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SHORT COMMUNICATION

Effects of Camptothecin, a Topoisomerase I Inhibitor, on *Plasmodium falciparum*

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ABSTRACT. Currently, the treatment of falciparum malaria is seriously compromised by spreading drug resistance. We studied the effects of camptothecin, a potent and specific topoisomerase I inhibitor, on erythrocytic malaria parasites *in vitro*. In *Plasmodium falciparum*, camptothecin trapped protein–DNA complexes, inhibited nucleic acid biosynthesis, and was cytotoxic. These results provide proof for the concept that topoisomerase I is a vulnerable target for new antimalarial drug development. BIOCHEM PHARMACOL 55;5: 709–711, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. *Plasmodium falciparum*; malaria; topoisomerase; camptothecin

Malaria afflicts over 300 million people, and the inexorable spread of drug-resistant *Plasmodium falciparum* has greatly increased the need to identify molecular targets for antimalarial chemotherapy [1, 2]. Among the possible candidates are the topoisomerases, enzymes that are recognized targets for clinically important antitumor and antibacterial agents [3, 4]. Topoisomerases are essential for nucleic acid metabolism: they relax supercoiled DNA by breaking one (type I) or both (type II) strands of duplex DNA, and then allowing the passage of a second strand through the break before resealing the break [5]. During the catalytic cycle, a transient covalent complex (the "cleavable complex") forms between the topoisomerase and its DNA substrate. Cleavable complexes may be trapped permanently by the addition of a strong protein denaturant, such as SDS, to yield covalent adducts comprised of denatured topoisomerase molecules linked to the cleaved DNA substrate. In the cell, the collision of DNA tracking machinery (e.g. during DNA synthesis or RNA transcription) with drug-promoted cleavable complexes initiates a series of cellular events that culminate in cell death.

Camptothecin, a naturally occurring plant alkaloid, is the parent compound of a family of drugs currently in clinical trials. The cellular target of camptothecin is DNA topoisomerase I (EC 5.99.1.2), and numerous analogs have been synthesized as potential therapeutic agents [6]. The remarkable specificity of camptothecin for topoisomerase I was elegantly demonstrated by gene deletion experiments in yeast [7]. For malaria parasites, work on topoisomerases has focused largely on the type II enzyme [8, 9]; however, topoisomerase I has been isolated from *P. berghei* [8], and the gene has been cloned from *P. falciparum* [10]. Here, we report the molecular and cytotoxic effects of camptothecin on *P. falciparum*.

MATERIALS AND METHODS

Cleavable Complex Formation

P. falciparum was maintained *in vitro* at 37°, in O^+ erythrocytes, under 3% O_2, 4% CO_2, 93% N_2 [11]. Parasitized cells (NF54 strain; 10 mL; 2.4% hematocrit; 4.5% parasitemia) were treated with 100 μM camptothecin or 0.33% DMSO, the drug solvent, and then were lysed with 0.83% SDS and 42 mM EDTA (final concentrations). The lysate was digested with 1.67 mg/mL proteinase K at 55° for 1 hr and then were extracted sequentially with phenol and 24:1 chloroform:isoamyl alcohol. DNA was precipitated with ethanol, resuspended, separated by electrophoresis [1.5% agarose; 90 mM Tris-borate (pH 8.3), 2.5 mM EDTA, 1 μg/mL ethidium bromide; 17 hr; 1.4 V/cm], visualized by ultraviolet transillumination, and photographed. Images were scanned (Adobe Photoshop 3.0), and the bands were quantitated (Fuji MacBas 2.2).

Inhibition of Nucleic Acid Biosynthesis

Parasitized erythrocytes (NF54 strain, 1.5% hematocrit, 0.15% initial parasitemia) were incubated with or without 100 μM camptothecin (Sigma) in the presence of [^{3}H]hypoxanthine (DuPont NEN; 2.8 μCi/mL, 16 Ci/mmol). At predetermined times, duplicate 100-μL aliquots of the cultures were removed and deposited onto Whatman 3MM filter paper, and the nucleic acids were precipitated with ice-cold 5% trichloroacetic acid. Simultaneously, duplicate 75-μL samples were lysed in 0.95% SDS, 24 mM EDTA

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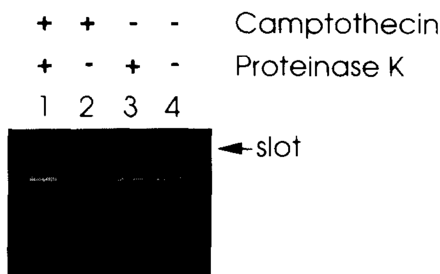

FIG. 1. Camptothecin-promoted cleavable complex formation in *P. falciparum*. Parasitized erythrocytes were treated with 100 μM camptothecin (lanes 1 and 2) or DMSO, the drug solvent (lanes 3 and 4), and then were lysed with SDS. Each lysate was divided; one half was extracted immediately with phenol (lanes 2 and 4); the other half was digested with proteinase K prior to extraction (lanes 1 and 3). Each lane contained DNA from 10^7 parasitized erythrocytes. In three experiments, the average intensity of bands for camptothecin-treated samples not digested with proteinase K (lane 2) was 62% of the proteinase K-treated control (lane 1).

and treated with 0.95 mg/mL RNase A and 1900 units/mL RNase T1 (37°, 60 min); then the remaining RNase-insensitive nucleic acids were precipitated with trichloroacetic acid. Incorporation into erythrocytes was less than 0.4% of that into infected erythrocytes. Results were analyzed with statistical tools provided in Microsoft Excel 5.0.

Cytotoxicity Assay

Camptothecin sensitivity of early log phase cultures of NF54 chloroquine-sensitive and FCR3 chloroquine-resistant *P. falciparum* was evaluated by monitoring hypoxanthine incorporation, as described previously [12], with the following modifications. Cell cultures (100 μL aliquots; 2.4% hematocrit; 0.25 or 0.5% parasitemia, for NF54 or FCR3, respectively) were placed into each well of a 96-well microtiter plate, already containing 100 μL of varied concentrations of camptothecin, 0.4 or 1.0% DMSO, or medium alone. Each drug concentration was tested in quadruplicate. The plates were incubated for 48 hr, then [^{3}H]hypoxanthine (25 μL of 25 μCi/mL stock) was added, and incubation was continued for 18 hr. The parasites were harvested (Brandel harvester) onto glass fiber filter paper, lysed with water, and label incorporated into nucleic acids adherent to the filters was measured. As described previously [13], the data were fit using the Marquardt algorithm to derive EC_{50}[‖] values.

RESULTS AND DISCUSSION

To obtain evidence for topoisomerase I inhibition by camptothecin, we used a DNA depletion assay [14] (Fig. 1). Parasitized erythrocytes were treated with camptothecin (to form intracellular cleavable complexes) and then were

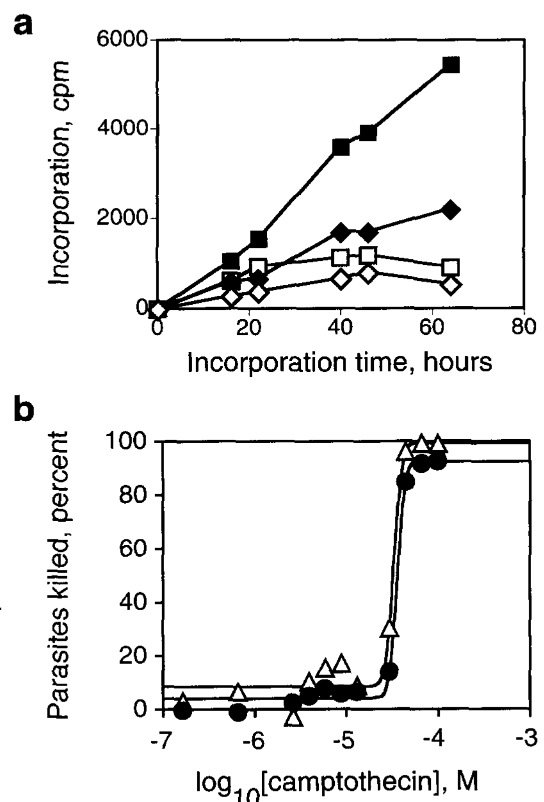

FIG. 2. Effects of camptothecin on nucleic acid biosynthesis and on survival of *P. falciparum in vitro*. (a) Incorporation of radioactivity from [^{3}H]hypoxanthine into nucleic acids was monitored as a function of time in the presence of 100 μM camptothecin (□, ◇) or 0.5% DMSO (■, ◆). Squares represent total incorporation and diamonds indicate RNase-insensitive incorporation (DNA). Results are standardized to cpm/100 μL of culture. Duplicates varied by less than 9% of their average; error bars for each point fall within the symbols. Based on an analysis of variance (two factor with replication), camptothecin inhibited total incorporation ($P < 0.0001$) and incorporation into RNase-insensitive nucleic acid ($P < 0.0001$). (b) Cultures of chloroquine-sensitive (△) and -resistant (●) parasites (2.4% hematocrit; 0.25 or 0.5% parasitemia, respectively) were exposed to camptothecin or DMSO for 48 hr. Data were fit to yield EC_{50} values of 32 μM (r^2, 0.983) and 36 μM (R^2, 0.995) for the sensitive and resistant strains, respectively. The standard deviation for each set of quadruplicate drug concentrations was ≤10% of the mean; in duplicate assays, EC_{50} values varied by less than 2% of the mean.

lysed with SDS (to generate a covalent linkage between topoisomerase I and its DNA substrate). The lysate was divided, and one half was not treated with proteinase K prior to phenol extraction. During extraction, DNA covalently linked to protein partitioned between the aqueous and organic phases and was not collected with the aqueous phase for further processing. Lysates containing covalent DNA–protein adducts were thus depleted of DNA. By this method, the sample treated with camptothecin but not digested with proteinase K exhibited a substantial reduction in DNA content (Fig. 1, lane 2), the expected result if camptothecin traps topoisomerase I in cleavable complexes

[‖] *Abbreviations:* EC_{50}, concentration that achieves a 50% effect.

in vivo. This finding indicates that the drug gains access to the enzyme in living cells, and that the parasites have drug-sensitive topoisomerase I activity.

In eukaryotic cells, inhibition of topoisomerase I activity reduces DNA synthesis and RNA transcription [3]. To determine whether camptothecin affects these processes in erythrocytic *P. falciparum*, we monitored the incorporation of label from [^{3}H]hypoxanthine into nucleic acids, in the presence and absence of camptothecin (Fig. 2a, compare open to closed symbols). In control samples, 38–48% of the total incorporated radioactivity was present in DNA (nucleic acid stable to digestion by RNases). Camptothecin virtually halted the synthesis of both RNA and DNA.

To determine whether camptothecin is cytotoxic to erythrocytic *P. falciparum*, we used chloroquine-sensitive (NF54 strain; EC_{50}, 5 nM) and chloroquine-resistant (FCR3 strain; EC_{50}, 45 nM) parasites, and monitored the incorporation into nucleic acids of radioactivity from [^{3}H]hypoxanthine. Camptothecin was cytotoxic to *P. falciparum*, with an EC_{50} value of 32 μM for NF54 (Fig. 2b) and 36 μM for FCR3. To ensure that the reduction in hypoxanthine incorporation was a faithful indicator of cell death, these results were confirmed by microscopy. In contrast to their well-characterized resistance to quinoline antimalarial drugs [15], the FCR3 parasites retained full sensitivity to camptothecin.

Our experiments indicate that camptothecin gains access to the erythrocytic forms of *P. falciparum*; promotes cleavable complex formation; inhibits nucleic acid biosynthesis; and ultimately kills the parasite. With an EC_{50} value in the micromolar range, camptothecin itself is an unlikely candidate for therapy. However, it may be regarded as a lead compound, which supports the concept that topoisomerase I is a susceptible target for antimalarial drug development. Some camptothecins avoid the cellular pumps involved in cross-resistance [16], which may provide additional leverage against multidrug-resistant parasites. Among the many analogs that have been synthesized, there are almost certainly some with greater potency, and perhaps selectivity, against *P. falciparum*.

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References

1. Butler D, Maurice J and O'Brien C, Time to put malaria control on the global agenda. *Nature* **386:** 535–536, 1997.
2. Hoffman SL, Artemether in severe malaria—Still too many deaths. *N Engl J Med* **335:** 124–126, 1996.
3. Schneider E, Hsiang Y-H and Liu LF, DNA topoisomerases as anticancer drug targets. *Adv Pharmacol* **21:** 149–183, 1990.
4. Drlica K and Kreiswirth B, 4-Quinolones and the physiology of DNA gyrase. *Adv Pharmacol* **29A:** 263–283, 1994.
5. Wang JC, DNA topoisomerases. *Annu Rev Biochem* **65:** 635–692, 1996.
6. Wall ME and Wani MC, Camptothecin and analogs: From discovery to clinic. In: *Camptothecins: New Anticancer Agents* (Eds. Potmesil M and Pinedo H), pp. 21–41. CRC Press, Boca Raton, 1995.
7. Nitiss J and Wang JC, DNA topoisomerase-targeting antitumor drugs can be studied in yeast. *Proc Natl Acad Sci USA* **85:** 7501–7505, 1988.
8. Riou J-F, Gabillot M, Philippe M, Schrevel J and Riou G, Purification and characterization of *Plasmodium berghei* DNA topoisomerase I and II: Drug action, inhibition of decatenation and relaxation, and stimulation of DNA cleavage. *Biochemistry* **25:** 1471–1479, 1986.
9. Cheesman S, McAleese S, Goman M, Johnson D, Horroks P, Ridley RG and Kilbey BJ, The gene encoding topoisomerase II from *Plasmodium falciparum*. *Nucleic Acids Res* **22:** 2547–2551, 1994.
10. Tosh K and Kilbey B, The gene encoding topoisomerase I from the human malaria parasite *Plasmodium falciparum*. *Gene* **163:** 151–154, 1995.
11. Trager W and Jensen JB, Human malaria parasites in continuous culture. *Science* **193:** 673–675, 1976.
12. Desjardins RE, Canfield CJ, Haynes JD and Chulay JD, Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. *Antimicrob Agents Chemother* **16:** 710–718, 1979.
13. Posner GH, González L, Cumming JN, Klinedinst D and Shapiro TA, Synthesis and antimalarial activity of heteroatom-containing bicyclic endoperoxides. *Tetrahedron* **53:** 37–50, 1997.
14. Bodley AL and Shapiro TA, Molecular and cytotoxic effects of camptothecin, a topoisomerase I inhibitor, on trypanosomes and *Leishmania*. *Proc Natl Acad Sci USA* **92:** 3726–3730, 1995.
15. Geary TG, Divo AA and Jensen JB, Activity of quinoline-containing antimalarials against chloroquine-sensitive and -resistant strains of *Plasmodium falciparum in vitro*. *Trans R Soc Trop Med Hyg* **81:** 499–503, 1987.
16. Chen AY, Yu C, Potmesil M, Wall ME, Wani MC and Liu LF, Camptothecin overcomes MDR1-mediated resistance in human KB carcinoma cells. *Cancer Res* **51:** 6039–6044, 1991.