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Model System to Define Pharmacokinetic Requirements for Antimalarial Drug Efficacy

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Abstract

Malaria presents a tremendous public health burden and new therapies are needed. Massive compound libraries screened against *Plasmodium falciparum* have yielded thousands of lead compounds, resulting in an acute need for rational criteria to select the best candidates for development. We reasoned that, akin to antibacterials, antimalarials might have an essential pharmacokinetic requirement for efficacy: action governed either by total exposure or peak concentration (AUC/C_{MAX}), or by duration above a defined minimum concentration (Time above Minimum Inhibitory Concentration, T_{MIC}). We devised an in vitro system for *P. falciparum*, capable of mimicking the dynamic fluctuations of a drug in vivo. Utilizing this apparatus, we find that chloroquine is T_{MIC}-dependent while the efficacy of artemisinin is driven by C_{MAX}. The latter was confirmed in a mouse model of malaria. These characteristics can explain the clinical success of two antimalarial drugs with widely different kinetics in humans. Chloroquine, which persists for weeks, is ideally suited for its T_{MIC} mechanism, whereas great efficacy despite short exposure (t_{1/2} in blood 3 h or less) is attained by C_{MAX}-driven artemisinins. This validated preclinical model system can be used to select those antimalarial lead compounds whose C_{MAX} or T_{MIC} requirement for efficacy match pharmacokinetics obtained in vivo. The apparatus can also be used to explore the kinetic-dependence of other pharmacodynamic endpoints in parasites.

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Author contributions

Designed research, conducted research, analyzed data and wrote the paper: RPB, EN, AKT, DJS, TAS

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INTRODUCTION

The public health burden of malaria is estimated to include half the world's population at risk for infection. About 250 million people have malaria (1), resulting in a million deaths per year with maximum mortality in children younger than 5 years (2). Although promising efforts are underway toward a clinically useful vaccine, current prevention and treatment of malaria are heavily dependent on chemotherapy. Unfortunately the utility of existing antimalarials, including artemisinins (the last major line of defense), has been undermined by diminished drug responsiveness and the emergence and spread of drug-resistant malaria parasites (3, 4). There is a pressing need for safe and effective new antimalarials.

The availability of the genome sequence for malaria parasites (5) and high throughput screens of compound libraries against intra-erythrocytic *Plasmodium falciparum* have led to identification of thousands of lead compounds with chemical structures and molecular mechanisms previously unrecognized in antimalarial therapies (6–8). Given the limited resources available to develop new antimalarials (9), the challenge now is to find rational and efficient criteria by which to select the best candidates for further development. For all experimental drugs, regardless of therapeutic indication, suitable pharmacokinetics is considered key in the preclinical proof-of-concept analysis of lead candidates and often provides the basis for pivotal GO/NOGO decisions (10).

After oral administration of a drug, its concentration in blood at any moment reflects the net effect of absorption, distribution, metabolism and elimination rates. This results in concentrations that peak and then decline with a given half-life (Fig. 1A). The Area Under the Concentration-time curve (AUC) is a measure of drug exposure. In general, drugs are administered so as to maintain blood levels above a defined minimum (T_{MIC}), and preclinical development efforts are commonly directed toward compounds with long half-lives, for which therapeutic levels can be sustained by conveniently infrequent (typically daily) dosing. This strategy is not always appropriate, however, because drug efficacy may be governed by AUC, C_{MAX} or T_{MIC} (11). Though not predictable on the basis of any recognized property (for example, 'static vs 'cidal action, reversible vs irreversible inhibition, molecular target, chemical structure), the governing pharmacokinetic/pharmacodynamic (PK/PD) relationship tends to be uniform within a given drug class, although exceptions have been noted (12). It may depend on the interaction of the drug and the target, as well as on the functional kinetics of the drug target in vivo.

Among the best-studied PK/PD relationships are those for anti-bacterial agents (11). Indeed, antibacterials are commonly classified and dosed on the basis of whether their efficacy is driven by AUC, C_{MAX} or T_{MIC} (13, 14). For T_{MIC} -governed drugs (such as the β -lactams), maximum benefit is obtained by prolonging drug presence. For others such as the aminoglycosides, however, efficacy is more dependent on AUC and to some extent, C_{MAX} , than on T_{MIC} . For AUC/ C_{MAX} -governed drugs the clinical consequence is the same: Dosing is designed to achieve high peak concentration. Aminoglycoside efficacy is maximized by high AUC/peak concentration rather than duration over a minimal concentration (11), and sustained blood levels increase toxicity but not efficacy (15, 16). This concept has also been applied to antitumor agents (17, 18). We call the concentration- or time-dependence of clinical efficacy the "essential PK/PD relationship". This fundamental empirical property underpins the rational dosing of drugs in the clinic and can be utilized to facilitate many stages of drug development. For example, if a lead compound is shown to be AUC/ C_{MAX} -dependent, synthetic chemistry efforts are best directed toward achieving rapid bioavailability, not toward prolonging half-life. Animal efficacy/toxicity and early human studies should be geared towards higher rather than more frequent doses.

Neither a suitable in vitro system nor a readily available animal model exists to assess the pharmacokinetic requirements for activity against *P. falciparum*. Most in vitro studies are performed with fixed concentrations of drug for defined time intervals, a protocol that does not reflect the dynamic fluctuation of drug levels in vivo. The humanized mouse model for *P. falciparum* is complex and costly, requiring severe immunodeficiency in the animals and daily infusions of human erythrocytes (19). Furthermore, animal models do not allow precise manipulation of drug pharmacokinetics.

PK/PD studies of experimental drugs in vitro have been made possible by commercially available hollow-fiber reactors (cartridges) that expose cells to dynamic drug concentrations without the constraints imposed by animal physiology (20, 21). Unfortunately such systems have not worked for antimalarials. To address this, we developed a cartridge that supports the continuous growth of malaria parasites, and, for these initial studies, a strain of *P. falciparum* that proliferates under standard tissue culture atmosphere (5% CO₂ in air). Using these, we have asked whether antimalarials, like antibacterials and some antitumor drugs, have an essential PK/PD relationship: Is their efficacy governed by T_{MIC} or by AUC/C_{MAX} (Fig. 1A)?

RESULTS

Development of *P. falciparum* HOX (High O₂)

The PK/PD apparatus we utilize is modular such that the primary loop that contains the cartridge can be placed within an incubator (Fig. 1B, fig. S1) (21). Since the tubing in the system is gas permeable, we maintained the usual hypoxic condition for *P. falciparum* growth (22) by purging the incubator chamber with the commercial custom low O₂ gas mixture that we routinely use for maintenance of these cultures (3% O₂/4% CO₂/93% N₂). However, parasite growth was impaired when the microaerophilic environment was disrupted by frequent PK sampling. To circumvent this we adapted *P. falciparum* NF54 parasites to survive in conventional tissue culture conditions of 5% CO₂ in air (~19% O₂). Changing from 3% to ~19% O₂ in a single step led to uneven and unreliable proliferation. Parasites were therefore adapted in a stepwise manner. Duration of flask sparging, normally 2 min, was reduced in steps of 30 sec, and at every step parasites were acclimatized for 2 – 3 wk until growth stabilized. Adaptation to 19% O₂ was accomplished in 12 wk.

Comparison of *P. falciparum* HOX and NF54 strains

Phenotypic comparison—Apart from being oxygen-tolerant, HOX appeared quite similar to its parent NF54. It proliferated in 5% CO₂ in air, with exponential growth indistinguishable from that of NF54 in 3% O₂/4% CO₂/93% N₂ (Fig. 2A). In 23 determinations over 6 months of time, incorporation of [³H]hypoxanthine metabolites into parasite nucleic acid polymers was comparable for NF54 and HOX (58,000 ± 34,000 and 49,000 ± 24,000 dpm, respectively; group mean ± SD, *P* = 0.3, two-tail t-test). Sensitivity to five antimalarial drugs with diverse molecular mechanisms (23) was also assessed (Table 1). Responses to chloroquine, atovaquone and pyrimethamine were not significantly different from those of its parent. Previous investigators have noted that the susceptibility of *P. falciparum* to some drugs (24), including the tetracyclines (25), is affected by acute changes in oxygen tension. We found that HOX was more sensitive to doxycycline and artemisinin, but by factors less than 3.5. These drug sensitivities fall well within the range seen for clinical isolates (26–28) and do not indicate that HOX has altered drug sensitivity (28).

Molecular comparison—A comparison of NF54 and HOX mRNA expression by microarray analysis revealed that the 18 transcripts that showed significant changes in expression (>1.5 fold increase or decrease compared to control, *P* < 0.01) comprised 13

variant surface antigens, 4 hypotheticals (sequences that do not have sufficient similarity to proteins in other organisms to justify provision of functional assignment), and 1 kinesin-like transcript (table S1). None of these are known to be related to antioxidant metabolism. Previous assessments of the effect of acute oxidative stress or drug pressure on *Plasmodium* gene expression indicated that the response of the transcriptome of this organism to external stresses is small, typically <2-fold (29–31). This, and the fact that HOX was acclimatized over a period of months, may account for the similarity between transcripts of NF54 and HOX.

Based on observed correspondence between HOX and NF54, in rates of growth and nucleic acid synthesis, drug susceptibilities, and expression analysis, and the fact that HOX drug susceptibilities fall well within the range reported among clinical isolates, we utilized HOX for our initial PK/PD studies of *P. falciparum*.

Development of the glass cartridge

A variety of hollow fiber cartridges are commercially available, and *P. falciparum* has been grown in a plastic cartridge with polyvinyl sulfone fibers at 5% O₂ and hematocrits >80% (32). Our attempts to culture HOX in commercial cartridges (polyvinyl sulfone or cellulose fibers; 20 to 200 kDa pore size) with various hematocrits (2.4 – 50%) and medium flow rates (20 or 40 ml/min through the primary loop) failed. In most cases cells perished within hours of inoculation. Consistent with findings of other investigators (33), we achieved parasite persistence, but not exponential growth, at 50% hematocrit and 40 ml/min medium flow. These requirements for large volumes of human erythrocytes and 1–2 liters/day of medium made PK/PD studies unfeasible. Subsequent experiments revealed that the polymer shell of the cartridge (and possibly also the fibers) was toxic to *P. falciparum*. We therefore devised a new cartridge, in which the plastic shell was replaced by glass and the hollow fibers by perforated dialysis tubing (fig. S1). The millimeter-sized perforations allowed free movement of drug and macromolecules between the compartments, thus providing rapid equilibration and eliminating protein binding of drug as a possible experimental confounder. In this apparatus *P. falciparum* HOX grew normally for many weeks (Fig. 2B). As a first test of our system and to demonstrate proof-of-principle, we elucidated the essential PK/PD relationships of the well characterized antimalarials chloroquine and artemisinin.

Experimental strategy to determine the essential PK/PD relationship

Many different aspects of PK/PD can be investigated with an in vitro system, including studies with kinetics that closely mimic those known to occur in vivo. For these initial studies, however, we used standardized kinetic parameters that would reveal whether a drug's efficacy against *P. falciparum* is driven by T_{MIC} or AUC/C_{MAX}.

Our cartridge studies were designed to relate directly to classic 96-well plate antimalarial assays, with respect to duration of drug exposure (72 h), starting parasitemia (0.25%, asynchronous) and hematocrit (1.2%). The 72-h interval encompasses 1.5 of *P. falciparum*'s 48 hr asexual intraerythrocytic lifecycle. From plate assays, MIC was set at a conservative EC₂₀. Crucial to the study design was the use of the same total dose (AUC) of drug, deployed in two extremely different regimens: a short-lived high concentration bolus (C_{MAX} regimen) versus a constant low concentration 'infusion' (T_{MIC} regimen). The latter concentration was set at the EC₄₀ obtained from the microtiter plate assay. This permitted cross-validation between microtiter plate and cartridge systems and provided maximum opportunity to detect a difference in efficacy between the two regimens. Finally, t_{1/2} of the bolus was set at 4 h. This clears 98% of the drug by 24 h, leaving parasites exposed to well below therapeutic levels of drug for the remaining 48 h.

Since the impact of C_{MAX} and T_{MIC} are independently examined within the confines of a constant AUC, this experimental design permits the clear and unambiguous assignment of control of the response to either C_{MAX} or T_{MIC} . Furthermore, since the design is unlikely to reflect actual in vivo pharmacokinetics, it can be used to study any compound without prior animal experiments. Instead, this strategy provides a prospective indication of the PKs that will maximize antimalarial efficacy in vivo. In cases where half-life is linked to PK/PD (12), this needs to be kept in mind when extrapolating results from lead compounds to actual candidate drugs.

Chloroquine efficacy linked to T_{MIC}

P. falciparum in cartridges was exposed to no drug (control) or to a fixed dose of chloroquine (1628 ng, $AUC_{72h} = 360 \text{ nM}\cdot\text{h}$) administered either as a rapidly cleared bolus or a constant infusion (Fig. 3A). On the basis of microtiter plate assays, MIC was set at the EC_{20} of 4 nM. The bolus regimen achieved a C_{MAX} of 63 nM and a T_{MIC} of 16 h; the infusion was maintained at a steady concentration 5 nM (EC_{40}) with a T_{MIC} of 72 h. [^3H]Chloroquine tracer confirmed that the desired kinetics were achieved in the PD compartment and ruled out any significant non-specific binding to the apparatus (Fig. 3B). At 72 h the infusion resulted in $47 \pm 4\%$ reduction in parasitemia, as expected from the plate assay (Fig. 3C). However, the same dose given as a bolus regimen was significantly less effective, at just $28 \pm 4\%$ reduction. Since the total drug exposure was equal in these two regimens, chloroquine's efficacy was clearly T_{MIC} -dependent. This essential PK/PD relationship is maintained at a higher AUC of $720 \text{ nM}\cdot\text{h}$ (67% reduction with bolus vs. 83% with infusion).

Artemisinin action is C_{MAX} -dependent

The same experimental strategy was used to determine the essential PK/PD relationship of artemisinin. Artemisinin (2126 ng, $AUC_{72h} = 864 \text{ nM}\cdot\text{h}$) was administered in one cartridge as a bolus ($C_{MAX} = 151 \text{ nM}$, $T_{MIC} = 18 \text{ h}$, $t_{1/2} 4 \text{ h}$) and in the other as a constant infusion (EC_{40} , 12 nM, $T_{MIC} = 72 \text{ h}$) (Fig. 4A). MIC or EC_{20} was determined to be 6 nM. Control cells in a third cartridge were not treated. We monitored PK profiles by sampling the PD compartment and verified artemisinin concentrations with the 96 well microtiter plate assay (Fig. 4B). As for chloroquine, the infusion was set at EC_{40} and served to validate concentrations obtained in the cartridge (infusion regimen resulted in $40 \pm 4\%$ reduction in parasitemia). In contrast to chloroquine, however, for artemisinin the bolus regimen was twice as effective as the infusion, indicating that the efficacy of artemisinin is governed by C_{MAX} (Fig. 4C). These findings predict that efficacy of the artemisinin class of drugs in vivo is C_{MAX} -dependent.

Confirmation of predicted PK/PD in an animal model

Unlike the in vitro cartridge system, the ability to manipulate PK parameters in animals is limited. For compounds with very long $t_{1/2}$ in vivo, such as chloroquine (34), it is not possible to evaluate the impact of C_{MAX} independent of T_{MIC} . However, such assessment is possible with short-lived compounds such as the artemisinins. To test the cartridge predictions in vivo we used artesunate, an artemisinin derivative with high aqueous solubility (35), in the *P. berghei* ANKA mouse malaria model. In animals the clinically used artemisinins and their rapidly formed, antimalarial dihydroartemisinin metabolite are briskly cleared with a half-life of ~30 min (36).

As for the in vitro studies, a dose-response curve was obtained, in this case with escalating single doses of artesunate and assessment of parasitemia 36 h after treatment (Fig. 5A, table S2). This efficacy endpoint covers 1.5 life cycles of *P. berghei* ANKA (37), corresponding to that of the cartridge and microtiter plate assays of *P. falciparum*. To evaluate the impact

of dosing regimen, we chose total doses of 0.8 and 1.32 mg/kg, expected to produce efficacy values on the linear portion of the dose response curve (Fig. 5A). Artesunate was administered as either a single large dose at 0 h, or as 0.25 doses at 0, 9, 18 and 27 h. The 9 h dosing interval (~18 half-lives) ensures that there is no accumulation of drug in the multiple dose regimen and that AUC_{36h} is the same for both regimens. Confirming the cartridge predictions and for both total doses, artesunate given as a single bolus was more efficacious than the same total dose divided over time (Fig. 5B and C). These findings demonstrate that C_{MAX} is the driver of artesunate's antimalarial activity in vivo.

DISCUSSION

We devised a preclinical model system that enables exposure of *P. falciparum* malaria parasites to dynamic drug concentrations in vitro. The system can simulate any PK profile without the limitations imposed by animal physiology. This in turn permits an unambiguous determination of the pharmacokinetic parameter that governs drug efficacy. Utilizing this system, we were able to determine the essential PK/PD relationships of chloroquine and artemisinin against *P. falciparum*. Our results indicated that chloroquine is T_{MIC}-dependent (Fig. 3C), whereas C_{MAX} governs the action of artemisinin (Fig. 4C). The latter was validated with the *P. berghei* ANKA mouse malaria model (Fig. 5B and C).

Our findings lead to several important conclusions. First, antimalarials, like antibacterials and some antitumor agents, have an essential PK requirement for PD efficacy, and we now have examples of both C_{MAX}- and T_{MIC}-dependence against *P. falciparum*. Second, the underpinning PK requirements of these agents afford a satisfying explanation for their tremendous clinical utility despite a more than 100-fold difference in plasma half-lives. The weeks-long t_{1/2} of chloroquine (34) ensures long persistence in the blood, ideal for its T_{MIC}-dependence. Conversely, the artemisinins are enormously effective antimalarials despite half-lives of single-digit hours or less and daily dosing regimens that result in many hours of sub-therapeutic drug concentration (38). Great efficacy despite short exposure is attainable, indeed expected, for a C_{MAX} drug. In keeping with this, for the same total dose of artesunate in patients with falciparum malaria, the efficacy of a once-daily regimen is not improved by twice daily dosing (39). Third, since artemisinin and chloroquine are both parasitocidal (40, 41), there appears to be no obligatory correlation between 'static or 'cidal action and T_{MIC}- or C_{MAX}-dependence. Finally, the essential PK/PD relationship of antimalarials may be class-wide. In this work we found that artemisinin and artesunate are both C_{MAX}-driven. Additionally, we re-analyzed previously reported data from a large cytotoxicity study, and for identical drug exposures (AUCs) the antimalarial activity of quinine, like that of its mechanistically similar structural analog chloroquine, is time-dependent (42).

In these experiments the difficulty of maintaining microaerophilic conditions with a custom gas mixture led to the development of *P. falciparum* HOX, which grows well in conventional 5% CO₂ in air (~19% O₂). Although the landmark breakthrough in continuous culture of malaria parasites hinged on the use of an hypoxic environment (22), and O₂ levels as low as 1% are still widely used, *P. falciparum* can be maintained in candle jar conditions (43), which at 14–18% O₂ (44), approach the ~19% O₂ environment of a CO₂ incubator. Within humans, circulating intraerythrocytic parasites experience rapid swings in PO₂, which range from ~100 mm Hg in arteries to ~25 mmHg in veins draining maximum-extraction tissues (45), and biochemical studies have revealed that intraerythrocytic *P. falciparum* possess a tremendous capacity to deal with oxidative stress (46). Close resemblance of HOX to parent NF54, and drug susceptibilities well within the spectrum of clinical isolates, indicated it was reasonable to initiate our PK/PD studies with HOX. This close similarity between strains also raises the question of the necessity or even desirability

of cultivating *P. falciparum* at O₂ levels less than the convenient and relatively inexpensive ~19% of a conventional CO₂ incubator.

The tools – the cartridge and *P. falciparum* HOX – we have developed allow rapid and unambiguous determination of the essential PK/PD relationship of antimalarial compounds in a more definitive and less resource-intensive manner than in animal models. Chloroquine and artemisinin are highly effective because their PK requirements match well to their actual half-lives in vivo. During drug development, a mismatch between the pharmacokinetic driver of efficacy and attainable half-life in vivo would signal concern about a candidate drug's likelihood of success.

Our apparatus makes it possible to study the PK dependence of many different PD endpoints for *P. falciparum* (e.g., intracellular accumulation of drug/metabolites; metabolomic changes; emergence of resistance). Conversely, various kinetic conditions, including those that mimic drug behavior in vivo, can be used to optimize dosing regimens within the limitations imposed by animal physiology. The apparatus is amenable to miniaturization, permitting maintenance of parasites in hypoxic conditions and enabling the study of non-adapted strains of *P. falciparum*, including recent clinical isolates. We believe, however, that the most important application for this new technology is in antimalarial drug development. The essential PK/PD relationship provides a new criterion for judging and selecting drug candidates for further development, and a rational basis for planning their developmental pathway.

MATERIALS AND METHODS

Experimental design

In vitro PK/PD determinations utilized human pathogen *Plasmodium falciparum*. The PK parameter governing efficacy was elucidated by dosing one AUC via two widely disparate regimens: A large rapidly cleared bolus or a constant infusion. These regimens provide a wide separation between C_{MAX} and T_{MIC}, the components of AUC. The AUC to be studied was chosen from the linear portion of a dose-response curve generated by microtiter plate assay. The efficacy of the each regimen was assessed by comparison with a no-drug control. Three independent experiments were conducted over a period of several weeks. Statistical comparisons were performed using a two-tailed t-test. A detailed rationale for the experimental strategy is described in the Results section and experimental details are described below.

In vivo PK/PD determinations utilized the *Plasmodium berghei* ANKA murine model of malaria. A dose response curve of artesunate versus *P. berghei* was generated, and using the linear portion of this dose-response curve we chose two test AUCs. Each AUC was dosed either as a single large bolus, or as 4 smaller doses. Assay length was adjusted for the shorter life cycle of the rodent malaria parasite, and dose intervals were designed to prevent drug accumulation. Three to five animals were used per group. Statistical analysis was performed using the two-tailed t-test. Experimental details are provided below.

Malaria parasite cultivation

P. falciparum NF54 and HOX were maintained at 37 °C in hypoxanthine-free RPMI (with 50 mM HEPES, 27 mM NaHCO₃, pH 7.3) in O⁺ erythrocytes and 0.5% AlbuMax (Invitrogen). Erythrocytes were collected weekly from healthy donors under an IRB-approved protocol. Cultures were propagated at 1.2 or 2.4% hematocrit, and parasitemia was maintained between 0.25 and 6%. *P. falciparum* NF54 was cultured with custom gas mixture (3%O₂/4%CO₂/93%N₂); HOX was kept in 5% CO₂ in air. For our incubators

maintained at 5% CO₂, 37 °C and 100% humidity, O₂ concentration measured by Drager Pac 3000 gas meter (Fisher Scientific) ranged from 18.8% to 19.1%.

Drug cytotoxicity assays

Assays were performed in 96-well plates as described (47, 48). Briefly, parasites (0.25% parasitemia, 1.2% hematocrit) in hypoxanthine-free medium were exposed to serial dilutions of drug; each concentration was tested in quadruplicate. After 48 h, [³H]hypoxanthine (PerkinElmer NET0177, 14 Ci/mmol) was added to each well (final concentration 0.25 µCi/ml) and plates were incubated 24 h. Cells were harvested and lysed, nucleic acids were captured on GF/C glass fiber filters (Brandel Inc.), and incorporation of radiolabel was detected by liquid scintillation quantitation. EC₅₀ values were calculated as described (48). For bioassay of artemisinin concentration, medium was obtained from the PD compartment at indicated times (Fig. 4B) and stored –80 °C until analysis. Samples from three independent cartridge experiments were each tested in quadruplicate, in the 96-well assay described above. Unknowns (group mean ± SD in Fig. 4B) all fell within the range of the standard curve ($r^2 = 0.9994$).

Microarray analysis

On three occasions, asynchronous cultures of NF54 and HOX with closely matched parasite lifecycle stages (80% rings + trophozoites) were harvested and processed. Parasites were extracted from erythrocytes with 1% acetone in phosphate-buffered saline, RNA was obtained (Trizol Reagent and PureLink Mini Kit system, Invitrogen), its suitable quality was verified by RNA Nano LabChip analysis on an Agilent BioAnalyzer, and 100 ng total RNA (per sample) was processed for hybridization to *Plasmodium/Anopheles* microarrays (Affymetrix) using the 3' IVT Express kit (Affymetrix). Processing, hybridization and signal amplification were according to manufacturer's instructions. Arrays were scanned at 570 nm and 2.5 µm resolution (GCS300 laser scanner, Affymetrix). Quality assessment was performed using Expression Console software and data were analyzed using Partek Genomics Suite Version 6.5.

In vitro PK/PD determinations

Construction of the apparatus (Fig. 1B, fig. S1) has been described previously (20, 21, 49). Commercial hollow-fiber cartridges were from FiberCell Systems; custom glass cartridges (7 cm length, 2 cm outer diameter; Adams and Chittenden Scientific Glass) were divided into PK and PD compartments by threading perforated dialysis tubing through the glass shell (Spectrapor 7, 12 mm flat width, Spectrum Laboratories). Prior to use tubing was boiled (10 min, 0.5% NaHCO₃) and washed extensively with distilled water. Processed tubing was perforated with a 23 gauge needle (~20 perforations/cm², 200 perforations total). Cartridge units were assembled, autoclaved (121 °C, 20 min, liquid cycle) and used within hours of preparation. Volume of the PD compartment (all within the cartridge) was 12.5 ml; of the PK compartment (dialysis tubing, primary loop and central reservoir) was 37.5 ml; total volume of the primary loop (which comprises PK + PD compartments and is the volume of distribution for the drug) was 50 ml.

For an experiment, three cartridges were loaded with the same culture of parasites and run simultaneously. Every cartridge had its own primary and dilution loops. Drug-free medium was circulated through the primary loop and parasites (0.25% parasitemia, 1.2% hematocrit, 12.5 ml) were introduced into the PD compartment and allowed to settle. Drug was then dosed to initiate the desired profiles, and subsequent PK simulations were executed by precalculated pump programs. Drug concentrations were chosen to provide identical AUC_{72h} for bolus and constant infusion regimens. The bolus was programmed to clear with t_{1/2} of 4 h, based on simulation calculations (Microsoft Excel). Infusion regimens were set at

EC₄₀ and MIC was set at EC₂₀ based on 96-well plate assay results. Actual concentrations of drug in the PD compartment were monitored by spiking the dosing preparation with 0.25 μ Ci [³H]chloroquine (1.6 Ci/mmol, Moravsek Biochemicals), or by bioassay of artemisinin in the [³H]hypoxanthine method (above). After 72 h, parasites in samples from the PD compartments were detected by fluorescence microscopy of fixed thin smears stained with acridine orange. At least 1000 erythrocytes were examined for each sample and % reduction in parasitemia was calculated as $(1 - (\% \text{ parasitemia}_{\text{treated}} / \% \text{ parasitemia}_{\text{no drug control}})) \times 100$.

In vivo PK-PD determinations

The *P. berghei* ANKA murine malaria model was used to test PK-PD predictions generated by the in vitro system. All studies were approved by the Institutional Animal Care and Use Committee (Protocol #MO12H321). C57/BL6 mice (~20 g, 3–5 mice per group; The Jackson Laboratory) were infected with *P. berghei* ANKA (average 1 million, intraperitoneally). When parasitemia reached 1–3% (3–4 days after infection) mice were dosed intraperitoneally with 5% NaHCO₃ or with artesunate (Sigma) in 5% NaHCO₃ (100 μ l injection volume) at the appropriate concentration (doses and regimens in table S2). At 36 h post-initial dose, blood was sampled from the tail vein and animals were euthanized. Parasites were visualized on Giemsa-stained thin smears and percent parasitemia was calculated as $(\text{parasitized RBCs} / \text{total RBCs}) \times 100$. For each mouse determination, a minimum of 500 RBCs were evaluated by two independent observers. Percent reduction in parasitemia was calculated as $[1 - (\% \text{ parasitemia}_{\text{treated}} / \% \text{ parasitemia}_{\text{control}})] \times 100$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Dynamic drug concentrations seen in vivo can be simulated by the preclinical model system. (A) When a single dose of drug is given orally, levels in blood rise, reach a peak (C_{MAX}) then fall (blue). Area under the curve reflects both concentration and time and is an index of overall drug exposure. The same total dose and AUC provided by a single large dose (blue) can be obtained by smaller repeated doses (red). MIC (dashed line) is an efficacy value obtained from dose-response curves, and T_{MIC} is the interval during which drug levels exceed MIC. Although dose and AUC are identical for the two depicted regimens, C_{MAX} and T_{MIC} differ greatly. Constant infusion is the extreme multidose regimen. (B) Our preclinical model system allows direct comparison of C_{MAX} versus T_{MIC} dosing regimens. A hollow cylinder cartridge traversed by semipermeable tubing divides the cartridge into two compartments between which molecules exchange freely. Cells are introduced, maintained, and sampled from the extra-tubing (PD) compartment, and medium containing desired concentrations of drug flows continuously through the tubing (PK compartment). Sterile ports allow sampling of both compartments. Primary loop (cartridge, fast pump, central reservoir) is mounted on an incubator shelf; dilution loop (input and output reservoirs, dilution pump) remains outside. Drug is pumped into the central reservoir and distributed through the primary loop via the fast pump. Desired PK profiles are achieved by programming pump rates.

Fig. 2.

Characterization of *P. falciparum* HOX proliferation. (A) Growth rates in tissue culture flasks of *P. falciparum* HOX (filled triangles; in 5% CO₂ in air) or NF54 (red open squares; in 3% O₂/4% CO₂/93% N₂). Both cultures were maintained at 2.4% hematocrit. (B) Over an extended period of time *P. falciparum* HOX proliferated as well in the cartridge with circulating medium (filled triangles) as in a tissue culture flask (red open squares). Both cultures were maintained in 5% CO₂ in air and 1.2% hematocrit. Each data point is the average of dilution-adjusted parasite counts, made in duplicate.

Fig. 3. Antimalarial activity of chloroquine is T_{MIC} -dependent. (A) Two PK profiles were programmed in the PK/PD system. The bolus regimen (blue line) had a C_{MAX} of 63 nM and T_{MIC} of 16 h while the infusion regimen (red line) was constant at 5 nM (EC_{40}), for a T_{MIC} of 72 h. Dotted line is the MIC (EC_{20}), determined to be 4 nM. AUC_{72h} for both profiles is 360 nM•h. (B) Chloroquine concentrations in the PD compartment, monitored by [3H]-labeled drug, (filled squares) were as expected from the programmed profile (open squares). Error bars are too small to be seen outside the data points. (C) Exposure to a high peak bolus of chloroquine (blue bar) was less effective against *P. falciparum* than the same dose provided by constant infusion (red bar). Each value shown above is a group mean $\pm$ SD, $N = 3$ experiments done over several weeks' time; $P = 0.003$ (two-tail t-test).

Fig. 4.

Artemisinin efficacy in vitro is driven by C_{MAX} . **(A)** PK profiles tested in the PK/PD system. The bolus regimen (blue line) had a C_{MAX} of 151 nM and T_{MIC} of 18 h while the infusion (red line) was constant for 72 h at 12 nM (EC_{40}). Dotted line is the MIC, set at the EC_{20} of 6 nM. AUC_{72h} for both profiles was 864 nM•h. **(B)** Artemisinin concentrations actually obtained in the PD compartment (filled squares) closely follow those expected from the programmed profile (open squares). Error bars on the 8 and 12 h time points are too small to be seen outside the points. **(C)** Relative to parasite counts in the untreated control cartridge, the artemisinin bolus regimen (blue bar) was more effective than the infusion (red bar). Each value shown above is a group mean $\pm$ SD, $N = 3$ experiments done over several weeks' time; $P = 0.002$ (two-tail t-test).

Fig. 5.

Artesunate efficacy in *P.berghei* ANKA-infected mice is driven by C_{MAX} . **(A)** Dose-response curve of artesunate in mice. Infected animals (3–5/group) were treated with a single dose of artesunate and assessed for parasites (doses and regimens detailed in table S2). Efficacy is reduction in parasitemia relative to that of vehicle-treated control mice ($r^2 = 0.96$, $ED_{50} = 1.4$ mg/kg). **(B and C)** Artesunate efficacy correlates with concentration. A total dose of 0.8 mg/kg artesunate **(B)** was administered to infected mice either as a single bolus of 0.8 mg/kg or as four doses of 0.2 mg/kg every 9 h. A total dose of 1.32 mg/kg artesunate **(C)** was administered to infected mice either as a single bolus of 1.32 mg/kg or as four doses of 0.33 mg/kg every 9 h. Both bolus regimens (blue bars) were more effective than the multidose regimens (red bars). Each value shown is a group mean $\pm$ SD of 3–5 animals; P values were obtained by two-tailed t-test. For all experiments, mice were at 1–3% parasitemia at the start of treatment, doses were administered intraperitoneally, and parasitemia was measured 36 h after the start of treatment.

Table 1Drug sensitivities of *P. falciparum* NF54 and *P. falciparum* HOX

Drug	Molecular Mechanism	Antimalarial Activity* EC ₅₀ (nM) Mean ± SD	
		NF54	HOX
Chloroquine	Inhibits heme crystallization	6.7 ± 0.3	7.6 ± 0.5
Atovaquone	Inhibits cytochrome b	0.51 ± 0.1	0.53 ± 0.04
Pyrimethamine	Inhibits folate reductase	19 ± 4.6	14 ± 4.1
Doxycycline	Inhibits apicoplast protein synthesis	7400 ± 960 [†]	2100 ± 250
Artemisinin	Free radical-mediated	28 ± 3.7 [†]	14 ± 4.1

* Obtained by microtiter plate-based [³H]hypoxanthine method; data from three or more independent determinations

[†] Statistically significant difference from that of HOX; *P* < 0.01 (two-tail t-test).