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ANTITRYPANOSOMAL ACTIVITY OF CAMPTOTHECIN ANALOGS

STRUCTURE–ACTIVITY CORRELATIONS

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Abstract—African trypanosomes (*Trypanosoma brucei* species) are parasitic protozoa that cause lethal diseases in humans and cattle. Previous studies showed that camptothecin, a potent and specific inhibitor of DNA topoisomerase I, is cytotoxic to African trypanosomes and related pathogenic hemoflagellates (Bodley AL and Shapiro TA, *Proc Natl Acad Sci USA* **92:** 3726–3730, 1995). In this study, a series of camptothecin analogs was tested against axenically cultured, bloodstream form, *T. brucei*. Modifications to the pentacyclic nucleus of camptothecin ablated antiparasitic activity. In contrast, activity could be increased by substituents added to the parent ring system (e.g. 10,11-methylenedioxy or ethylenedioxy groups; alkyl additions to carbon 7; or 9-amino or 9-chloro substituents). Cytotoxicity was correlated with the level of cleavable complexes in trypanosomes, implicating topoisomerase I as the intracellular target for these compounds. To obtain some indication of selective toxicity, ten compounds were also tested against L1210 mouse leukemia cells. The 9-substituted-10,11-methylenedioxy analogs caused a disproportionate increase in antiparasitic activity, compared with mammalian cell toxicity. These findings provide a basis for designing further structural modifications and for selecting camptothecin analogs to test in animal models of trypanosomiasis.

Key words: camptothecin; *Trypanosoma brucei;* topoisomerase I; structure–activity relationship; antitrypanosomal activity

20(*S*)-Camptothecin, a novel pentacyclic alkaloid from the stem wood of *Camptotheca acuminata*, was isolated and structurally characterized based on its powerful antitumor properties [1]. Although studies attempting to determine the molecular mechanism of action of this drug began in the early 1970s, its cellular target, type I topoisomerase (EC 5.99.1.2), remained a mystery until 1985 [2]. Topoisomerase I catalyzes changes in DNA topology by breaking one strand of a DNA duplex, allowing the second strand of the duplex to pass through the break, and then resealing the nick. This relaxation activity is required for removal of the supercoils generated during cellular replication and transcription of DNA (see Refs. [3–6] for a review of topoisomerases). Camptothecin inhibits topoisomerase I by trapping the enzyme in a complex with DNA, termed the cleavable complex, which produces covalent protein–DNA linkages upon exposure to a protein denaturant ([2]; reviewed in [7]). The cytotoxic action of camptothecin is specific for topoisomerase I [8]; it is S phase specific [9, 10], and it appears to require an interaction between the cellular replication machinery and the drug-trapped, cleavable complex [11, 12]. By the mid-1980s, a substantial number of camptothecin analogs had been prepared and evaluated for antitumor activity, and these efforts were greatly stimulated by elucidation of the molecular mechanism of action of camptothecin [13–18].

Our research has focused on the interaction of camptothecin with African trypanosomes (*Trypanosoma brucei* species). These unicellular parasites, transmitted by the bit of a tsetse fly, cause sleeping sickness in humans and a related disease in cattle [19]. Untreated African trypanosomiasis is ultimately fatal. Four drugs (suramin, pentamidine, melarsoprol and eflornithine) are currently available to treat trypanosomiasis (reviewed in [20]). Of these, only melarsoprol and eflornithine are effective against the meningoencephalitis that develops in the late stage of the disease. All four drugs require lengthy, parenteral administration, and all but eflornithine have frequent, severe, toxic side-effects. Moreover, drug resistance is increasingly reported. Clearly, the development of new agents to kill trypanosomes is urgently needed.

Topoisomerase I activity has been isolated from *Trypanosoma cruzi* [21] and other kinetoplastids (*Leishmania donovani* [22] and *Crithidia fasciculata* [23]). In previous studies with African trypanosomes, we found that treatment with 20(*S*)-camptothecin produced covalent adducts with nuclear and mitochondrial DNA, inhibited DNA replication, and resulted in cell death [24]. Topoisomerase I thus appears to be a suitable target for antitrypanosomal chemotherapy. We also found that camptothecin effectively killed *T. cruzi* and *L. donovani*, closely related hemoflagellates that cause Chagas' disease and leishmaniasis, respectively. These observations suggest that topoisomerase I-targeting agents may have broad spectrum antiprotozoal activity.

In light of the scant financial resources available to develop new antiparasitic drugs [19, 25], we hoped that the enormous battery of camptothecin analogs, already synthesized as potential antitumor drugs, might prove useful against trypanosomes. To explore this possibility, we studied the molecular and cytotoxic effects on *T. brucei* of twenty-nine camptothecin analogs. These com-

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pounds were carefully selected to represent a spectrum of antitumor activity that correlates well with inhibition of purified mammalian topoisomerase I ([26–29] and §). With these analogs, we have been able to delineate some of the structural features of camptothecin that are essential for antiparasitic activity, and to identify substituents that increase this activity nearly 40-fold. We also demonstrate that antitrypanosomal activity, as compared with mammalian cell toxicity, was selectively enhanced by the 9-substituted-10,11-methylenedioxy-20(*S*)-camptothecin analogs.

MATERIALS AND METHODS

Parasites

Bloodstream form *T. brucei* (MiTat 1.2, strain 427) were grown axenically in HMI-9 medium containing phenol-red free Iscove's modified Dulbecco's medium (Mediatech, Inc.) [30]. L1210 mouse leukemia cells were cultured in Dulbecco's modified minimal medium containing 4.5 g/L glucose, supplemented with 10% heat-inactivated horse serum and 20 mM L-glutamine. All cells were grown at 37° in a humidified incubator, maintained at 5% CO_2.

Camptothecin analogs

Camptothecin analogs, listed in Tables 1 and 2, were synthesized as described ([28, 29, 31, 32], and *). The compounds were analyzed for purity by TLC and HPLC, and characterized by i.r. UV, and NMR spectroscopy. For cytotoxicity assays, each analog was dissolved in DMSO (Aldrich, 99 + %) at stock concentrations ranging from 2 to 20 mM, depending on the solubility of the compound, and stored at −80°. Serial dilutions were made in DMSO.

Cytotoxicity assay

A modification of the acid phosphatase cytotoxicity assay was used† [33]. Briefly, exponentially growing *T. brucei* (199 μL, 10^5 cells/mL) or L1210 cells (199 μL, 7×10^4 cells/mL) were added to each well of a 96-well microtiter plate containing inhibitor solutions for DMSO (1 μL/well). Each concentration was tested in quadruplicate. Plates were incubated at 37° for 24 hr (*T. brucei*) or 48 hr (L1210). Acid phosphatase activity in the surviving cells was assayed by adding *p*-nitrophenyl phosphate (20 μL of a 20 mg/mL solution in 1 M sodium acetate, pH 5.5, 1% Triton X-100), and then continuing the incubation at 37° for 4–6 hr. The enzymatic product (*p*-nitrophenol) was measured at 405 nm on a microtitre plate reader (Molecular Devices). The data were fit to the equation for the sigmoidal E_{max} model [34] to obtain concentration-response curves and EC_{50} values.

Precipitation of topoisomerase–DNA complexes

The precipitation of topoisomerase–DNA covalent complexes was performed essentially as described previously [35]. *T. brucei* were suspended in medium (3×10^6 cells/mL) and radiolabeled with [^{3}H]thymidine (340 μCi/mL; 3 hr; 37°). Washed cells (0.75 mL; 2.7×10^6 cells/mL) were incubated with drug (30 min, 37°), and then were lysed with an equal volume of 2.5% SDS, 0.8 mg/mL sheared calf thymus DNA, 10 mM EDTA. Covalent DNA-protein complexes were precipitated with KCl and counted. Samples were assayed in duplicate or triplicate. To measure total incorporation of [^{3}H]thymidine (typically 33,000 cpm/10^6 cells), an aliquot of untreated cells was transferred to Whatman 3MM filters; the DNA was precipitated with trichloroacetic acid, washed, and counted.

RESULTS

Antitrypanosomal activity of camptothecin analogs

We tested camptothecin (**19**, Table 1) and twenty-nine of its analogs for cytotoxic activity against axenically cultured, bloodstream form *T. brucei*. All drug concentrations were assayed in quadruplicate, and EC_{50} determinations were repeated two to five times. The R^2 values obtained for curves fitted to the concentration–response data were always greater than 0.97, and the standard deviations in multiple determinations of EC_{50} values were less than 22% of the means. The compounds are numbered in order of antitrypanosomal potency, (**1**) being the most potent.

The analogs were divided into two major groups: those that retain the parent ring system (Table 1), and those with a change in the parent ring system or at C20 (Table 2). Analogs that retain the parent ring system were subdivided further into those without or with a 10,11-methylenedioxy group (Table 1, **a** and **b** series, respectively). As we reported previously [24], the EC_{50} for camptothecin is 1.6 μM (Table 1, **19**). The largest increase in antitrypanosomal activity occurred upon the addition of a 10,11-methylenedioxy group to the A ring. Regardless of other substituents, the 10,11-methylenedioxy moiety caused a 10- to 20-fold increase in activity (Table 1, compare the **a** and **b** series). A 10,11-ethylenedioxy addition (**5**) afforded a similar increase in cytotoxicity. Two- to four-fold enhancement of activity resulted from the addition of an alkyl group to the 7 position (**12, 13, 2, 4**), or of a chloro- or amino-substituent at carbon 9 (**14, 15, 1, 6**). Nitro-substitution at carbon 9 (**20, 9**) or small substituents added to position 10 (**22, 18, 17, 21**) generally had no significant effect. Substituents at carbon 11 (**25, 28**) or carbon 12 (**24**) markedly diminished activity.

Modification of the pentacyclic nucleus of camptothecin resulted in a substantial reduction, or total loss, of antitrypanosomal activity (Table 2). Analogs that possess a ring D benzene substitution (**27**), that lack the A ring (**29**), or that have a lactam substitution in the E ring (**30**), were essentially inactive at soluble concentrations. Similarly, conversion of the 20(*S*)-hydroxy-group to an amino-group (**26**) abolished activity.

Analogs trap cleavable complexes in trypanosomes

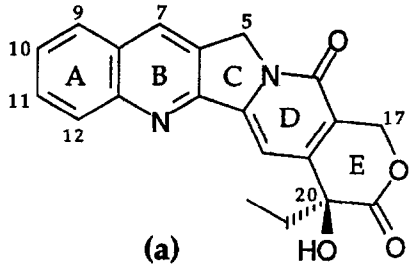
To test whether cytotoxicity correlates with the proposed molecular mechanism of action, we used the KSDS method [35] to assess six compounds, with a wide range of antitrypanosomal activity, for their ability to promote the formation of covalent protein–DNA adducts (Fig. 1). Covalent adducts arise from cleavable complexes that form in the intact cell, and they reflect the intracellular inhibition of topoisomerase I activity [26, 36]. We found that the cytotoxicity of an analog correlated well with its ability to trap cleavable complexes (Fig. 1), affirming the notion that in trypanosomes,

§ Wall ME and Wani MC, unpublished data.

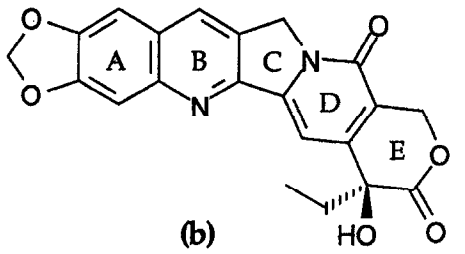
* Wall ME and Wani MC, unpublished data.

† Bodley AL and Shapiro TA, Drug cytotoxicity assay for African trypanosomes and *Leishmania*. *J Infect Dis*, in press.

Table 1. Antitrypanosomal activity of camptothecin analogs that retain the parent ring system



(a)



(b)

Modification	(a) 20(*S*)-Camptothecin			(b) 10,11-Methylenedioxy-20(*S*)-camptothecin		
	EC_{50} (μM)	Ratio*	Number†	EC_{50} (μM)	Ratio*	Number†
Parent compound	**1.6**	**1.0**	**19**	0.16	10	**7**
7-methyl				0.044	36	**2**
7-ethyl	0.63	2.5	**12**	0.060	27	**4**
7-propyl	0.80	2.0	**13**			
7-ethyl-9-amino	0.86	1.9	**16**	0.057	28	**3**
7-ethyl-9-nitro	2.7	0.59	**23**	0.17	9.4	**8**
7-ethyl-10-amino	0.62	2.6	**11**			
7-ethyl-10-nitro	0.60	2.7	**10**			
9-chloro	0.81	2.0	**14**	0.041	39	**1**
9-amino	0.84	1.9	**15**	0.074	22	**6**
9-nitro	1.6	1.0	**20**	0.40	4.0	**9**
10-methyl	2.3	0.70	**22**			
10-chloro	1.5	1.1	**18**			
10-amino	1.2	1.3	**17**			
10-nitro	2.1	0.76	**21**			
10,11-dimethoxy‡	>100.0	<0.02	**28**			
11-amino	18.0	0.09	**25**			
12-amino	12.0	0.13	**24**			
7-methyl-10,11-ethylenedioxy	0.070	23	**5**			

* EC_{50} of 20(*S*)-camptothecin/EC_{50} of modified analog.
† Compound number, ranked from the most potent (**1**) to the least potent (**30**).
‡ Mixture of 20(*RS*) enantiomers.

topoisomerase I is the cellular target for camptothecin analogs. The basis for the log-linear relationship is not clear, but it may reflect limitations of the cleavable complex assay, at the extremes of activity.

Selective toxicity

When searching for an antiparasitic drug, selective toxicity against the parasite relative to the host is essential. To identify structural features that might prove more toxic to trypanosomes than to mammalian cells, we selected for comparison a variety of mono-, di-, and trisubstituted camptothecin analogs (Fig. 2). The antiparasitic activity of these compounds ranged from 2- to 40-fold greater than that of camptothecin (the potency ratio; black bars, Fig. 2). To obtain comparable values for mammalian cells, we used our assay to determine activity against murine L1210 leukemia cells. L1210 was chosen because it is a mammalian cell line against which many camptothecin analogs have already been tested. Our results with L1210 are comparable to those reported previously for **7**, **15**, and **19** [32, 36, 37] (EC_{50} values for camptothecin and its 10,11-methylenedioxy analog were 0.12 and 0.016 μM, respectively). In contrast to the results with trypanosomes, against mammalian cells, none of these analogs was more than 20-fold more active than camptothecin (gray bars, Fig. 2). Recent studies indicate that camptothecin and its analogs are substantially more toxic to malignant, in comparison with normal, mammalian cells [38–40]. The EC_{50} values we found for L1210 cells are therefore likely to overestimate the cytotoxicity to normal mammalian tissues.

To obtain some measure of relative toxicity, we compared the potency ratios for trypanosomes with those for L1210 cells. This comparison (depicted as a number above the columns in Fig. 2) provides a means to identify structural modifications that improve antitrypanosomal activity relative to mammalian cell toxicity. Increasing numbers indicate an increase in the relative toxicity against trypanosomes. No compound had a ratio of less than 1, i.e. no analog showed a greater increase in toxicity (relative to camptothecin) to mammalian cells than to trypanosomes. Most compounds had a ratio of 2 or less. Four of the analogs had ratios of 4 or more (Fig. 2; **1**, **3**, **6**, **8**). All four of these have both the 10,11-methylenedioxy group and a substitution at carbon 9. Interestingly, selective toxicity appeared to be unrelated to antitrypanosomal activity, i.e. the most potent inhibitors were not necessarily the most selective.

DISCUSSION

Camptothecin, a well-characterized topoisomerase I inhibitor [7], has molecular and cytotoxic effects on Af-

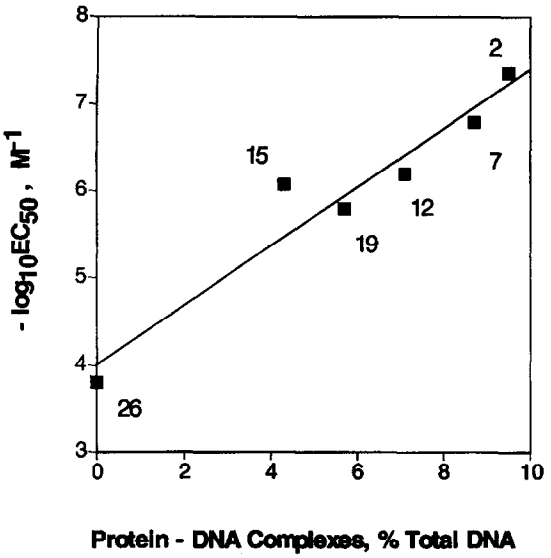
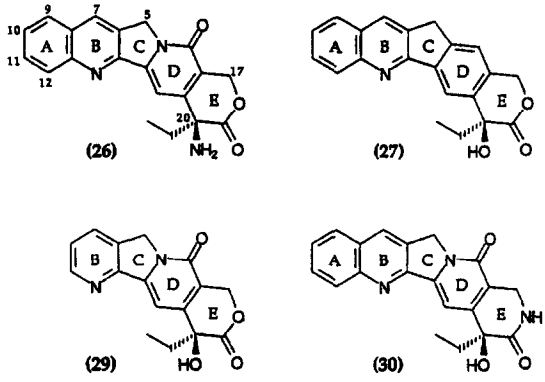


Fig. 1. Correlation of cytotoxicity with cleavable complex formation in *T. brucei*. Camptothecin (**19**) and five of its analogs (**2, 7, 12, 15, 26**) with a range of antitrypanosomal activity (Tables 1 and 2) were chosen for analysis. Each compound was assayed at 0.5 μM for its ability to promote covalent protein-DNA complex formation, by the KSDS method [35]. Increasing levels of cleavable complex formation correlated well with increasing antitrypanosomal activity (R^2 = 0.929). The level of cleavable complexes from no drug control cells has been subtracted from the data. This value (12.9% total DNA) is a measure of naturally occurring cleavable complexes, and is comparable to those reported previously [24].

rican trypanosomes and closely related pathogens [24]. To extend this work we have now tested an array of camptothecin analogs for their ability to trap protein–DNA complexes and to kill trypanosomes *in vitro*. Many

Table 2. Antitrypanosomal activity of camptothecin analogs with modifications in the parent ring system or at C20



Compound	EC_{50} (μM)	Number*
Camptothecin	**1.6**	**19**
20(*S*)-Amino-camptothecin	160	**26**
Ring D-benzo-20(*S*)-camptothecin	>100	**27**
Tetracyclic-20(*S*)-camptothecin	Inactive	**29**
Ring E-lactam-20(*S*)-camptothecin	Inactive	**30**

* Compound number, ranked from the most potent (**1**) to the least potent (**30**).

of these analogs showed large increases in potency, ranging up to 40-fold more active than camptothecin (Table 1). Antiparasitic activity correlated well with the ability to promote the formation of covalent protein–DNA adducts (Fig. 1), in accord with the concept that the cellular target of these agents is topoisomerase I.

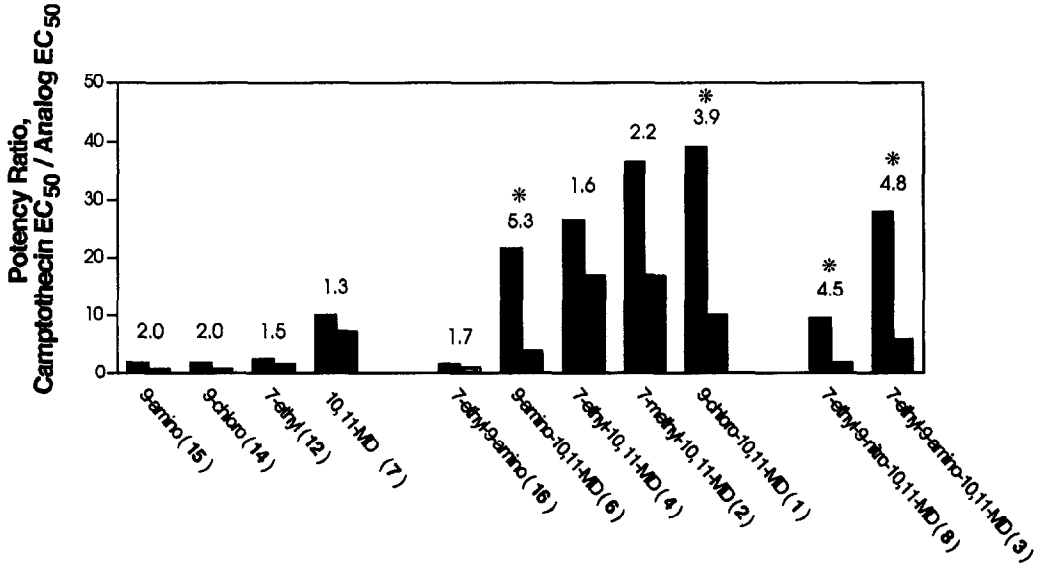


Fig. 2. Effect of substituents on potency and selectivity. The cytotoxicity of selected analogs, relative to camptothecin, against *T. brucei* (black bars) or L1210 cells (gray bars) was determined. Compounds are arranged in groups with one, two, or three substituents, and within each group are displayed in order of increasing antiparasitic activity. Selective toxicity (numbers above the bars) is a ratio of the relative toxicities: [(camptothecin EC_{50}/analog $EC_{50})_{T.\ brucei}$]/[(camptothecin EC_{50}/analog $EC_{50})_{L1210}$]. Increasing numbers indicate an increase in relative potency against trypanosomes, compared with L1210 cells; compounds with the greatest increase are marked with an asterisk. MD = 10,11-methylenedioxy.

To identify structural modifications that might impart selective toxicity, we assayed ten of the analogs against mammalian L1210 leukemia cells. Interestingly, activity against trypanosomes (black bars, Fig. 2) and against mammalian cells (gray bars, Fig. 2) did not entirely parallel one another. The discrepancy was most notable in the 9-substituted-10,11-methylenedioxy analogs. These were ten to forty times as effective as camptothecin in killing trypanosomes, yet caused less than a 10-fold increase in mammalian cell toxicity (Fig. 2). Although these effects were not great enough to make any analog more toxic to trypanosomes than to mammalian leukemia cells, this discrimination between the two cell lines recommends the 9-substituted-10,11-methylenedioxy structural motif as a starting point for attaining selective toxicity. Tumor cells, relative to their nonmalignant counterparts, are unusually sensitive to the toxic effects of camptothecin analogs [38–40]. Indeed, nonmalignant cells in culture appear unaffected by concentrations of camptothecin analogs that kill tumor cells. Hence, we anticipate that normal mammalian cells would yield significantly higher EC_{50} values than those obtained for L1210 cells, providing a real margin of selective toxicity. However, the most meaningful test of selective toxicity obviously lies in animal studies.

Camptothecin lactone and at least four of its analogs, 9-amino-20(*S*)-camptothecin, 9-[(dimethylamino)methyl]-10-hydroxy-camptothecin (Topotecan), 7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxy-20(*S*)-camptothecin (CPT-11, Irinotecan), and 7-(4-methylpiperazinomethylene)-10,11-ethylenedioxy-20(*S*)-camptothecin (G1147211) are currently in clinical trails [41–44]. It is hoped that the structure–activity information provided by *in vitro* antiparasitic assays will help us to identify truly selective inhibitors, and in conjunction with the encouraging data emerging from clinical trials, will provide an efficient route toward developing much-needed new drugs for trypanosomiasis and related parasitic diseases.

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