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Targeting the gyrase of *Plasmodium falciparum* with topoisomerase poisons

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ABSTRACT

Drug-resistant malaria poses a major public health problem throughout the world and the need for new antimalarial drugs is growing. The apicoplast, a chloroplast-like organelle essential for malaria parasite survival and with no counterpart in humans, offers an attractive target for selectively toxic new therapies. The apicoplast genome (plDNA) is a 35 kb circular DNA that is served by gyrase, a prokaryotic type II topoisomerase. Gyrase is poisoned by fluoroquinolone antibacterials that stabilize a catalytically inert ternary complex of enzyme, its plDNA substrate, and inhibitor. We used fluoroquinolones to study the gyrase and plDNA of *Plasmodium falciparum*. New methods for isolating and separating plDNA reveal four topologically different forms and permit a quantitative exam of perturbations that result from gyrase poisoning. In keeping with its role in DNA replication, gyrase is most abundant in late stages of the parasite lifecycle, but several lines of evidence indicate that even in these cells the enzyme is present in relatively low abundance: about 1 enzyme for every two plDNAs or a ratio of 1 gyrase: 70 kb DNA. For a spectrum of quinolones, correlation was generally good between antimalarial activity and gyrase poisoning, the putative molecular mechanism of drug action. However, in *P. falciparum* there is evidence for off-target toxicity, particularly for ciprofloxacin. These studies highlight the utility of the new methods and of fluoroquinolones as a tool for studying the *in situ* workings of gyrase and its plDNA substrate.

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1. Introduction

Among the world's top infectious disease killers (along with HIV/AIDS and tuberculosis), malaria infects an estimated 207 million people and kills 630 thousand annually, most of whom are children in Africa under the age of 5 [1–3]. The eukaryotic protozoan parasite that causes malaria belongs to the genus *Plasmodium* and is transmitted by female anopheline mosquitoes. Many *Plasmodium* species cause malaria, but *Plasmodium falciparum*, subject of the current work, is most lethal. Malaria is preventable and controllable through a combination of vector control methods, limitation of mosquito and human interaction, and antimalarial drugs. Unfortunately, malaria vaccines remain experimental and many of the available chemotherapies now face widespread resistance. There is a pressing need to identify novel drug targets in the malaria parasite and to develop new antimalarial drugs.

Although a human may simultaneously harbor multiple stages of the lifecycle of *P. falciparum*, it is the cycling, asexually replicating obligate intraerythrocytic parasites that cause symptomatic clinical disease [1]. After entering a red cell, the initial ring form contains one haploid copy of the nuclear genome. As it evolves into a trophozoite, the parasite enlarges, importing large amounts of glucose, ingesting host cytoplasm and having highly active metabolism. Finally, during schizogony the parasite undergoes multiple rounds of DNA replication and organellar division before cytokinesis. Within a single erythrocyte, a mature schizont may comprise 8–32 progeny, each with a haploid copy of the nuclear genome. The erythrocyte ruptures and releases progeny

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merozoites, which invade other erythrocytes and re-initiate the cycle. For *P. falciparum* the erythrocytic cycle lasts ~48 h.

The malaria parasite has three genomes. Sequence of the 23 Mb, 14 chromosome, nuclear genome of *P. falciparum* was reported in 2002 [4]. It is exceptionally AT rich, averaging 81% overall and nearly 90% for introns and intergenic regions. At just 6 kb, the mitochondrial genome is the smallest mtDNA known and is arranged in head-to-tail tandem arrays [5]. The third genome is housed in the apicoplast, an organelle intensely studied as a therapeutic target because it has no counterpart in humans [6,7]. The apicoplast is related to chloroplasts and is the site of biosynthetic pathways for isoprenoids, heme, iron-sulfur clusters and fatty acids. Most apicoplast proteins are encoded in the nucleus and imported, but critical components are also provided by its own DNA. The 35-kb circular apicoplast genome (plDNA) has an AT content of ~87% and is reportedly present in 1–15 copies per parasite [8–10]. Both strands of plDNA encode genes required for transcription and translation, and a large inverted repeat accounts for one-third of the genome, coding for 9 duplicated tRNAs and the large- and small-subunit rRNA genes [11,12].

Believed to be of prokaryotic origin, the apicoplast is the target for several antibacterials that have antimalarial action, including doxycycline, clindamycin, azithromycin, and ciprofloxacin. Although parasites treated with these drugs are themselves apparently unaffected, their progeny fail to proliferate even after removal of drug, leading to a phenomenon termed "second cycle effect" [13,14] or, more recently, "delayed cell death" [15].

Topoisomerases are enzymes that change the topology of DNA and are essential for orderly replication and metabolism of nucleic acids and for cell survival [16]. Type II enzymes make a double-stranded break in substrate DNA by tyrosine-mediated cleavage of the phosphodiester backbone of both strands of the helix. The 5′-ends are held in transient covalent linkage to the active site tyrosines, and topology of the DNA is altered by transferring segments of DNA through the double-strand break. The enzyme then religates strands to complete the reaction. Given its three structurally different genomes in distinct subcellular compartments, it is not surprising that *P. falciparum* encodes multiple type II topoisomerases. One, with homology to mammalian topoisomerase II, is thought to service the nuclear genome. Two additional nuclear genes were found to encode proteins homologous to the A- and B-subunits of DNA gyrase, a prokaryotic type II enzyme capable of negatively supercoiling DNA. Both subunits contain apicoplast-targeting signals and the B subunit has been shown to immunolocalize to the apicoplast [17–19].

The type II topoisomerases are targets for clinically valuable anti-tumor and anti-infective agents (*e.g.*, etoposide, ciprofloxacin). These drugs belong to an important subset of topoisomerase inhibitors, termed poisons, that bind only to a preformed enzyme-DNA Michaelis complex, forming a ternary "cleavable complex," comprised of DNA, protein and poison [20]. The presence of a poison prevents ligation of the DNA strands, rendering the topoisomerase inert and bound to DNA. *In situ*, collision of DNA-tracking machinery with fixed cleavable complexes creates the lethal double-stranded breaks that underpin drug efficacy. In the laboratory, addition of a rapid denaturant such as sodium dodecyl sulfate (SDS), leads to protein-DNA adducts with enzyme covalently linked to the 5′-ends of its substrate DNA *via* phosphotyrosine bonds, a phenomenon that has provided valuable insights into the enzyme's function within the cell [20–22]. Previous studies have demonstrated the sensitivity of malaria parasites and their apicoplast gyrase to various quinolones [23,24].

To further study the effect of quinolones on *P. falciparum* we developed methods that provide reproducibly accurate parasite counts and that entail minimal manipulation of the parasites or of their plDNA. Using these techniques we treated intraerythrocytic malaria parasites with quinolones, studied the relationship of drug structure with antimalarial activity and with poisoning of apicoplast or nuclear DNA, and asked whether gyrase poisoning correlates with parasite death.

2. Materials and methods

2.1. Fluoroquinolones and topoisomerase-targeting compounds

Compounds were stored desiccated at −20 °C. Quinolones were dissolved in autoclaved HPLC-grade water or NaOH (both JT Baker, Phillipsburg, NJ) solution; etoposide (Sigma, St. Louis, MO) was dissolved in DMSO (Sigma, St. Louis, MO). Tested were: ciprofloxacin HCl, clinafloxacin (Santa Cruz Biotechnology, Santa Cruz, CA); potassium fleroxacin (Hoffman-La Roche, Basel, Switzerland); gatifloxacin (Selleck Chemicals, Houston, TX); levofloxacin, sodium nalidixate, oxolinic acid, piromidic acid (Sigma, St. Louis, MO); norfloxacin (Merck Sharpe & Dohme, Whitehouse Station, NJ); ofloxacin (Robert Wood Johnson Pharm, NJ); and pefloxacin methanesulfonate (Rhône Poulenc Rorer now Sanofi-Aventis, Bridgewater, NJ).

2.2. Cultivation and synchronization of P. falciparum

P. falciparum NF54 was maintained in O+ erythrocytes in RPMI, 25 mM HEPES, 27 mM sodium bicarbonate (all Sigma, St. Louis, MO) and 10% heat-inactivated O+ serum (Interstate Blood Bank, Inc., Memphis, TN) in non-vented flasks under 3% O_2, 4% CO_2, 93% N_2. All experiments were done at 37 °C. Human erythrocytes were obtained weekly from healthy donors under an IRB-approved protocol. Parasites were maintained in 1.2% or 2.4% hematocrit. To synchronize, cells were pelleted (1500 × *g*, 10 min), resuspended in 5% sorbitol (Sigma, St. Louis, MO) (5 min), resedimented, and resuspended in fresh medium [25].

2.3. Parasites, counts, imaging

To determine number of parasitized erythrocytes per mL of culture, 25 μL culture was fixed with 25 μL 10% formalin (3.7% formaldehyde) (Sigma, St. Louis, MO) and stained with 200 μL 0.1% gentian (crystal) violet (Sigma, St. Louis, MO) in 0.8% NaCl (Fisher, Fair Lawn, NJ), 0.02% Na_2EDTA (J.T. Baker, Phillipsburg, NJ). Rings, trophozoites and schizonts were counted by hemocytometer. To confirm parasitemia thin smears were fixed with methanol (J.T. Baker, Phillipsburg, NJ), stained with 100 μg/mL acridine orange (Sigma, St. Louis, MO) in 1× PBS (Quality Biologicals Inc., Gaithersburg, MD), visualized (ParaLens Fiber Optic Illuminator, Becton Dickinson), and ~1000 erythrocytes were examined to determine the percent infected. Thin smears were stained with Giemsa (Accustain, Giemsa Stain, Modified, Sigma St. Louis, MO). Photomicrographs were obtained with a Zeiss Axiphot and Prog Res C14 plus camera.

2.4. Determination of antimalarial activity

A modified microtiter plate-based assay of [^{3}H]hypoxanthine metabolic labeling was used [26]. Briefly, 100 μL *P. falciparum* at 0.25% parasitemia was incubated for indicated times with 100 μL medium containing solvent or serially diluted drug; each concentration was assayed in quadruplicate. At 40–48 h, 0.64 μCi [^{3}H]hypoxanthine (10–30 Ci (370GBq-1.11TBq)/mmol; Perkin Elmer, Boston, MA) was added to each well, the plate was incubated (20–24 h), samples from each well were harvested, and tritium counts were measured. All tested compounds achieved 100% efficacy. Curve fitting and EC_{50} were obtained with the E_{max} model [27] and DeltaGraph Pro 3.5. Outliers identified by

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