DNA-damaging agents. They were also potent cytotoxic drugs. Nonbridged compounds **3** (dechlorinated rebeccamycin), **13** (disubstituted at positions 3 and 9 with a hydroxy group), and **18** (bearing an exocyclic methylene group on the sugar ring) were capable to bind to the DNA, and to inhibit topoisomerase I and Chk1.

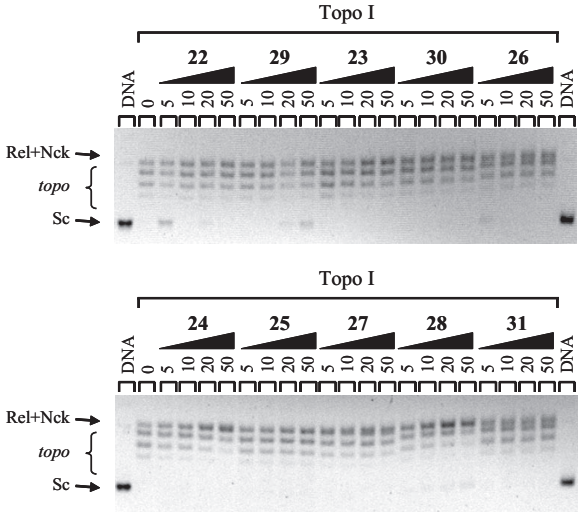


Fig. 5. Relaxation of DNA mediated by topoisomerase I. Native supercoiled pUC19 plasmid (0.13 μg, lanes DNA) was incubated with 4 units of topoisomerase I (Topo I) in the absence (lanes 0) or presence of increasing amount of the various rebeccamycin compounds specified on the lop of the lanes and at the indicated concentrations (micromolar). After treatment, the DNA samples were separated under electrophoresis on a 1% agarose gel without ethidium bromide. The gel was stained in a bath containing ethidium bromide after migration of the DNAs to visualize the various DNA conformers. *Sc* and *Rel* correspond to the supercoiled and relaxed forms of the plasmid DNA, respectively, whereas *topo* refers to the various topoisomer products generated from partially induced topoisomerase I DNA relaxation.

In the aza series (Fig. 2, Table 1), compound **19**, with the sugar moiety attached to the indole nitrogen, inhibits Chk1 significantly. Compound **19** can also induce DNA lesions by intercalation and topoisomerase I-mediated DNA single strand breaks. Like other aza rebeccamycins not described in this study, the cytotoxicity of compound **19** was selective toward the various tumor cell lines tested (Marminon, 2003a). We previously observed major differences in DNA binding properties according to the position of the sugar unit. Compounds with the sugar linked to the indole exhibited high affinity for DNA whereas, when the sugar was attached to the azaindole, DNA binding properties were abolished (Marminon, 2003a). In this series, a parallel was observed between DNA binding properties and topoisomerase I inhibition.

The activities toward either topoisomerase I, Chk1, or DNA interactions varied dramatically with the rebeccamycin derivatives. Rebeccamycin **1** and compound **15** with a dimethyloxycarbonyl were inactive against the three targets, whereas other compounds with moderate activity were specifically directed against topoisomerase I only (**14**), Chk1 only (**25**), or DNA only (**16, 21**). In addition, some compounds were either poorly soluble or not taken up by cells because they had no activity in cell-based cytotoxicity tests, despite having topoisomerase I inhibitory activity (**17**, **23**, **24**) or showed no activity in any test (**29**). With all these parameters in mind, we observed some differences in cytotoxicity that might be due to the sum of Chk1 inhibitory activity combined with either topoisomerase I activity or DNA activity. For example, compound **20**, which has both DNA damage activity and Chk1 inhibitory activity, is more cytotoxic than compound **21**, which has DNA damage activity alone. Compound **28**, which has high topoisomerase I activity and high Chk1 activity, is 10 times more cytotoxic than compound **24**, which has low topoisomerase I activity and high Chk1 activity, suggesting that the combination of these two activities may have a cytotoxic benefit. These activities would need to be further studied in detail to determine the precise contribution of each cytotoxic component. One step toward this goal was to determine whether other protein kinases could be inhibited by these compounds, therefore, we selected several representative examples for further analysis in protein kinase profiling.

To get an insight into the kinase selectivity, the inhibitory activities of bridged compounds **20**, **24**, **25**, and **28** were evaluated on a panel of 14 kinases (Chk1, AMP-activated protein kinase, Ca^{2+}/calmodulin dependent protein kinase II, casein kinase I, fibroblast growth factor receptor 3, lymphocyte-specific protein tyrosine kinase, MAP kinase 1, mitogen-activated protein kinase-activated protein kinase 2, kinase responsible for site specific phosphorylation of BAD, protein kinase A, protein kinase C β, α, and ε isoforms) in Upstate's kinase profiler screening panel. The percentages of enzyme activity were determined at a drug concentration of 1 μM (Table 2). In addition, most of the compounds described in this study were also tested for Src tyrosine kinase inhibi-

tion at a drug concentration of 1 μM, and their IC_{50} values toward six other kinases (CDK1/cyclin B, CDK5/p25, PIM1, PKCζ, ERK1, GSK3) and a phosphatase (Cdc25A) (Table 3) were determined.

It is noteworthy that in Table 2, the best inhibitory activities were found toward Chk1 for the four bridged compounds tested. In Table 3, concerning Src tyrosine kinase inhibition, the strongest Src inhibition (66.4%) was found with com-

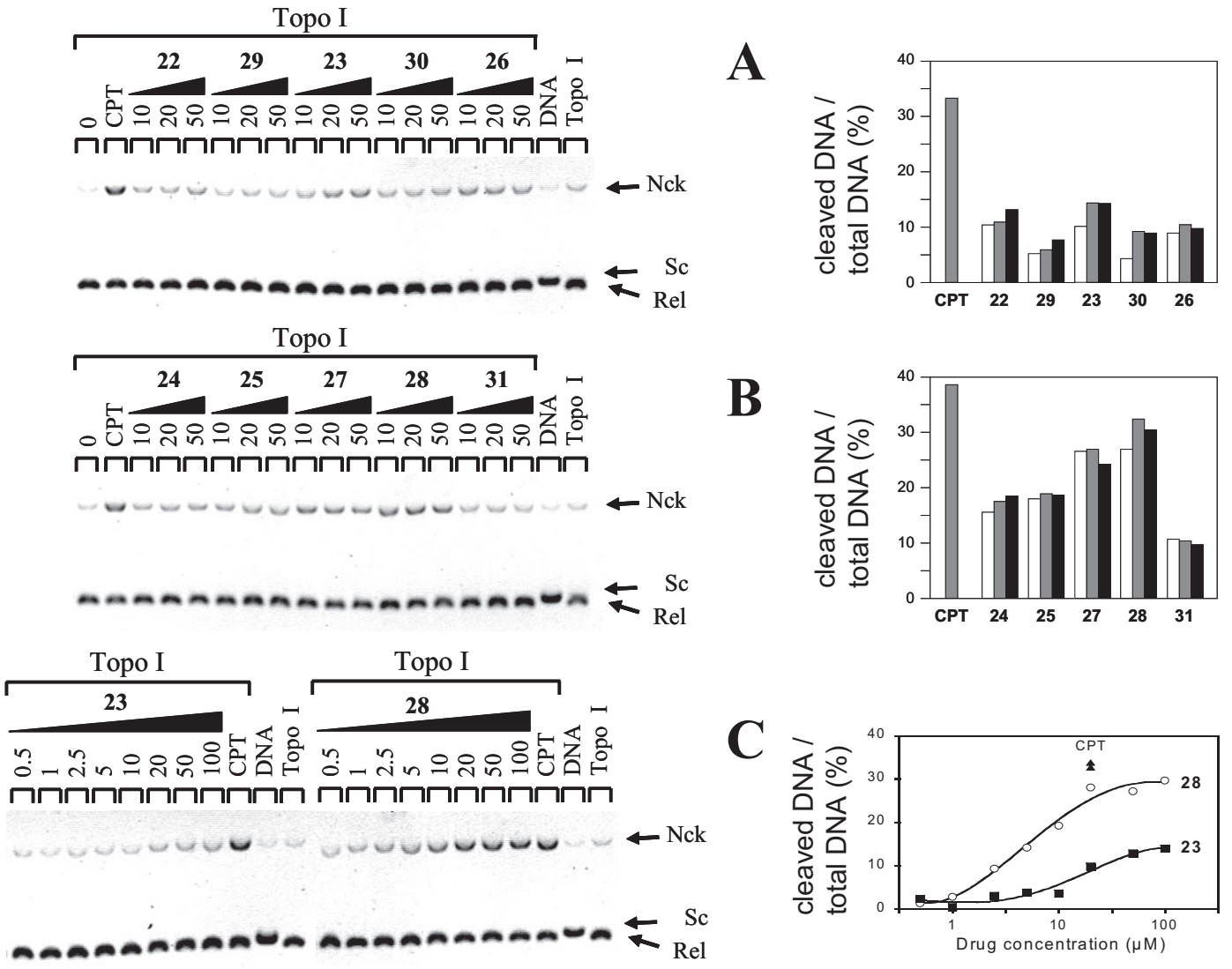


Fig. 6. Topoisomerase I-mediated DNA cleavage. Native supercoiled pUC19 plasmid (0.13 μg, lanes DNA) was incubated with topoisomerase I in the absence (lanes Topo I or 0) or presence of increasing concentrations of compounds **22** to **31** (A and B) at the concentration specified on the top of the lanes (micromolar) or CPT (20 μM) used as a reference drug. The DNA samples were separated using electrophoresis on a 1% agarose gel containing ethidium bromide. A larger range of molecule concentration from 0.5 to 100 μM was also used for compounds **23** (■) and **28** (○) to be visualized on gel (C). Both gels were subsequently quantified as the percentage of cleavable complex formation over drug concentration (right). *Sc*, *Rel*, and *Nck* refer to the supercoiled, relaxed, and open circular forms of the plasmid DNA, respectively. The percentages of Topo I/drug/DNA cleavable complex formed in the presence of 20 μM CPT as internal controls for each gels are located on the graph (▲). White, gray, or black histograms refer to 10, 20, or 50 μM, respectively.

TABLE 2

Inhibitory activity of compounds **20**, **24**, **25**, and **28** toward 14 kinases

Percentage of kinase activity at a drug concentration of 1 μM

Compound	Chk1	AMPK	CAMKII	CDK2/CyclinA	FGFR3	CKI	MAPKI	MAPKA	P70S6K	PKA	PKCβ	PKCα	PKCϵ	LCK
							%							
20	1	30	22	14	83	92	102	91	11	34	65	12	28	67
24	3	16	23	6	20	45	83	93	23	19	82	26	24	49
25	26	90	79	80	102	108	115	75	51	98	79	75	86	95
28	17	62	44	39	42	101	82	102	37	77	93	25	59	76

AMPK, AMP-activated protein kinase; CAMKII, Ca^{2+}/calmodulin dependent protein kinase II; FGFR3, fibroblast growth factor receptor 3; CKI, casein kinase I; MAPK1, mitogen-activated protein kinase 1; MAPKAPK2, mitogen-activated protein kinase-activated protein kinase 2; P70S6K, kinase responsible for site specific phosphorylation of BAD; PKA, protein kinase A; PKCβ, -α, -ϵ, protein kinases C β, α, ϵ isoforms; LCK, lymphocyte-specific protein tyrosine kinase.

pound **18** bearing an exocyclic methylene. In the aza series, compound **19** was a poor Src inhibitor. None of the rebeccamycin analogs of this study significantly inhibited PKCζ, ERK1, or the phosphatase Cdc25a. In contrast, GSK3 was inhibited by these compounds. Compound **13**, bearing two hydroxyl substituents at positions 3 and 9 on the indole moieties, was a strong GSK3 inhibitor with an IC_{50} value of 6 nM. Compound **17**, with 3,9-dihydroxymethyl, exhibited an IC_{50} value toward GSK3 of 30 nM. Most of the compounds inhibited PIM1 significantly. Compounds **6**, **13**, **18**, and **28** are multitarget compounds; they inhibited Chk1, CDK1/cyclin B, CDK5/p25, PIM1, GSK3, and topoisomerase I and were able to bind to the DNA. Nevertheless, compound **28** was approximately 10-fold more active toward Chk1 than toward CDK1. Compounds **8**, **20**, and **24** were selective toward Chk1. In particular, of the compounds presented here, compound **20**, a bridged compound with an amino function at the 6′-position of the carbohydrate unit, was the strongest Chk1 inhibitor with an IC_{50} value of 2.8 nM. It was approximately 2000-fold more active toward Chk1 than toward CDK1, CDK5, and GSK3. Compound **20** did not inhibit topoisomerase 1, although it bound strongly DNA.

To conclude, rebeccamycin derivatives represent a very interesting family of compounds of which some members have dual action of targeting DNA, either directly or via topoisomerase I inhibiting and target Chk1. Because of the complementary nature of these two activities in one compound, they might present an advantage in chemotherapy as single drugs, in contrast with the other families of Chk1 inhibitors that need to be used in combination with a DNA-damaging agent. The in vitro activities reported in this work have to be confirmed in vivo by further studies.

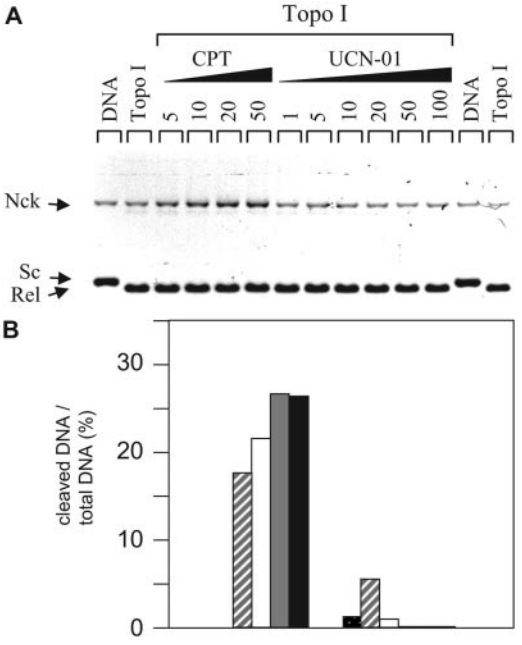


Fig. 7. Topoisomerase I-mediated DNA cleavage by UCN-01 and camptothecin. The conditions are identical to those described for Fig. 6. Ranges of molecule concentration from 5 to 20 μM for CPT and from 1 to 100 μM for UCN-01 were used.

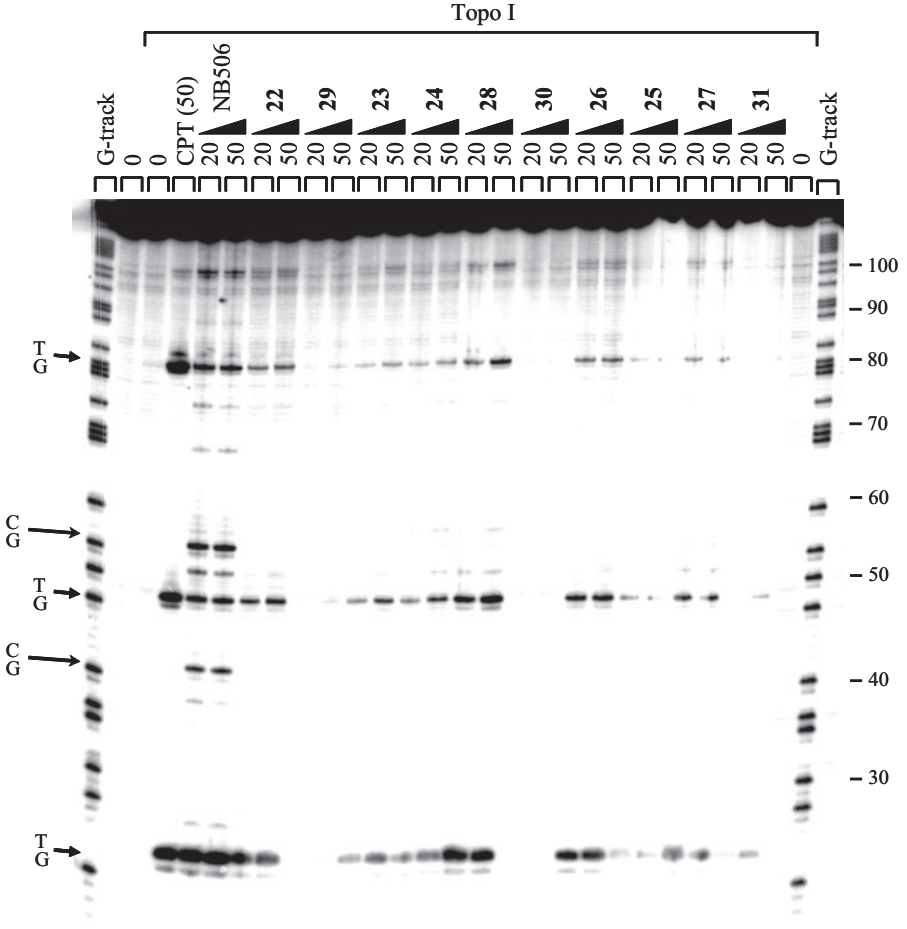


Fig. 8. Sequence localization of the topoisomerase I/DNA complexes formed in the presence of the various **22** to **31** compounds. CPT (50 μM), NB506, and compounds **22** to **31** (20 or 50 μM) were incubated with a 117-base pair radiolabeled DNA fragment before further incubation with human recombinant topoisomerase I enzyme. The DNA samples were separated on a 8% denaturing polyacrylamide gel containing urea. "*G-track*" lanes reveal the position of the guanines within the radiolabeled 117-base pair DNA fragment and are used as a marker to locate the nucleotides from the known sequence. Lanes labeled "0" do not contain any drug and were treated or not with topoisomerase I depending whether the lane is encompassed by the bracket. Short arrows indicate cleavage sites common to each topoisomerase I inhibitor and correspond to 5′-TG sequence, whereas long arrows indicate cleavage sites specific for NB506 (like the 5′-CG dinucleotide).

TABLE 3

Percentage of Src inhibition at a drug concentration of 1 μM

Inhibition of CDK1/cyclin B, CDK5/p25, PIM1, PKCζ, Cdc25a, GSK3 (IC_{50}, μM)

Compound	Inhibition Src at 1 μM	CDK1/Cyclin B	CDK5/p25	PIM1	PKCζ	ERK1	Cdc25a	GSK3
	%							
1	inactive	>5	>5	0.5–5	>5	>5	>5	<5
2	49.5	40	>100	0.5–5	>5	>5	>5	0.07
3	13.1	>10	>5	0.25	>5	5	>5	0.7
4	29.8	1	0.2	0.5–5	>5	>5	>5	0.15
5	40.4	0.2	0.07	0.5–5	>5	>5	>5	0.13
6	47.3	0.6	0.05	0.3	>5	>5	>5	0.4
7	49.6	>10	4	0.5–5	>5	>5	>5	0.4
8	5.99	6	1.6	0.5–5	>5	>5	>5	0.2
9	42.1	>10	5	0.5–5	>5	>5	>5	4
10	36.3	>5	>5	0.5–5	>5	>5	>5	4
11	28.8	>5	>5	>5	>5	>5	>5	>5
12	46.8	10	>5	0.5–5	>5	>5	>5	3
13	N.D.	0.08	0.04	0.1	<5	>5	>5	0.006
14	N.D.	>5	>5	0.5–5	>5	>5	>5	>5
15	N.D.	>5	>5	0.5–5	>5	>5	>5	>5
17	N.D.	>5	>5	0.5–5	>5	>5	>5	0.03
18	66.4	0.035	0.013	0.5–5	>5	<5	>5	0.15
19	4.9	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20	38.4	0.55	0.5	N.D.	N.D.	N.D.	>10	0.6
21	Inactive	>10	>10	N.D.	N.D.	N.D.	>10	>10
22	N.D.	>5	>5	0.5–5	>5	>5	>5	>5
23	32.4	>5	>5	>5	>5	>5	>5	4
24	46.3	>10	0.6	0.5–5	>5	>5	>5	7
25	32.1	>5	>5	>5	>5	>5	>5	>5
26	27.3	>5	>5	>5	>5	>5	>5	>5
27	5.4	>5	>5	0.5–5	>5	>5	>5	>5
28	Inactive	0.12	3	0.25	>5	>5	>5	0.2
29	47.1	>5	>5	0.5–5	>5	>5	>5	>5
30	N.D.	3	2	0.5–5	>5	>5	>5	0.13
31	18.5	>5	>5	>5	>5	>5	>5	>5

N.D., not determined.

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