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Effect of Mitonafide Analogs on Topoisomerase II of *Leishmania chagasi*

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Mitonafide (4-nitro-benzoisoquinolinedione) and a number of structural analogs were synthesized and studied in order to determine the structural requirements for inhibition of leishmanial nuclear and kinetoplast topoisomerase II and human topoisomerase II. The structure-activity relationship studies with the mitonafide analogs demonstrated that there was selective targeting of leishmanial nuclear topoisomerase II and human topoisomerase II and differential targeting of kinetoplast over nuclear topoisomerase II in the parasite. Mitonafide analogs appeared to have multiple mechanisms of action leading to death of leishmanias, but several compounds that affected kinetoplast but not nuclear topoisomerase II were not cytotoxic as determined by short-term assays. These studies provide new insight into the differential sensitivities of leishmanial nuclear and kinetoplast topoisomerase II to topoisomerase II-targeting drugs.

Leishmania species cause a spectrum of human diseases ranging from cutaneous skin lesions to more severe visceral infections. Traditional first-line therapy involves pentavalent antimonial compounds which must be delivered intravenously or intramuscularly over long periods of time, have a number of adverse side effects, and are associated with increasing therapeutic failure (22). In unresponsive cases of leishmaniasis, several other drugs, such as pentamidine and amphotericin B, have been utilized; however, they are also associated with substantial toxicities. More effective and specific antileishmanial chemotherapy is therefore needed.

DNA topoisomerase II (Topo II) is a fundamental enzyme in both prokaryotic and eukaryotic cells which alters the topology of DNA through a catalytic sequence involving induction of a double-strand cut, passing of a second strand through the break, and resealing of the DNA strand break. Topo II has been targeted therapeutically by various antibacterial and antitumor agents. A number of prokaryotic Topo II (gyrase) inhibitors, including ciprofloxacin, ofloxacin, and norfloxacin, have been identified and are used as antibacterial agents (21). There is a large structural diversity among eukaryotic Topo II-targeting drugs, and they include the following classes of agents: anthracyclines, ellipticines, epipodophyllotoxins, anthracenediones, and bisantrenes (4, 6, 11). Some of these agents are among the most widely used antitumor drugs.

Topo II-directed agents act by stabilizing a reversible drug-DNA-enzyme ternary cleavable complex and thereby disrupt the cleavage-religation equilibrium of Topo II, resulting in the formation of a concomitant covalent association between the Topo II and the broken strand(s) of the duplex DNA. These lesions can create a cytotoxic event and eventually lead to cell death (4, 11). As Topo II is present in all organisms and targeting of Topo II has been successful in cancer chemotherapy and antibacterial therapy, it is believed that Topo II in leishmanias may be a viable target for antiparasitic therapy.

It has been shown previously in our laboratories and in others that leishmanial and trypanosomal Topo II may be a cytotoxic target of known eukaryotic and prokaryotic Topo II inhibitors (5, 8, 13, 19, 20, 23, 24). Topoisomerases have been identified in the nuclear matrix as well as in the kinetoplast of *Leishmania donovani* (3). Shapiro et al. have shown that differential sensitivity to some mammalian topo II inhibitors and antibacterial agents exists between kinetoplast Topo II and nuclear Topo II (19, 20). It has been shown previously in our laboratory that anilinoacridine compounds stabilize cleavable complexes with both nuclear and kinetoplast Topo II in *Leishmania chagasi*; however, these compounds also exhibit cytotoxicity against the human enzyme and are not selective agents. We postulate that differential sensitivity between parasitic and human Topo II exists and could be exploited for chemotherapy. We also hypothesize that distinct activities may exist between nuclear and kinetoplast Topo IIs in leishmanias and that drugs which target one enzyme over the other could be developed.

Amonafide (4-amino-benzoisoquinolinedione) (Fig. 1) [R_1 = NH_2, R_2 = H, and R_3 = $(CH_2)_2N(CH_3)_2$] and a structural analog mitonafide, 1 (Table 1), have been shown to intercalate with DNA and inhibit both DNA and RNA synthesis (2). Hsiang et al. demonstrated that these drugs stabilize Topo II-cleavable complexes in vitro (9). We have synthesized a number of structural analogs of mitonafide, shown in Table 1 and Fig. 1, in order to determine the structural requirements for inhibition of nuclear and kinetoplast Topo II in *L. chagasi* and human Topo II in order to develop compounds with potential selectivity for the inhibition of leishmanial Topo II.

MATERIALS AND METHODS

Chemicals. Mitonafide and its structural analogs, shown in Table 1 and Fig. 1, were synthesized by refluxing the appropriate naphthalic anhydride with the

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TABLE 1. Effect of various mitonafide analogs on human and leishmanial Topo II[a]

Compound	R_1	R_2	R_3	Stimulation of human Topo II-cleavable complex	Stimulation of *L. chagasi* nuclear Topo II-cleavable complex	Linearization of kDNA	C_{50} (μM)	*L. chagasi* LD_{90} (μM)
1	NO_2	H	$(CH_2)_2N(CH_3)_2$	++++	+++(+)	++++	11	88
2	NO_2	H	$(CH_2)_3N(CH_3)_2$	++++	+++	+++	16	75
3	NO_2	H	$(CH_2)_2N(CH_2CH_3)_2$	++	+++	++++	35	20
4	NO_2	H	$(CH_2)_2NH_2$	+++	−	(+)	>100 (27%)	>100
5	NO_2	H	$(CH_2)_2NHCH_3$	++++	−	++++	14	>100
6	NO_2	H	$(CH_2)_2OH$	+++	−	++++	>100 (25%)	>100
7	NO_2	H	$(CH_2)_2X$	++++	+++(+)	+++	10	38
8	NO_2	H	$(CH_2)_2Y$	+	+(+)	+	>100 (10%)	>100
9	NO_2	H	$(CH_2)_2N(CH_2CH_2)_2N$	−	−	++	>100 (39%)	>100
10	H	NO_2	$(CH_2)_2N(CH_3)_2$	−	−	+++(+)	>100 (33%)	95
11	H	NO_2	$(CH_2)_3N(CH_3)_2$	−	−	++++	>100 (36%)	>100
12	H	NO_2	$(CH_2)_2N(CH_2CH_3)_2$	+	+++	+++(+)	28	13
13	H	NO_2	$(CH_2)_2NH_2$	+	−	++++	17	18
14	H	NO_2	$(CH_2)_2NHCH_3$	+	−	++++	19	19
15	H	NO_2	$(CH_2)_2OH$	++	−	−	>100 (34%)	42
16	H	NO_2	$(CH_2)_2X$	++	−	−	11	13
17	H	NO_2	$(CH_2)_2Y$	−	−	(+)	>100 (0%)	>100
18	H	H	$(CH_2)_2N(CH_3)_2$	+	−	++	100	>100
19	H	H	$(CH_2)_2N(CH_2CH_3)_2$	−	+++++	+++	100	37
20	H	H	$(CH_2)_2NH_2$	+	+	(+)	50	20
21	H	H	$(CH_2)_2NHCH_3$	+	+++	++(+)	>100 (41%)	54
22	H	H	$(CH_2)_2OH$	+	+(+)	−	>100 (12%)	>100

[a] The stimulation of human Topo II-cleavable complex was assessed with purified human Topo II and pUC 18 DNA. The amount of cleavage was determined qualitatively on an agarose gel and compared with azatoxin and etoposide, which are designated +++++, and *m*-AMSA, designated ++++. The stimulation of *L. chagasi* nuclear Topo II-cleavable complex and the linearization of *L. chagasi* minicircles were determined as described in Materials and Methods and were compared with *m*-AMSA, which has been designated +++++. The number in parentheses for the compounds with a C_{50} value of >100 indicates the percentage of decrease in fluorescence at 595 nm at a drug concentration of 100 μM. LD_{90}, 90% lethal dose. For structures of the R groups, please see Fig. 1.

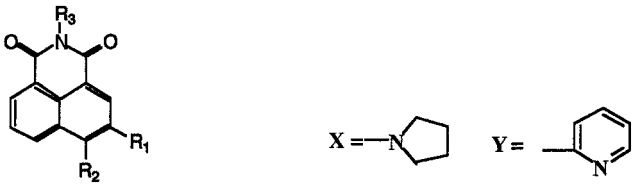

FIG. 1. Chemical structure of mitonafide analogs.

appropriate amine containing the desired R_3 side chain in absolute ethanol as described previously (2). 4′-(9′-Acrinylamino)methanesulfon-*m*-aniside (*m*-AMSA) was provided by the National Cancer Institute in Bethesda, Md. All drugs were dissolved in dimethyl sulfoxide at 10 mM and stored at 4°C. Restriction enzymes and a random primer DNA labeling kit were obtained from Gibco BRL. pUC 18 plasmid DNA was purified from *Escherichia coli* DH5α with a Midi plasmid kit (Qiagen, Chatsworth, Calif.). Kinetoplast DNA (kDNA) was isolated from *L. chagasi* with cesium chloride gradients as described previously by Englund (7).

Parasites. *L. chagasi* (strain MHOM/BR/86/L669) was maintained by intracardiac inoculation of amastigotes into Syrian hamsters. The amastigotes were isolated from the hamster spleen and allowed to convert into promastigotes in modified HO-MEM media containing 10% fetal calf serum, penicillin, and gentamicin (1, 17).

Cytotoxicity studies in vitro. The lethal dose was defined as the concentration of drug that reduced the number of motile promastigotes by 90% as assessed microscopically with a hemocytometer (15, 16).

Drug-DNA binding assay. The binding of mitonafide analogs to DNA was determined by a decrease in fluorescence of an ethidium bromide-DNA solution as described previously (14). C_{50} values were calculated as the concentration of drug at which a 50% decrease in fluorescence at 595 nm occurred.

Whole-cell KCl-sodium dodecyl sulfate (SDS) precipitation of protein-associated DNA strand breaks in *L. chagasi* promastigotes. Promastigotes (5×10^6 parasites per ml) were treated with 20 μCi of [^{3}H]thymidine in 20 ml of HO-MEM for 12 h at 25°C. The labeled parasites were treated with drugs for 1 h at 25°C. The cells were lysed with 0.625% SDS by the method of Shapiro et al. (20), and the protein-associated strand breaks were precipitated with 35 μM KCl as outlined by Rowe et al. (18).

Preparation of a radiolabeled linearized kDNA probe. The kDNA network (5 μg) was completely digested with 10 U of *Taq*I in REact 2 buffer (provided by Gibco BRL) for 1 h at 65°C. The linearized kDNA was labeled with [α-^{32}P]dATP with the random primer kit.

Drug treatment of *L. chagasi* promastigotes and isolation of DNA. The basic protocol with slight modifications was developed from methods published by Shapiro et al. (20). Log-phase *L. chagasi* promastigotes were incubated with 20 μM drugs dissolved in 0.2% dimethyl sulfoxide for 3 h. Cells were lysed in 5 mM Tris-HCl–37.5 mM EDTA–0.5% SDS (pH 8)–1 mg of proteinase K per ml at 37°C for 2 h. The samples were then digested with 100 μg of RNase A per ml at 37°C for 30 min. Samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitated with 0.25 volumes of 10 M ammonium acetate and >2 volumes of ice-cold ethanol.

Nuclease digestions. Samples after protease and RNase A treatment were extracted with phenol and precipitated as described above. Samples to be treated with exonuclease II were dissolved in 66 mM Tris (pH 8.0)–5 mM $MgCl_2$–10 mM 2-mercaptoethanol and digested with 130 U of exonuclease III. Digestion with 10 U of lambda exonuclease was carried out in 67 mM glycine (pH 9.4)–25 mM $MgCl_2$–50 μg of bovine serum albumin per ml. The reaction mixtures were incubated at 37°C for 30 min, and reactions were terminated with 2 μl of 0.5 M EDTA.

Gel electrophoresis and hybridization. Samples treated as above were dissolved in loading buffer and electrophoresed on a 1.5% agarose gel (20 by 13 cm) containing 1 μg of ethidium bromide per ml for 5 h at 100 V. The gel was destained for 1 h and photographed. The appropriate section of gel containing the desired DNA bands was treated with 0.25 M HCl and 0.5 M NaOH, dried on a gel dryer, and hybridized with the radiolabeled linearized kDNA probe as described previously (12). Autoradiography was carried out at −70°C with Kodak X-Omat 50 film.

RESULTS

KCl-SDS precipitation of nuclear Topo II-cleavable complexes in *L. chagasi*. Topo II-cleavable complexes are reversible in nature but can be irreversibly frozen with a strong detergent, such as SDS, or a strong base. The Topo II is covalently attached to the 5′ ends of the DNA, and these protein-associated strand breaks can be precipitated with potassium chloride. Several of the mitonafide analogs induced Topo II cleavage of DNA in the nucleus of *L. chagasi* (Table 1; Fig. 2). As a comparison, the human nuclear Topo II activities,

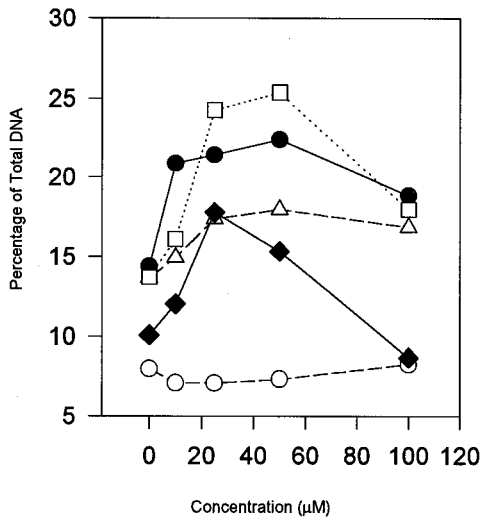

FIG. 2. Whole-cell KCl-SDS precipitation of protein-associated DNA strand breaks in *L. chagasi* promastigotes by representative mitonafide analogs. *L. chagasi* cells were labeled with [^{3}H]thymidine and treated with *m*-AMSA (●) and compounds 12 (◆), 13 (○), 19 (□), and 21 (△). The DNA strand breaks were isolated as described in Materials and Methods.

which have been discussed elsewhere (14), are included in Table 1.

An increase in cleavable complex formation occurred in a dose-dependent manner reaching a maximum between 25 and 50 μM drug; however, a decrease in DNA-cleavable complex occurred at higher drug concentrations. We have proposed that Topo II-targeting drugs interact with the DNA in such a fashion that a local distortion which mimics an intermediate in the catalytic cycle of the enzyme occurs. At higher concentrations of drug, the distortion increases so that Topo II no longer recognizes the DNA template and cleavage decreases. Also, we have previously shown that this assay targets the nuclear Topo II on the basis of densitometry data which showed that only approximately 2% of the total DNA is in the kinetoplast, while the precipitated DNA-cleavable complexes with mitonafide analogs accounted for approximately 20% of the total DNA (24).

Linearization of kinetoplast minicircles from drug-treated *L. chagasi*. Linearization of minicircle DNA is an indication of the effect of drugs on kinetoplast Topo II in *L. chagasi*. A majority of the mitonafide analogs induced the formation of linear (form III) DNA (Table 1; Fig. 3). As mentioned previously, Topo II is bound covalently to the 5′ ends of linear DNA in a stabilized cleavable complex. In order to verify that the induction of form III in the hybridization assay was due to stabilization of Topo II-cleavable complex by mitonafide analogs, exonuclease digestions of the linearized kDNA were performed. Exonuclease III digests DNA from the 3′ to the 5′ end whereas λ exonuclease digests DNA from the 5′ to the 3′ end. The linear DNA (form III) and nicked circular form of kDNA (form II), released from parasites treated with 50 μM compound 3, were digested entirely by exonuclease III (data not shown). Lambda exonuclease had no effect on form II or III of kDNA. When proteinase K treatment was omitted, the solubility of the cleaved minicircles was affected and the protein-bound linear species remained at the phenol-water interface during extractions. Taken together with the exonuclease data, these studies indicate that a protein is linked covalently at the 5′ ends of the form III DNA and provide confirmation that the minicircle linearization is due to Topo II inhibition.

Cytotoxicity studies in vitro and drug-DNA binding studies. The cytotoxicity of mitonafide analogs against *L. chagasi* varied. The 90% lethal dose values are summarized in Table 1. The binding of mitonafide analogs to DNA was determined by an ethidium bromide displacement assay, and the C_{50} values are given in Table 1. The numbers in parentheses indicate the percentage of displacement of ethidium bromide by 100 μM drug.

DISCUSSION

The structure of the mitonafide analogs had a major effect on leishmanial nuclear and kinetoplast and human Topo II activity. Differences were observed between stimulation of cleavable complexes with purified human nuclear Topo II and leishmanial nuclear Topo II (Table 1). (5, 19). Some analogs, such as compounds 1 to 3, exhibited similar activities with the two nuclear enzymes; however, other analogs, compounds 12 and 19, affected the leishmanial Topo II in a distinct manner. A comparison of leishmanial nuclear Topo II with kinetoplast Topo II demonstrated that two distinct activities may exist. While a majority of the mitonafide analogs stimulated linearization of kDNA, only a subset of this drug class affected the human or leishmanial nuclear enzymes. These results contrast with the effects of anilinoacridines, which affected leishmanial kinetoplast and nuclear enzymes in similar fashion as well as human nuclear Topo II activity. In summary, within the mitonafide series selective inhibition between human and leishmanial Topo II as well as selective inhibition between leishmanial nuclear and kinetoplast Topo II was observed.

The position and presence of the nitro group appear to dictate the activity of the analogs. It is presumed that the planar aromatic region intercalates into the DNA, the side chain R_3 lies in the minor groove, and the nitro group dictates the position within the DNA. We have proposed that Topo II-targeting drugs interact with DNA in such a fashion that a local distortion which mimics an intermediate in the catalytic cycle of the enzyme occurs. It has been proposed that a drug may take on several conformations of interaction with the DNA but only one of these association modes can stabilize cleavable complex formation.

With the nitro in the 4 position ($R_1 = NO_2$), there appears to be a correlation between the tertiary substitution with small moieties on the nitrogen in R_3 and the formation of cleavable complex with leishmanial nuclear Topo II. The orientation of the side chain in the minor groove may affect drug-DNA conformation and thus affect cleavable complex formation. There is less of an apparent correlation between the structure of the side chain and the linearization of kDNA by the analogs in this series, possibly because of the different structure of DNA in

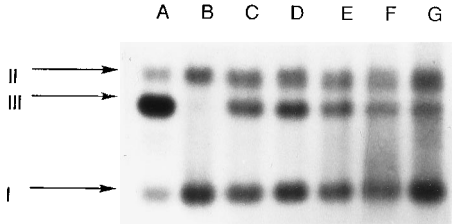

FIG. 3. Induction of minicircle linearization in drug-treated *L. chagasi* by representative mitonafide analogs. Lane A, *Taq*-restricted kDNA; lane B, dimethyl sulfoxide-treated parasites; lane C, parasites treated with 20 μM *m*-AMSA; lane D, parasites treated with 20 μM compound 1; lane E, parasites treated with 20 μM compound 2; lane F, parasites treated with 20 μM compound 10; lane G, parasites treated with compound 13.

the kinetoplast, which includes catenated mini- and maxicircles.

For the series with the nitro in the 5 position (R_2 = nitro), the human Topo II activity follows a trend opposite of the 4-nitro series and smaller, less bulky side chains appear to be preferred, possibly because of an orientation within a smaller cleft in the minor groove. The analogs possessing a nitro in the 5 position exhibit differential activity between human nuclear and leishmanial nuclear Topo II-cleavable complex formation. Also, a distinction between the two Topo II activities, nuclear and kinetoplast, within the parasite is manifested in this series. The nuclear leishmanial Topo II appears to be less sensitive, with only compound 12 having an effect on cleavable complex formation.

The series without a nitro group has diminished activity and appears to follow similar trends as the 4-nitro series. It has been previously proposed with the human activity that this series lacks the directing influence of the nitro group. Without the force to lock the drug into a particular conformation within the DNA, the drug can take on various association modes. We postulate that only one conformation induces the correct deformation which induces cleavable complex formation. As with the human Topo II activity, the nitro group appears to play a crucial role in dictating the activity of the mitonafide analogs in leishmanias.

The results with compound 16, which is cytotoxic but does not affect leishmanial Topo II, indicate that the mitonafide analogs have multiple mechanisms of action against leishmanias. As various mechanisms leading to cytotoxicity exist for mitonafide analogs, a correlation between targeting of Topo II and lethality cannot be ascertained. Several analogs including compounds 5, 6, and 11 do not exhibit cytolytic activity and affect only the kinetoplast Topo II. These results indicate that the kDNA is not necessary for the survival of the parasite, but the targeting of kinetoplast Topo II may have long-term consequences for the organism not evaluated in this study. It has been previously observed that dyskinetoplastic leishmanias are unable to replicate (10). In contrast to the kinetoplast Topo II, targeting of the leishmanial nuclear Topo II by mitonafide analogs results in a lethal event. It is also interesting to note that the mode of DNA binding does not appear to correlate strongly with Topo II activity, and one of the suggested alternative mechanisms of action is the inhibition of other DNA-processing enzymes.

The results of the structure-activity relationship studies with the mitonafide analogs indicate that selective inhibitors can be synthesized to target leishmanial Topo II. This research also demonstrates for the first time that drugs can specifically target the kinetoplast Topo II over the nuclear enzyme in leishmanias, but in the studies, inhibition of kDNA was not lethal. The drawback to mitonafide analogs is the undefined multiple mechanisms of action. This result supports the hypothesis that selective nuclear and kinetoplast Topo II-targeting drugs can be developed, and further investigations may produce a selective Topo II inhibitor which could be used for chemotherapy.

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