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Hollow Fiber Methodology for Pharmacokinetic/ Pharmacodynamic Studies of Antimalarial Compounds

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Abstract

Knowledge of pharmacokinetic/pharmacodynamic (PK/PD) relationships can enhance the speed and economy of drug development by enabling informed and rational decisions at every step, from lead selection to clinical dosing. For anti-infective agents in particular, dynamic in vitro hollow fiber cartridge experiments permit exquisite control of kinetic parameters and the study of their consequent impact on pharmacodynamic efficacy. Such information is of great interest for the cost-restricted but much-needed development of new antimalarial drugs, especially since major human pathogen *Plasmodium falciparum* can be cultivated in vitro but is not readily available in animal models. This protocol describes the materials and procedures for determining the PK/PD relationships of antimalarial compounds.

Keywords

Plasmodium falciparum; pharmacokinetics; pharmacodynamics; dynamic PK/PD; hollow fiber cartridge system; drug development; malaria

INTRODUCTION

Malaria is a leading cause of death in many tropical and subtropical countries, reaching highest mortality in young children in Africa (World Health Organization, 2014). Resistance to existing antimalarials, including the last major line of defense artemisinins, makes the need for new drugs greater than ever. High throughput screenings of massive compound libraries against *Plasmodium falciparum* have identified thousands of potential leads (Gamo et al., 2010, Guiguemde et al., 2010; Anthony et al., 2012) and there is a need for stringent and rational criteria by which to identify the best candidates for further development, especially given the limited resources available for this indication. The importance of pharmacokinetic/pharmacodynamic (PK/PD) relationships in early GO/NOGO decisions is

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Conflict of Interest

No conflict of interest for any of the authors.

generally acknowledged (Cartwright et al., 2010), and anti-infective programs in particular rely on dynamic in vitro hollow fiber PK/PD studies (Drusano, 2004; Ambrose et al., 2007). Once human PKs are defined, in vitro models based on actual clinical data have proven valuable in predicting best dosing regimens or best drug combinations (Brown et al., 2011; Drusano et al., 2014). Such data can substantially shortcut time and expense. Unfortunately, the ability to study and exploit PK/PD for antimalarials has been limited by lack of a suitable in vitro system, a problem remedied by the apparatus and methods described here.

Several species of *Plasmodium* cause disease in humans, but *falciparum* is most pathogenic and the only one reliably cultivated in vitro. The methods described herein focus on the intraerythrocytic forms of *P. falciparum* that evolve through a 48 hr cycle, and cause clinically apparent disease (Shapiro and Goldberg, 2006). The described methods are tailored for economical and standardized discovery of the PKs that govern a compound's antimalarial efficacy. Several aspects of this methodology differ from those used for other anti-infectives. First, and key to the success of this work, was development of a glass cartridge system to replace commercial hollow fiber cartridges that are toxic to *P. falciparum* (Bakshi et al., 2013). Second, *P. falciparum* was acclimated to grow in conventional 5% CO₂ incubator conditions rather than the more typical 2–5% O₂ (Bakshi et al., 2013). Third, since the potency of a compound may vary as the parasite progresses through its 48 hr cycle (Delves et al., 2012), these studies utilize asynchronous cells akin to most *falciparum* populations in vivo (Fairhurst and Welles, 2010; Dondorp and von Seilein, 2010), and run for 72 hr to cover 1.5 lifecycles. Finally, while the applied PKs (peak concentration, half-life) can be programmed to simulate those in animals or humans, they need not do so. Indeed, as described below a distinct advantage of the system is that artificial conditions, independent of those imposed by animal physiology, can be applied so as to discern the fundamental PK driver of efficacy.

Orally administered drugs are subject to absorption, distribution, metabolism, and elimination (Rowland and Tozer, 2004). These result in blood levels that rise to a peak then fall with a given half-life (Fig. 1A). Area under the concentration-time curve (AUC) is a measure of total drug exposure. By varying the dosing regimen, the same AUC can be obtained with concentration-time curves of widely differing shapes. At the extremes are a concentration-intensive short-lived high peak versus a time-intensive long-lived constant lower concentration infusion (Fig. 1B). As perhaps best characterized for anti-bacterials, efficacy for a given drug class is preferentially driven by either concentration or time of exposure (Ambrose et al., 2007). This unit describes the methods for discerning the PK governance of antimalarial activity.

STRATEGIC PLANNING

Experimental Strategy

Each experiment involves three glass cartridges: two drug-treated and one drug-free control ('drug' may denote any compound of interest). Malaria parasites are exposed to two dosing regimens that differ widely in their concentration-time profile, but nevertheless provide the same total exposure (AUC, Fig. 1B). The governing PK parameter (C_{MAX} or T_{MIC}) is identified based on the antimalarial efficacy of a large rapidly cleared peak (C_{MAX} -driven

regimen) versus that of a constant infusion (T_{MIC} -driven regimen; see Video 1 for simulation of dynamic PK/PD experiment). By definition, half-life of drug in the constant infusion regimen is infinity. In contrast, C_{MAX} studies are performed with a half-life of just 4 hr. This eliminates 98% of drug by 24 hr (one-half of a life cycle) and creates large disparities between the two regimens, with respect to concentration and time. At 72 hr parasites from each cartridge are counted and the result for each dosing regimen is compared to that of untreated control. The regimen providing greater efficacy reveals the governing PK parameter. If non-specific drug binding and release from system components affect the kinetics, the apparatus is pre-incubated to block nonspecific binding and decay rates are kept as rapid as possible (typically ~8 hr) within this limitation.

Workflow

The experimental workflow follows a series of steps (Fig. 2). In Basic Protocol 1 the dose-response relationship of the drug is determined in a conventional 96-well microtiter plate assay. The resulting curve is used to select concentrations for the dynamic hollow fiber studies. Nonspecific binding of drug to system components is assessed in Basic Protocol 2, and Basic Protocol 3 is used to verify that the desired PK profiles are obtained in the cartridge system. These experiments are done with medium but no cells. If necessary, dose adjustments and experimental modifications are made to achieve the desired PK. These adjustments are specific to the drug being tested and may require iteration. Finally, in Basic Protocol 4 parasites are exposed to the desired AUC, deployed as a C_{MAX} or T_{MIC} regimen, and compared with a drug-free control. Experimental conditions are usually designed to allow direct comparison of the infusion regimen to the 96-well assay from Basic Protocol 1. Basic Protocol 4 is repeated three times to obtain statistical validation of results. Straightforward variations of Basic Protocol 4 include study of a different total dose (AUC), use of a different half-life for the peak regimen, or a total exposure time other than 72 hr.

Design of the PK/PD Apparatus

Our apparatus uses the combination of a previously described system of bottles and pumps that generates dynamically changing drug concentrations (Blaser et al., 1985; Moore et al., 1994), and a newly-fashioned glass cartridge that supports the continuous and robust growth of malaria parasites (Bakshi et al., 2013). The hollow glass cylinder cartridge is traversed by semipermeable, macroscopically perforated dialysis tubing (Fig. 3). This creates two compartments within the cartridge (intra- and extra-tubing) and allows transmembrane movement of drugs and macromolecules. Cells settle and remain at the bottom of the glass cartridge in the extra-tubing space, which serves as the PD compartment. The intra-tubing PK compartment of each cartridge is linked to its cognate primary loop bottle by platinum-cured silicone tubing (Fig. 4). Medium with or without drug is continuously and briskly pumped around the primary loop to effect mixing within the primary loop bottle and rapid exchange between the PK and PD compartments of the cartridge. PK samples for measurement of drug concentration can be obtained from multiple locations but most important to assay are those in the PD compartment where parasites reside.

Medium with or without drug enters and exits the system via the primary loop bottle. Input bottles provide fresh medium with no drug (control and peak regimen cartridges), or with

the desired constant concentration of drug (infusion regimen cartridge). Pumped at the programmed rate, these solutions drip into the primary loop bottles while fluid simultaneously drawn from the meniscus is pumped to the output bottles. For the peak regimen, medium in the primary loop is loaded with drug at peak concentration, which is then diluted at the desired half-life with drug-free input medium.

Each cartridge assembly has three associated segments of tubing that pass through the peristaltic pump: the primary loop segment exiting the cartridge (Fig. 5, purple), the segment between the input bottle and primary loop bottle (Fig. 5, green), and the segment from the primary loop bottle to the output bottle (Fig. 5, yellow). During an experiment, medium is pumped from the input bottle into the primary loop bottle. Volume in the primary loop bottle is kept constant by withdrawing fluid from the meniscus and transferring it to the output bottle. For the C_{MAX} regimen, replenishment of drug-containing medium with drug-free medium, while keeping total volume constant, results in a controlled decrease in drug concentration in the primary loop (see Video 1). For the T_{MIC} regimen, dilution medium contains drug at the infusion concentration, resulting in no net change in drug concentration.

The most critical flow rate in the experiment is dictated by the inner diameter of the pump tubing between the input and primary loop bottles. It governs the clearance (Cl), and hence $t_{1/2}$, of drug in the C_{MAX} regimen. A matching output rate from the primary loop bottle (and a fixed circulating volume) are achieved by using output module pump tubing whose inner diameter exceeds that of the input module, and by withdrawing fluid from the meniscus of the primary loop bottle. Relatively rapid equilibration between the primary loop bottle and cartridge is obtained by circulating medium through the primary loop significantly faster than the rate of dilution. To achieve this, primary loop pump tubing has an inner diameter 4–5 times greater than that of the input tubing. All three lines are run at the same pump setting. Actual pump tubing diameters should be chosen to fit the desired half-life and system volume of a given experiment.

For convenience and consistency, system components are grouped and classified as follows.

Primary loop: Closed circuit containing cartridge, connecting and pump tubings, and primary loop bottle. This does not include the peristaltic pump.

Cartridge assembly: Primary loop attached to input and output tubings. This does not include the peristaltic pump, input or output bottles.

Rig: Cartridge assemblies connected to input and output bottles and the pump, organized and transported on an incubator shelf.

BASIC PROTOCOL 1: ANTIMALARIAL DOSE-RESPONSE BY MICROTITER PLATE ASSAY

For purine auxotroph and obligate pyrimidine-synthesizing *P. falciparum*, metabolic incorporation of [3H]-hypoxanthine forms the basis of this conventional assay for the antimalarial activity of a drug against intraerythrocytic asexual parasites (Desjardins et al., 1979). The concentration that achieves a given antimalarial effectiveness (e.g., EC_{40} for 40% efficacy) is determined through nonlinear curve fitting of the data (Posner et al., 1997).

Unless specified otherwise, all operations described below must be performed inside a biological safety cabinet (BSC).

Materials

See Support Protocols for preparation of RBCs and cultivation and counting of parasites.

100 µg/ml acridine orange (Sigma) in phosphate buffered saline pH 7.4 (Quality Biological, Inc.)

P. falciparum HOX (ATCC)

Uninfected erythrocytes (red blood cells, RBCs)

Medium

Drug stock solutions (typically in DMSO or water)

[³H(G)]-Hypoxanthine monohydrochloride (14 Ci/mmol, 574 GBq/mmol; PerkinElmer NET0177)

96-well microtiter plate, flat-bottomed wells (Costar 3596)

50 ml sterile disposable conical tubes (Sarstedt Part No. 62-557-205)

Humidified incubation chamber for 96-well plates (CBS Scientific, catalog no. M-312)

37°C humidified 5% CO₂ incubator

Fluorescence microscope or light microscope fitted with Paralens Illuminator (Becton Dickinson)

Cell harvester (Brandel Inc., Model MB48)

GF/C glass fiber filters (Brandel Inc.)

Microsoft Excel, GraphPad Prism, or similar software for nonlinear curve fitting

1. Prepare serial dilutions of drug stock in medium at 2× final concentration. Transfer 100 µl of each concentration into each of four wells of a 96-well plate. Ensure final DMSO concentration 0.2%.

Two drugs are tested per plate (see layout in Table 1). We recommend that each concentration be assayed in quadruplicate, and typically conduct three independent assays to obtain EC₅₀ values. Each plate should include appropriate controls as described in Table 1.

2. Add 100 µl of 2.4% hematocrit uninfected RBCs to wells D9-12 (final hematocrit 1.2% v/v).
3. Prepare an experimental culture of 0.25% parasitemia and 2.4% hematocrit (Support Protocols 2 and 3).
4. Add 100 µl of culture to the remaining wells using a multichannel pipettor, for a final hematocrit of 1.2% v/v in each well. After each addition pipet up/down 3–4

times to mix contents of the wells. Rock the reservoir 10–15 times between additions to ensure cells remain uniformly suspended.

5. Place the plate in a humidified incubation chamber to minimize evaporation. Transfer the chamber to an incubator maintained at 37°C and 5% CO₂. The chamber must allow gas exchange to enable CO₂-mediated buffering of the medium.
6. Incubate 48 hr.
7. In a 50 ml conical tube add 81 µl [³H]-hypoxanthine to 3.2 ml medium, transfer into a reservoir, and using a multichannel pipettor dispense 25 µl into each well (final concentration 0.25 µCi/ml).
8. With a multichannel pipettor set to 100 µl, resuspend the cells and thoroughly mix content of the wells by pipetting up/down 12–15 times.
9. Return the plate to the humidified chamber and incubate at 37°C and 5% CO₂ for an additional 24 hr.
10. Harvest the cells onto glass fiber filter paper, dry overnight on benchtop or 20 min under a heat lamp, and measure radioisotope incorporation by liquid scintillation counting.
11. Transfer the raw data to Microsoft Excel or a similar data analysis program.
12. Calculate the mean and standard deviation of disintegrations per min (dpm) for each quadruplicate group.
13. Compare the means for parasite only wells (H9-12) with those of each vehicle control (D5-8 and H5-8) to identify any vehicle toxicity.
14. For each drug (A or B), calculate percent effect at each concentration (x) using the average dpm from quadruplicate determinations calculated in step 12 and equation 1.

$$\% \text{ Effect} = 100 - \frac{(dpm \text{ Conc } A_x - dpm \text{ RBC only})}{(dpm \text{ Vehicle A} - dpm \text{ RBC only})} \times 100 \quad (1)$$

15. Generate a dose-response curve by fitting the experimental data to a generalized sigmoidal function by the Marquardt algorithm (Bard, 1974). Plot percent effect versus drug concentration using nonlinear regression analysis in GraphPad Prism or a similar data analysis program.
16. Solve for the concentration of drug that reduces hypoxanthine incorporation by 40% relative to control (EC₄₀) and analyze for goodness of fit (R² value).

See Desjardins et al. (1979) and Posner et al. (1997) for additional details and representative plots.

BASIC PROTOCOL 2: ASSESSMENT OF NON-SPECIFIC DRUG BINDING AND RELEASE

Basic Protocols 2 and 3 describe the preliminary PK experiments, which are done without parasites. These studies ensure that the desired PK profile is actually obtained experimentally, for both the C_{MAX} and T_{MIC} regimens. Non-specific drug binding to hardware components is a major factor that must be accounted for when using this in vitro test system. Such binding varies based on the affinity of the compound for system components, and must be assayed every time a new compound is studied or a system component is modified. There are two aspects to the non-specific binding phenomenon: drug binding to the system and drug release from this bound pool. Both processes will affect final drug concentrations and the PKs that are achieved. As described below these experiments are most conveniently performed using radiolabeled drug as a tracer, but if this is not feasible drug concentrations may be analyzed by another method (*e.g.*, LC-MS/MS or bioassay).

In Basic Protocol 2 the extent and kinetics of drug binding and release are assessed. Two primary loops are charged with medium containing a high or low level of drug (corresponding to the desired peak or infusion concentration, respectively) and spiked with radiolabeled tracer. To measure drug adherence, the solutions are circulated around the primary loops and samples taken from the cartridge PD compartments over 24 hr are assayed via liquid scintillation. Differences between concentrations at start and end of the experiment reveal the extent, if any, of drug adherence to system components. To measure drug release, a portion of the circulating fluid is replaced with drug- (and radioisotope-) free medium. The time-dependent appearance of radioactivity in subsequent cartridge samples reveals the extent and rate of drug release. If the difference between desired and expected results is >10%, experimental conditions must be adjusted. Knowing the extent of drug binding in the low concentration system is sufficient to finalize design of the T_{MIC} regimen for Basic Protocol 4. However, to adjust the peak regimen both binding and release results from the high concentration conditions are required, and these more extensive modifications must be validated in Basic Protocol 3.

Materials (also see Basic Protocol 1 and .)

Radiolabeled drug tracer (1 μ Ci/ μ l; 10 μ l/50 ml medium is sufficient for the experiments described below; tracer should contribute 1% of total drug mass)

Spectra/Por 7, 28 mm flat width, 50kD MWCO (Spectrum Laboratories, part no. 132129)

Ismatech IP 12-channel standard-speed digital dispensing pump (Cole Parmer EW-78001-22)

Glass cartridges (custom from Adams & Chittenden Scientific Glass)

18-gauge blunt reusable syringe needles

Aluminum foil

Sewing needle and thread

31-gauge syringe needles

Sterile syringe filters, 0.22 μ m (Olympus)

Injection ports (Interlink® system injection site; Baxter 2N3399)

Curity 4" x 4" All Purpose Sponges (Covidien)

Slide clips (Qosina, part no. 95868)

Sterility requirements—Meticulous sterile technique is paramount for success of these protocols. Only steps 1–10 (primary loop construction) of Basic Protocol 2 may be performed on the bench. **All other steps described in Basic Protocols 2–4 must be performed in a biological safety cabinet (BSC).** Do not use paper products (*e.g.*, laboratory wipes or paper towels) in the BSC since these are known to introduce contamination. Instead, use Curity sponges (or a similar product) and 70% ethanol for cleaning the BSC and equipment.

Prepare two primary loops

- 1 Cut two 16 cm lengths of dialysis tubing, rinse thoroughly with distilled, deionized water (ddH₂O) then soak in 1 liter ddH₂O for at least 1 hr. Until experienced with the method, it may be helpful to cut an additional spare length of tubing.
- 2 While the dialysis tubing is soaking, secure a primary loop bottle cap on a Qorpak bottle beaker to serve as a placeholder primary loop bottle during the autoclaving process. Connect the 2-stop tubing and its associated connecting tubings to ports on the caps of the primary loop bottles (see Support Protocol 4). Close all ports and line ends with the proper male or female luer cap.
- 3 Wrap all caps and joints with aluminum foil, as these connection points are most susceptible to contamination.

Steps 4–8 below are demonstrated in Video 2.

- 4 Pierce 5 lines of 20 holes (evenly distributed) through each piece of flattened dialysis tubing using a 31-gauge needle.
- 5 Holding the dialysis tubing horizontally, fold in half making sure the outside edges are aligned. Then fold in half again forming a quarter fold. Preparing the dialysis tubing in this manner facilitates its insertion into the glass cartridge.
- 6 Thread a sewing needle with ~25 cm of thread and double knot the ends together. Wrap the knotted end of the thread once around the dialysis tubing, ~1cm from one end. Cinch and knot the thread in place. Hold the glass cartridge so its inlet and outlet are oriented vertically. Insert the needle through the top opening and use gravity and a little tapping to get the needle with attached thread to drop through the glass cartridge and exit from the lower end. Use the needle and thread as a guide to pull the cinched end of the dialysis tubing

through the length of the glass cartridge. With ~2 cm of dialysis tubing extending beyond each end of the cartridge, loosen the knot and remove the guiding needle and thread.

- 7 Open and flare out each end of the dialysis tubing. Reflect each end back over the lip of the inlet and outlet to create the channel that will serve as the flow path through the cartridge (Fig. 3, Video 2). While firmly holding one end of the dialysis tubing in place, secure the opposite end by pushing cartridge inlet (or outlet) tubing over it. Repeat this process on the other end of the cartridge.
- 8 Run ddH₂O through each end of the cartridge to test the flow path. The aim is to have a steady constant drip exiting at the opposite side. If the flow is not a steady constant drip, the dialysis tubing is bunched and occluding the pathway. If this occurs, start over using a new piece of dialysis tubing, repeating steps 4–8.
- 9 Construct a primary loop by connecting cartridge, tubing and bottle (Support Protocol 4), and cap the two sample ports on the cartridge. Wrap the connections with aluminum foil. Clip the tubing at the inlet and outlet ends of the cartridge using slide clips to prevent the dialysis tubing from drying out during autoclaving.
- 10 Wrap each primary loop, as well as three sets of two primary loop bottles and 18-gauge needles, in aluminum foil and autoclave for 40 min on liquid cycle.

Note: It is important to use a liquid cycle of autoclave (without a drying step) to avoid desiccating the dialysis tubing.

Program PK parameters and prepare drug solutions

- 11 Using the EC₄₀ value obtained from Basic Protocol 1 as the infusion concentration, calculate the matching peak concentration using equation 2, where T is the total time of the experiment (72 hr) and $t_{1/2}$ is the half-life.

$$[Peak] = T \times [Infusion] \times \frac{\ln 2}{t_{1/2}} \quad (2)$$

See Rowland and Tozer (2004) for description of mathematical concepts underlying calculation of PK parameters.

- 12 The total volume of distribution (V_d) of the primary loop – cartridge, bottle, and tubing – is 50 ml. The primary loop bottle accounts for 32 ml while the cartridge and the tubing make up the balance. Hence, concentration of the 32 ml drug solution added to the primary loop bottle must be adjusted for its ultimate dilution into 50 ml. Calculate the infusion or peak concentration for each primary loop bottle by dividing the values obtained in step 11 by the dilution factor (32 ml/50 ml).
- 13 Prepare 33 ml of the adjusted infusion and peak solutions (as calculated in step 12) in sterile 50 ml conical tubes using a fresh aliquot of drug stock prepared from Basic Protocol 1.

Cap and set these drug solutions aside until steps 14–21 are complete.

Connect primary loops to pump

- 14 Using sterile sponges sprayed with 70% ethanol, wipe the surfaces of the pump and pump cassettes and place them in the BSC.
- 15 Unwrap the first primary loop and remove the foil from each joint and cap. Tighten all connections to avoid leakage during the experiment. Replace the cap on the top of the primary loop bottle with a sterile syringe filter.
- 16 Fit the purple-white 2-stop tubing into a pump cassette, pulling tight from both ends to ensure it is positioned correctly, and clip the cassette into the pump (Fig. 4). Secure the glass cartridge to the floor of the BSC by taping between the upright sampling ports.
- 17 Repeat steps 15–16 for the second primary loop.
- 18 Remove the cap from the right sample port of each cartridge (always the distal end of the cartridge flow path) and replace it with a sterile injection port adapter. Do not screw in the injection adapter tightly.
- 19 Aliquot 12.5 ml of medium into each of two sterile 50 ml conical tubes. Spray the sample ports of each cartridge with 70% ethanol before proceeding. Loosen the luer cap of the proximal port (without the injection adapter) so it may be removed with one hand. Using a sterile 18-gauge needle and 20 ml syringe, transfer 12.5 ml medium into the cartridge, replace the luer cap, and then tighten both the cap and the injection adapter. Repeat for the second cartridge.
- 20 Using one set of autoclaved primary loop bottles, aliquot 32 ml of medium into each bottle. Swap these filled bottles for the placeholders used in autoclaving. Unclip the inlet and outlet tubing at both ends of each cartridge.

Keep the placeholder bottles sterile as these are needed for the disassembly process.
- 21 Power on the pump, adjust settings to achieve a half-life of 2 hr (0.289 ml/min for a V_d of 50 ml) and start the flow. Run the pump until all tubing is filled with medium.

Determine non-specific binding

- 22 Add sterile radiolabeled drug tracer to each of the solutions prepared in step 13. Mix well by gentle inversion so as to avoid bubbles.

The required amount of labeled drug may vary based on its specific radioactivity.
- 23 Transfer 32 ml of the infusion and peak drug solutions into the second set of sterile primary loop bottles. Keep the remainder to serve as the first PK samples ($t = 0$ hr).

- 24 Power off the pump and replace the drug-free primary loop bottles with those containing the tracer infusion and peak drug solutions.
- 25 Power on the pump, confirm settings, start flow and timer.
- 26 For the first sample, $t = 0$ hr, transfer 100 μ l from each 50 ml conical tube in step 23 to a scintillation vial containing scintillation fluid. Cap vials and shake to mix.
- 27 Using a sterile 18-gauge needle and 1 ml syringe, withdraw a sample from each cartridge at time points throughout the beginning of the experiment. We recommend sampling frequently for the first 8 hr. The required sample volume, typically 250 μ l for early time points, will depend on the amount of radioactivity present.
- Spray the septum of each cartridge with 70% ethanol before sampling. With care not to damage the dialysis tubing, carefully insert the needle through the septum and around the dialysis tubing. Ideally, samples should be taken from the space below the dialysis tubing.
- 28 Transfer 200 μ l of each PK sample into a scintillation vial containing scintillation fluid. Cap the vials and shake to mix. Allow vials to sit in the closed scintillation counter for at least 1 hr before counting.
- Larger sample volumes for counting are needed at later time points.

Off-rate determination

- 29 Continue running pump overnight in BSC, circulating the primary loops.
- 30 The next morning, stop the pump flow and timer, and withdraw a ~500 μ l sample from each cartridge as described in step 27.
- 31 Process 400 μ l of the above samples as described in step 28.
- 32 Using the third set of sterile primary loop bottles, aliquot 32 ml of drug-free medium into each bottle. Swap out the drug-containing bottles for those with drug-free medium. Take a sample from each of the removed bottles to compare concentrations in the cartridges with those in their respective primary loop bottles.
- 33 Restart the pump flow and timer and allow the primary loops to circulate the drug-free medium. Run the experiment at least for 24 hr.
- 34 As described in steps 27 and 28, samples should be taken at a minimum of six time points, typically at 2, 4, 6, 8, 20, and 24 hr, with sufficient volume to exceed the lower limit of quantification of the scintillation counter.
- 35 After 24 hr and at least six time points, stop the pump flow and timer.

Dismantle apparatus

- 36 Clip the inlet and outlet tubing of each cartridge with slide clips to retain contents within the cartridges.

- 37 Swap out the primary loop bottles for placeholder bottles (step 20).
- 38 Unclip the cassettes from the pump and remove the 2-stop tubing from the cassettes. Remove the primary loops from the BSC.
- 39 Power off the pump and remove from the BSC.
- 40 At a sink designated for disposal of small amounts of radioactive waste, disassemble the primary loops. Rinse the cartridges, primary loop tubings, and primary loop bottles thoroughly with ddH₂O.
- Run ddH₂O through each end of each piece of tubing for at least 10–20 seconds. This process is tedious but critical so as to prevent clogging and contamination in future uses. Do not skip this step.

Analyze PK data

- 41 For assessment of non-specific drug binding, enter data obtained in steps 26–31 into a spreadsheet. For $t = 0$ hr, remember to multiply the dpm by the dilution factor (32 ml/50 ml) to obtain the initial value. Normalize all dpm values for volumes counted.
- 42 For each cartridge, plot dpm versus time (hr) and compare dpm values at $t = 0$ hr (obtained in step 26) and after overnight equilibration (steps 30–31). The difference between initial and final concentrations gives the percent non-specific drug binding.
- A decrease in dpm over time is due to adsorption of the drug to the primary loop components. On-rate data are collected over time to ensure that equilibrium has been achieved by the end of the experiment.
- 43 For the cartridge run with a constant low (corresponding to infusion) concentration of drug, if $\sim 10\%$ of drug is bound after overnight circulation, no further adjustment is necessary. Dynamic PK/PD studies (Basic Protocol 4) may be performed using the EC₄₀ from Basic Protocol 1 as the concentration for the T_{MIC} regimen.
- 44 If, however, $>10\%$ is bound, a pre-incubation step is needed to saturate non-specific binding. Divide the EC₄₀ by the percent free drug (measured in step 42) to obtain the concentration of drug for the pre-incubation solution.
- Off-rate testing is not relevant for the low concentration cartridge, since drug concentration is kept constant in the C_{MIC} regimen in Basic Protocol 4.
- 45 For the cartridge run with a constant high (corresponding to peak) concentration of drug, if $\sim 10\%$ of drug is bound after overnight circulation, neither off-rate calculation nor pre-incubation is necessary, and PK verification of the C_{MAX} regimen (Basic Protocol 3) may be performed directly.
- 46 If, however, binding is $>10\%$, a pre-incubation to saturate non-specific binding is needed. The concentration of drug in the pre-incubation solution is determined by dividing the desired peak concentration by the percent free drug (determined

in step 42). Design of the C_{MAX} PK/PD regimen also requires assessment of the off-rate of bound drug, by the following process.

- 47 Using off-rate data for the high concentration cartridge, plot dpm versus time (hr). From this, calculate half-life for release of non-specifically bound drug.
- 48 If the half-life for release of non-specifically bound drug is ≤ 4.5 hr, Basic Protocol 3 (and Basic Protocol 4) may be performed using a 4 hr half-life. If, however, the half-life of release is >4.5 hr, Basic Protocols 3 and 4 must be performed using a half-life = release half-life. Using the new half-life value, the peak (and corresponding pre-incubation) concentration must be recalculated to ensure AUC equivalence with the infusion regimen.
- 49 New or infrequent users may wish to repeat Basic Protocol 2 to ensure reproducibility of results before proceeding to Basic Protocol 3.

BASIC PROTOCOL 3: VALIDATION OF DYNAMIC PK FOR C_{MAX} REGIMEN

This protocol tests, in duplicate, whether the desired peak concentration and $t_{1/2}$ for the C_{MAX} regimen are indeed obtained. Medium, with peak drug concentration and spiked with radiolabeled tracer, is loaded into the primary loop, then diluted over time with drug-free medium by means of input and output lines and bottles (see Video 1 for depiction of the dilution process). Timed samples are taken from each cartridge and quantitated by liquid scintillation counting. Plots of dpm versus time show the fall in drug concentrations over time. Comparison of the duplicate results with one another and with the desired kinetics will reveal whether further adjustments are necessary. If so, AUC equivalence must be maintained between the infusion and peak dosing regimens, and the adjusted C_{MAX} conditions must be tested in a repeat of this Basic Protocol 3.

Note: The 4 hr default half-life for our PK/PD determinations (see Experimental Strategy) can be obtained only if drug binding is $\leq 10\%$ or if the release half-life is ≤ 4.5 hr. If the release half-life determined in Basic Protocol 2 exceeds 4.5 hr, then the drug clearance rate in Basic Protocols 3 and 4 must be programmed to be longer than the non-specific release half-life. Additionally, peak concentration must be recalculated for the new half-life so as to maintain AUC equivalence.

Materials (see Basic Protocol 2 and Support Protocol 4)

Prepare two cartridge assemblies

- 1 Prepare primary loops as described in steps 1–9 of Basic Protocol 2. Complete the steps below for each primary loop.
- 2 Assemble input and output lines as shown in Support Protocol 4.
- 3 Connect the input and output lines to their respective primary loop bottle ports.

We make all tubing connections and attach tubing to the primary loop bottle cap prior to autoclaving to minimize the chance of contamination. After autoclaving, the only connections that need to be made in the BSC are those between the input line and input bottle, and between the output line and output bottle.

Note the directionality of placement of the 2-stop tubings. Make use of the colored tabs to keep directionality uniform. Mark one stop on the red-red tubing with a permanent marker for this purpose. Consistent directionality of the inlet 2-stop tubing is required for reproducible flow rates and kinetics.

1. For the primary loop, purple-white 2-stop tubing (2.79 mm i.d.) is used to circulate medium rapidly through the primary loop.
2. For the input line, orange-white 2-stop tubing (0.64 mm i.d.) is used to draw medium slowly from the input bottle into the primary loop bottle.
3. For the output line, red-red 2-stop tubing (1.14 mm i.d.) is used to draw medium from the primary loop bottle out to the output bottle.
4. Close all ports and lines with luer caps. Wrap all covered ports and joints with aluminum foil, as these breakpoints are most susceptible to contamination.
5. Wrap each cartridge assembly, as well as three sets of two primary loop bottles and 18-gauge needles, in aluminum foil. Also cap and wrap the ports of input and output bottles. Autoclave for 40 min on liquid cycle.

Complete construction inside BSC (see Fig. 6 and Video 3)

6. Using sterile sponges sprayed with 70% ethanol, wipe the surfaces of the pump and pump cassettes and place them in the BSC.
7. Transfer input and output bottles into the BSC and remove the foil. Replace the luer cap on the top of each bottle with a sterile syringe filter. Place the bottles behind the pump.
8. Unwrap the first cartridge assembly and remove the foil from each joint and cap. Tighten all connections to avoid leakage during the experiment. Replace the cap on the top of the primary loop bottle with a sterile syringe filter.
9. Fit the purple-white 2-stop tubing into a pump cassette, pulling tight from both sides to ensure it is positioned correctly. Clip the cassette into the first slot of the pump (Fig. 4).

It is important to be sure the direction of flow through the primary loop is correct and as follows: out of the cartridge, through the pump, into the primary loop bottle and back into the cartridge.

10. Fit the orange-white 2-stop tubing into a pump cassette, pull tight, and clip cassette into the second slot of the pump. Carefully remove the caps from a port of the input bottle and from the input line, and connect them together maintaining sterility.

Verify direction of flow is as follows: out from the input bottle, through the pump, and into the primary loop bottle.

11. Fit the red-red 2-stop tubing into a pump cassette, pull tight, and clip cassette into the third slot of the pump. Carefully remove the caps from a port of the

output bottle and from the output line, and connect them together maintaining sterility.

Verify direction of flow is as follows: out of the primary loop bottle, through the pump, and into the output bottle.

- 12 Repeat steps 8–11 for the second cartridge assembly.
- 13 Load the primary loops with medium as described in steps 18–20 of Basic Protocol 2. Unclip pump cassettes for input and output lines. Run pump until primary loop tubing is filled with medium. Stop pump flow and power off pump.
- 14 Using the EC_{40} value obtained from Basic Protocol 1 as the infusion concentration, and equation 2, calculate the matching peak concentration. The two cartridge assemblies prepared above will be used for duplicate half-life determinations by dynamic dilution of peak concentration with drug-free medium.

For drugs with >10% non-specific binding, pre-incubate the system and confirm PK (For drugs with negligible non-specific binding, go directly to step 21)

Pre-incubation to block nonspecific binding

- 15 Increase the peak concentration by dividing by the percent free drug, obtained in step 46 of Basic Protocol 2. This correction accounts for the mass of drug that will adhere during the pre-incubation, and results in an equilibration within the primary loop of the free drug concentration required at the start of the C_{MAX} regimen.
- 16 Account for the dilution of 32 ml into the total 50 ml V_d of the primary loop by dividing the corrected concentration by the dilution factor (32 ml/50 ml). This yields the corrected, concentrated pre-incubation solution.
- 17 Prepare 70 ml of corrected, concentrated pre-incubation solution, using a fresh aliquot of drug stock prepared in Basic Protocol 1.
- 18 Using the second set of autoclaved primary loop bottles, aliquot 32 ml of corrected, concentrated pre-incubation solution into each. Swap in these filled bottles.
- 19 Power on the pump and set flow rate (clearance, ml/hr) using equation 3 to obtain the desired half-life.

$$Cl = V_d \times \frac{\ln 2}{t_{1/2}} \quad (3)$$

Only the primary loops circulate during pre-incubation, but the input and output lines are in place and ready to run. Start pump flow and timer; run overnight in the BSC.

Prepare and load solutions for PK testing

- 21 Divide peak concentration (step 14) by the dilution factor to account for the 50 ml V_d of the primary loop.
- 22 Calculate the volume of medium needed for the input bottle based on two primary loops, the flow rate set for the given half-life, and the duration of the experiment.
- 23 Prepare the input bottle by aliquoting the volume of drug-free medium calculated in step 22 into a sterile bottle.
- 24 Prepare 70 ml of peak concentration drug solution, accounting for the dilution factor, as calculated in step 21.
- 25 Add radiolabeled drug tracer and mix well.
- 26 Transfer 32 ml of this solution to each of the third set of sterile primary loop bottles. Reserve the remainder to serve as the first PK sample ($t = 0$ hr).
- 27 If pre-incubating, stop pump flow and timer. Clip the inlet and outlet of each cartridge with the slide clips.
- 28 Swap in primary loop bottles containing drug and radiolabeled tracer, and an input bottle containing drug-free medium.

Perform PK testing and data analysis

- 29 Clip in all cassettes, making sure the tubing is properly aligned in each cassette. Unclip the inlet and outlet tubings on each of the cartridges. Verify that the pump settings are correct.
- 30 Start pump flow and timer. Observe that medium is being pulled into each primary loop bottle and that medium is exiting into the output bottle.

Note that the flow of medium into the output bottle is intermittent because the inner diameter of its 2-stop tubing is greater than that of the input line.
- 31 Using a sterile 18-gauge needle and 1 ml syringe, withdraw a sample from each cartridge through the injection port at a minimum of 6 time points (2, 4, 6, 8, 20, and 24 hr).

Spray the septum of each cartridge with 70% ethanol before sampling. With care not to damage the dialysis tubing, carefully insert the needle through the septum and around the dialysis tubing. Ideally, samples should be taken from the space below the dialysis tubing.
- 32 For the sample $t = 0$ hr, transfer 100 μ l of loading solution (step 26) to a scintillation vial containing scintillation fluid. For the 2, 4, 6, and 8 hr samples, transfer 200 μ l; for the 20 and 24 hr time points, transfer 400 μ l. Cap the vials, shake to mix, and allow them to sit in the closed scintillation counter for at least 1 hr before counting.

Larger sample volumes for counting are needed at later time points.

- 33 Measure dpm of all samples. For $t = 0$ hr, multiply the dpm by the dilution factor (32 ml/50 ml) to obtain the initial value. Normalize all dpm values for volume counted.
- 34 For each cartridge, plot dpm versus time (hr) to obtain a decay curve.
- 35 Transform the raw data at each time point by taking the natural log (ln) of the dpm value and plot $\ln(\text{dpm})$ versus time (hr).
- 36 Calculate the slopes of the lines generated step 35. The negatives of the slopes are the elimination rate constants k . Use k to calculate the observed half-lives based on equation 4.

$$t_{1/2} = \frac{\ln 2}{k} \quad (4)$$

- 37 Using the dpm value at $t = 0$ hr as initial concentration C_0 , calculate desired concentration C_t at each sampling time point (t) for the desired elimination rate constant k (and hence desired half-life) by using equation 5.

$$C_t = C_0 e^{-t \frac{\ln 2}{t_{1/2}}} \quad (5)$$

- 38 Compare observed and desired decay curves, and observed and desired half-lives to assess drug release rates. Perform the comparison for each cartridge separately. Half-lives obtained from individual cartridges should be within 0.5 hr of each other and of the desired value.

If half-lives for individual cartridges are not within 0.5 hr of each other, there is a problem in the experiment, likely in cartridge construction or integrity/positioning of 2-stop tubing, and Basic Protocol 3 must be repeated.

If half-lives of the two cartridges are within 0.5 hr of each other, but differ by >0.5 hr from the desired half-life, adjustment of the programmed half-life (and hence peak concentration) is necessary to ensure AUC equivalence. Basic Protocol 3 must be repeated with these revised parameters to verify the new PKs.

If half-lives match the desired value, Basic Protocol 4 may be performed.

Dismantle apparatus

- 39 After 24 hr and minimum 6 time points, power off the pump and timer. Clip the inlet and outlet tubing of each cartridge, tubing exiting the input bottle, and tubing entering each primary loop bottle.
- 40 Swap out the primary loop bottles for the sterile placeholder bottles.
- 41 Disconnect the input and output tubing from the input and output bottles, closing all ends and ports with the proper luer caps. Remove the input and output bottles from the BSC.

- 42 Unclip all six cassettes from the pump and remove the 2-stop tubing from the cassettes. For each cartridge, disconnect the input and output tubing from the primary loop bottle and close all ends and ports with the proper luer caps. Remove from BSC keeping the parts of each cartridge assembly grouped together.
- 43 Remove the pump from the BSC.
- 44 At a sink designated for disposal of small amounts of radioactive waste, disassemble the primary loops. Using ddH₂O, rinse each cartridge, every piece of tubing, and each primary loop bottle thoroughly.

Run ddH₂O through each end of each piece of tubing for at least 10–20 seconds. This process is tedious but critical so as to prevent clogging and contamination in future uses. Do not skip this step.

BASIC PROTOCOL 4: DYNAMIC HOLLOW FIBER STUDIES FOR ANTIMALARIAL PK/PD

The pharmacokinetic requirement for efficacy, also known as the “essential PK/PD relationship,” underpins the rational dosing of drugs and is useful for many stages of drug development (Bakshi et al., 2013). This protocol describes an experimental strategy to determine the essential PK/PD relationship of a compound against *P. falciparum*. It is important to note that this same experimental strategy can be used not only to study artificial kinetics, but also to model the known kinetics of a drug in animals or humans. Furthermore, though the described end point (parasite density) is taken just once (at 72 hr), many different PD endpoints may be assayed, at intervals other than 72 hr.

We strongly advise running a set of flask controls with each PK/PD run to monitor inter-experiment variation in drug susceptibility of cells. Note that while the infusion cartridge is designed to match the 96-well assay, drug susceptibility may differ between the constantly nutrient-replenished cartridge and the microtiter plate. Flasks mimic plate conditions, afford the opportunity to use the identical culture aliquoted into the cartridges, and provide comparable samples for pharmacodynamic analysis at the end of the experiment.

Materials (Also see Basic Protocol 3)

T25 vented flasks

Incubator shelf (which conveniently serves as a tray for transferring apparatus between BSC and incubator)

Prepare three cartridge assemblies—For simplicity, in all cartridge experiments we denote cartridge 1 as the T_{MIC} infusion, cartridge 2 as the C_{MAX} peak, and cartridge 3 as the control. This is critical in terms of assembly since the peak and control cartridges share the same input and output bottles.

- 1 Follow steps 1–4 of Basic Protocol 3 to construct a total of three cartridge assemblies.

- 2 Wrap each cartridge assembly, as well as three additional sets of three primary loop bottles and 18-gauge needles, in aluminum foil. Also cap and wrap the ports of two input bottles and two output bottles. Autoclave for 40 min on liquid cycle.

Construct rig inside BSC (Fig. 6 and Video 3)

- 3 Follow steps 6–13 of Basic Protocol 3 for setup of cartridge assemblies in BSC except increase to three cartridge assemblies with the following additions/alterations to setup:
 - a. Using sterile sponges sprayed with 70% ethanol, wipe all surfaces of an incubator shelf and place inside BSC. Complete construction of the rig on this shelf.
 - b. In step 10, connect the input lines for cartridges 2 (peak) and 3 (control) to the same input bottle so both share the input of drug-free medium.
 - c. In step 11, connect the output lines for cartridges 2 (peak) and 3 (control) to the same output bottle.

For drugs with >10% non-specific binding, pre-incubate the system—For drugs with negligible non-specific binding, skip to step 6

- 4 Pre-incubate primary loops as described in steps 15–20 of Basic Protocol 3. For cartridge 1, use the pre-incubation concentration determined in Basic Protocol 2, step 44. For cartridge 2, use the pre-incubation concentration derived in Basic Protocol 3, step 38.
- 5 Pre-incubate cartridge 3 with drug-free medium.

Prepare system for addition of malaria parasites

- 6 Using the infusion concentration (EC_{40} from Basic Protocol 1) and AUC-equivalent peak concentration (Basic Protocol 3, step 14), calculate the concentrations needed to account for dilution of 32 ml into the 50 ml V_d of the primary loop, by dividing by the dilution factor (32 ml/50 ml).
- 7 Calculate the volume of medium needed for an input bottle for a single cartridge based on the flow rate set for the given half-life and duration of the experiment.
- 8 Fill the input bottles with appropriate medium solutions. For the infusion, prepare a drug solution at the infusion concentration using the volume of medium calculated in step 7. For the C_{MAX} and control, aliquot the necessary volume of drug-free medium in a sterile bottle.

Cartridge 1 has its own input bottle (medium with drug), while cartridges 2 and 3 share an input bottle (drug-free medium). The volume of medium for the input bottle for cartridges 2 and 3 should be twice that for the cartridge 1 input bottle.

- 9 In 50 ml conical tubes, prepare medium for the primary loop bottles. Prepare 33 ml of infusion and peak concentrations as calculated in steps 6. Add 33 ml drug-free medium to the third tube, for the control primary loop bottle.
- 10 If PK confirmation of the C_{MAX} regimen is desired, add an adequate amount of radiolabeled drug tracer to the tube containing the peak solution. Mix well by gentle inversion to avoid bubbles.
- 11 Transfer 32 ml of each solution to the appropriately labeled primary loop bottle. Retain the residual peak drug solution to serve as the first sample ($t = 0$ hr) if PK confirmation is desired.
- 12 If system has been pre-incubating overnight, stop pump flow and timer to end the pre-incubation period. Clip the inlet and outlet tubing on both sides of the cartridges.
- 13 Swap in the experimental primary loop and input bottles.

Prepare *P. falciparum* culture for cartridges and flasks

- 14 Aliquot 160 ml pre-warmed medium into a sterile container. For a 1.2% RBC preparation, add 4 ml 50% RBCs (Support Protocol 1) and mix well.
- 15 Place the container with the RBC suspension in the incubator.
- 16 Prepare a thin smear of *P. falciparum* HOX stock culture (maintained at 2.4% hematocrit, Support Protocol 2) and count total RBCs and parasites to determine percent parasitemia (Support Protocol 3).
- 17 Add an appropriate amount of counted stock culture to the 164 ml RBC suspension (step 14) to obtain a parasitized RBC suspension at 0.25% parasitemia and 1.2% hematocrit.
- 18 Spray the sample ports of each cartridge with 70% ethanol before proceeding. On each cartridge, loosen the fittings on both sample ports. *Make sure the inlet and outlet of each cartridge are clipped closed.* Using a sterile 18-gauge needle with a 20 ml syringe, withdraw the medium from each cartridge.
- 19 Aliquot 12.5 ml of the parasitized RBC suspension into each of three 50 ml conical tubes, swirling before sampling to ensure uniformity. Use a new 18-gauge needle and 20 ml syringe to transfer the cells. For each cartridge, swirl the contents of a tube thoroughly, withdraw the suspension, remove a loosened sample port fitting, and load the cells into the cartridge. Replace luer fitting and tighten fittings on all sample ports before proceeding to next step.
- 20 Clip off tubing that exits the input bottles to avoid possible siphoning. Check to make sure all connections are tight before removing tray from BSC. Carefully transfer the tray from the BSC to the incubator and allow the cells to settle for 3–4 hr.

***Note:** When cells are settling, parasites are not exposed to any significant amount of drug. Exposure begins once pumping starts and drug-containing medium begins to circulate through the cartridge.*

Prepare flask controls

- 21** Set up ten sterile T25 vented flasks, each containing 10 ml of parasitized RBC suspension (prepared in step 17), in a series of drug concentrations that span the dose response curve generated in Basic Protocol 1. Transfer the remaining 10 ml of parasitized RBC suspension to a new sterile T25 vented flask to serve as the parasitized RBC (no drug) control.

One flask should contain the infusion drug concentration as a comparative measure of drug sensitivity.

- 22** Incubate flasks at 37°C and 5% CO₂ for the duration of the cartridge experiment.

Perform PK testing and data analysis

- 23** Once RBCs have settled to the bottom of the cartridges, unclip the inlet and outlet tubings of each cartridge and the tubings that exit the input bottle. Clip in all cassettes making certain the 2-stop tubing is properly aligned in each cassette. Verify that pump settings are correct.

- 24** Start pump flow and timer. Observe that medium is being pulled into each primary loop bottle and that medium is exiting into the output bottles. Run the experiment for at least seven half-lives. (See Video 1 for a simulation of the flow of solutions and the dynamically changing drug concentrations in cartridge 2.)

Note that the flow of medium into the output bottles is intermittent due to the output 2-stop tubing having a larger i.d. than that of the input 2-stop tubing.

- 25** If PK time points are desired, the rig must be taken from the incubator and returned to the BSC. We leave the pump running during transfers between incubator and BSC, and during PK sampling in BSC. Volumes of samples withdrawn, timing of sampling, and data analysis are described in Basic Protocol 3.

Since PK sampling will require repeated transfer of rig between incubator and BSC, an extension cord long enough to easily accommodate the movement of the pump between incubator and BSC is required.

Dismantle rig

- 26** After the experiment has run for at least seven half-lives, remove the rig from the incubator and transfer it to the BSC. Stop pump flow and timer. Clip the inlet and outlet tubings of each cartridge, the tubing exiting each input bottle, and the inlet tubing leading into each primary loop bottle.

- 27 Shake cartridge 3 back and forth 30–40 times to completely resuspend the settled RBCs. Spray both sampling ports with 70% ethanol then loosen the luer cap and septum. Withdraw contents of the cartridge using a sterile 18-gauge needle and 20 ml syringe. Transfer the contents to a sterile vented T25 flask. Place used needle in a beaker of ddH₂O and draw up some water to avoid clogging the needle with RBCs.

Note: After harvesting the cartridges, conditions are frozen: the peak regimen has cleared the drug and the infusion regimen stays constant.

- 28 Repeat this process for cartridges 2 and 1. Incubate the flasks at 37°C for remainder of the 72 hr experiment.
- 29 Replace the primary loop bottles with sterile placeholder bottles. Once removed from the rig, incubate the primary loop bottles and their contents at 37° C overnight, and check for signs of contamination the next morning.

See Troubleshooting if contamination occurs.

- 30 Disconnect the input and output tubing from the input and output bottles, closing all ends and ports with the proper luer caps. Swap the input and output bottles with sterile placeholder bottles. Remove the experimental input and output bottles from the BSC, incubate at 37°C overnight, and check for signs of contamination the next morning.

See Troubleshooting if contamination occurs

- 31 See steps 42–44 in Basic Protocol 3 for the remaining steps to dismantle the rig.
- 32 After checking for contamination, measure the volume remaining in each of the primary loop bottles. Do the same with the input and output bottles to confirm that medium was flowing at the set flow rate.

Perform pharmacodynamic testing and data analysis

- 33 At the end of the experiment, make 5 thin smears for each cartridge and 4–5 thin smears for each flask control. Fix the slides with methanol and store in a slide box.
- 34 For each cartridge and flask, stain and assess parasitemia as described in Support Protocol 3.
- 35 Calculate the percent efficacy for the infusion and peak regimens using equation 6.

$$\% \text{ Efficacy} = \left(1 - \left(\frac{\% \text{ parasitemia}_{\text{regimen}}}{\% \text{ parasitemia}_{\text{control}}} \right) \times 100 \right) \quad (6)$$

- 36 Compare percent efficacy of the infusion and peak regimens to determine the governing PK driver: concentration or time.

SUPPORT PROTOCOL 1: PREPARATION OF WASHED RBCs

A reliable and continuous supply of RBCs is essential for successfully propagating *P. falciparum* in the laboratory. We obtain citrate-anticoagulated O+ human blood weekly from healthy donors under an IRB-approved protocol. Donors cannot have hemoglobinopathy, G6PD deficiency, or abnormalities on routine clinical lab tests; must test negative for HIV, hepatitis B or C; may not routinely take any prescribed or over-the-counter drugs; and may not take any drug within three days of donation. For researchers without access to such a resource, RBCs may also be obtained from commercial suppliers (e.g., The Interstate Companies Laboratory, Memphis, TN). Human blood poses a biohazard; proper registration and precautions are necessary. All RBC processing must be performed in aseptic conditions within a BSC.

Materials

Human whole blood

Incomplete medium

15 ml sterile capped conical centrifuge tubes (Sarstedt Part No. 62-554-205)

1. Dispense whole blood in equal aliquots of ~10 ml into tubes.
2. Centrifuge $1500 \times g$, 10 min, room temperature.
3. Remove and discard supernatant plasma and buffy coat layer. Some RBCs will be lost at this step.
4. Resuspend RBCs in incomplete medium to a final volume of 10 ml. Mix well.
5. Centrifuge at $1500 \times g$, 10 min, room temperature. Remove and discard supernatant.
6. Repeat steps 4–5 twice. There should be no buffy coat visible after the final centrifugation.
7. Note the volume of the RBC pellet and resuspend to 50% hematocrit (volume cells/total volume) using incomplete medium.
8. Mix well by inversion until homogenous.
9. Store at 4°C and use for up to 14 days.

SUPPORT PROTOCOL 2: MAINTAINING *P. FALCIPARUM* HOX IN CONTINUOUS CULTURE

P. falciparum HOX was adapted from the NF54 drug-sensitive strain of parasites to survive in the high oxygen content (~19% O₂) of conventional 5% CO₂ incubator conditions (Bakshi et al., 2013). HOX is available from ATCC and is relatively simple to maintain. *P. falciparum* is a human pathogen; proper registration and precautions are necessary.

Materials

Medium

Washed RBCs at 50 % hematocrit

P. falciparum HOX (ATCC)

T25 vented tissue culture flasks

1. Add 0.5 ml washed RBCs at 50% hematocrit (Support Protocol 1) to 10 ml pre-warmed medium in a vented tissue culture flask to obtain a 2.4% hematocrit suspension.
2. Using the acridine orange staining method (Support Protocol 3), measure parasitemia of an existing stock culture.
3. Add an aliquot of counted culture to a flask of 2.4% RBCs to achieve a final parasitemia of 0.25%.
4. Close the flask and maintain in a humidified incubator at 37°C with 5% CO₂.
5. At 72 hr post-inoculation, measure parasitemia using acridine orange staining.
Under normal conditions, the culture should have achieved a parasitemia of 3–6%.
6. Dilute the culture back to 0.25% using fresh medium and RBCs as described above.

SUPPORT PROTOCOL 3: MEASURING PARASITEMIA

This describes staining *P. falciparum* with acridine orange dye and the counting RBCs and parasites using fluorescence microscopy.

Materials

100 µg/ml acridine orange (Sigma) in 1X phosphate buffered saline pH 7.4 (Quality Biological, Inc.)

P. falciparum HOX (ATCC)

Glass microscope slides (Fisher Catalog No. 12-550-15)

Microscope cover glass (Fisher Catalog No. 12-542-B)

100% methanol

Slide storage box

Fluorescence microscope or light microscope fitted with Paralens Illuminator. For a conventional fluorescence microscope, set excitation and emission parameters to visualize DNA-bound dye (Darzynkiewicz et al., 2004).

1. Suspend the cells by gently swirling, take a ~20 µl sample, deposit on an appropriately labeled microscope slide, and with the edge of a second slide create a thin smear of the culture. Air dry. (Detailed in http://www.cdc.gov/dpdx/resources/pdf/benchAids/malaria/Malaria_procedures_benchaid.pdf.)
2. Fix the cells by washing once with methanol; air dry. Preparations can be processed immediately or stored away from light indefinitely.

3. Add 13 µl acridine orange to the slide and place a coverslip on top. Apply a liberal drop of microscope oil on the center of the coverslip.
4. Position the slide so the objective is close to the edge of the cover slip and focus on the RBCs.
5. Count the number of infected RBCs and total RBCs in the field.
6. Count at least 1000 total RBCs. If the parasitemia is very low, we recommend counting at least 50 parasites or up to 2000 total RBCs.
7. Calculate percent parasitemia using equation 7.

$$\% \text{ Parasitemia} = \frac{\# \text{ infected RBCs}}{\text{total \# RBCs}} \times 100 \quad (7)$$

SUPPORT PROTOCOL 4: SYSTEM COMPONENTS AND CONSTRUCTION

Most components of the PK/PD system are custom-built. This provides a brief overview of hardware construction. Detailed instructions and diagrams are beyond the scope of this manuscript; researchers are encouraged to contact the authors directly if further information is needed.

Materials

Standard connecting tubing—Masterflex platinum-cured silicone tubing 96410-14 (1.6 mm i.d., 1/16" hose barb; Cole-Parmer EW-96403-14)

Cartridge tubing (for cartridge sampling ports & inlet/outlet)—Masterflex platinum-cured silicone tubing 96410-15 (4.8 mm i.d., 3/16" hose barb; Cole Parmer EW-96410-15)

2-stop tubing—Purple-white 2-stop platinum-cured silicone tubing (2.79 mm i.d.; IDEX SC0634); Orange-white 2-stop platinum-cured silicone tubing (0.64 mm i.d.; IDEX SC0621); Red-red 2-stop platinum-cured silicone tubing (1.14 mm i.d.; IDEX SC0625A)

Fittings

Cole-Parmer Part Number	Description
T-45505-08	Large Male Luer 3/16"
EW-45505-02	Male Luer 3/32"
T-45502-08	Large Female Luer 3/16"
EW-45502-02	Female Luer 3/32"
EW-06365-25	Elbow Connector 3/32"
EW-06365-11	Straight Connector 1/16"
SI-30800-12	Female Luer Cap
EW-30800-30	Male Luer Cap

Primary loop bottle—Qorpak bottle beaker, 60 mL (Fisher 2992601)

Input/output bottle—Glass media storage bottle with GL45 screw cap (Corning 1395-1L)

Input/ output bottle caps—GL45 media bottle cap, low temperature orange (Pyrex 1395-45LTC)

DAP Auto/Marine Sealant 100% RTV Silicone: (Amazon or local hardware store)

1. Use the specified materials to construct each system component (Fig 7).
2. For all bottle caps, use a liberal amount of DAP auto/marine sealant to secure hardware to the top of the bottle cap.
3. Allow sealant to cure completely (at least 2 days) before autoclaving.

REAGENTS AND SOLUTIONS

Use distilled, deionized water (ddH₂O) in all recipes and protocol steps. Individual chemicals can be purchased from Sigma unless otherwise specified.

Medium

RPMI 1640 powdered medium with L-glutamine, without phenol red or sodium bicarbonate

25 mM HEPES

4.4 μM hypoxanthine

27 mM NaHCO₃

Adjust to pH 7.4 with 1 M NaOH

0.5% (w/v) Albumax II (Life Technologies)

Filter sterilize

Store at 4°C and use up to 1 month

Incomplete medium (for RBC preparation)

RPMI 1640 powdered medium with L-glutamine, without phenol red or sodium bicarbonate

25 mM HEPES

367 μM hypoxanthine

Adjust to pH 7.0 with 1 M NaOH

Filter sterilize

Store at 4°C and use up to 1 month

COMMENTARY

Background Information

Multidrug resistance in *P. falciparum* malaria has created a heightened interest in the identification and development of novel drug candidates. Various methods have been devised to judge the promise of new antimalarial drug candidates, including microtiter plate-based in vitro cytotoxicity assays (some suitable for high throughput screens) and modifications of these methods that focus on time-kill parameters (Sanz et al., 2012; Le Manach, 2013; Sherlach and Roepe, 2014 and references therein). However, most in vitro studies use fixed drug concentrations for a defined time interval, and hence do not capture the effect of dynamic fluctuations of drug concentrations in vivo. Since *P. falciparum* survives only in humans, in vivo models typically involve non-*falciparum* species that infect rodents or nonhuman primates. While these do provide dynamic drug concentrations, neither the host nor the parasite species is directly relevant. The biology and pathophysiology of infection can vary substantially from those of *P. falciparum* in humans, as can the metabolism and kinetics of the drug. Partially addressing this is the humanized mouse model for *P. falciparum*, which is costly and requires severe immunodeficiency in the animals as well as daily infusions of human RBCs (Jimenez-Diaz et al., 2009).

To overcome these major obstacles and facilitate PK/PD analysis, we developed a preclinical model system in which *P. falciparum* malaria parasites are exposed to dynamic drug concentrations in vitro. This system can be used to study the PK dependence of many different PD endpoints (e.g., parasite growth – including time-kill, intracellular accumulation of drug metabolites, metabolomics changes, and emergence of drug resistance), thus facilitating drug development at a number of points. Since PK profiles can be simulated without the limitations imposed by animal physiology, the PK parameter that governs efficacy can be unambiguously identified. Comparing the time- or concentration-governance (elucidated with our system) with PKs subsequently obtained in animals provides an additional rational basis for selection of lead candidates, and gives guidance for their further testing in the drug development pipeline.

The system can also be programmed to mimic known kinetics in humans. For anti-infective drugs in development, this capability has proven valuable in selecting the best dosing regimens for clinical trials (Brown et al., 2011), thus saving considerable time and expense. Moreover, using in vitro dynamic PK/PD allows the anti-infective efficacy and prevention of resistance by combination drug regimens to be evaluated in a systematic manner, an approach simply not possible in clinical trials (Drusano et al., 2014).

Critical Parameters

PK validation—Before beginning the dynamic PK/PD cartridge studies described in this unit, it is important to understand the underpinning PK/PD concepts (Rowland and Tozer, 2004). Two kinetic regimens are being compared: the constant infusion held above a minimum inhibitory concentration versus the high peak concentration that is rapidly eliminated - the T_{MIC} and C_{MAX} regimen, respectively - in order to determine the PK parameter governing drug efficacy. For a given experiment, it is critical that the two

regimens produce the same overall total drug exposure, or AUC. Adjusting for non-specific drug binding to and release from system components ensures that the desired pharmacokinetics can, in fact, be achieved. Furthermore, some drugs may not be stable in aqueous solution for the 72 hr duration of these experiments. Final verification that expected PKs are actually obtained requires quantitative analysis of drug concentrations in timed samples taken from the PD compartment, where cells reside.

Apparatus assembly—In order to successfully complete Basic Protocols 2–4, several critical factors must be taken into account. The preparation and assembly of cartridges needs to be as uniform as possible. This can be challenging at first; however, Video 2 provides a visual aid and guided step-by-step instructions to achieve consistently reliable results. Construction of the apparatus for Basic Protocols 3 and 4 becomes relatively straightforward once direction of flow is understood (see Fig. 5 and Video 3). Aseptic technique throughout these experiments is of paramount importance. The considerable time, manpower, and expense of completing all four protocols is too great to risk contamination because of inadequate sterile technique.

Malaria strains and replication requirements—The drug-sensitive NF54 HOX strain used in these protocols can be substituted with other strains that have not been adapted to relatively high O₂ atmosphere, provided the apparatus is maintained in an incubator with microaerophilic (2–5% O₂) conditions. Given the biological variation between strains, it is critical to be consistent with strain choice throughout all four protocols. To account for intrinsic variation in malaria cell culture in vitro and to obtain statistical validity, Basic Protocols 1 and 4 should be run independently at least three times.

Troubleshooting

Mechanical problems—The major mishaps in these experiments are overgrowth by contaminating microbes and failure of various system components. Our low rate of <10% cartridge contamination, despite not using antibiotics in the medium, is attributable to meticulous sterile technique, thoroughly autoclaving the system components, banning paper products (laboratory wipes, cardboard) from the BSC area, and ensuring all connections are tight. Once contaminated, everything but the glass cartridges must be discarded, and the cartridges should be soaked overnight in 3% sodium hypochlorite (household bleach).

The most common mechanical problems involve pump tubing and the glass cartridge. If the 2-stop tubing is not properly positioned in the cassettes, medium will not flow through the system correctly, affecting drug clearance and making the peak and infusion regimens incomparable. This problem can be avoided by ensuring that the placement of all 2-stop tubings is secure, and monitoring the system periodically to detect and correct tubing slippage. If tubing misalignment does occur, we advise stopping the pump, realigning the tubing within the cassette, then continuing the run. The narrow diameter tubings are also susceptible to clogging. This is minimized by the use of Albumax rather than human serum, and can further be avoided if the system is dismantled promptly and thoroughly rinsed after each run. Deformation of the 2-stop tubing after repeated use and autoclaving will lead to

erroneous flow rates. We recommend discarding it after three to four uses, regardless of apparent condition.

Although uncommon, leaks do occur, usually due to an improper seal at the inlet or outlet of the cartridge. It is important to check the cartridge periodically after addition of the parasitized RBC suspension. Leakage may reflect improper assembly of the dialysis tubing, or deformities in the inlet/outlet tubing due to overuse. We recommend discarding inlet/outlet tubing after six uses regardless of apparent condition. Treating a leaking joint with 70% ethanol and tightly wrapping it in parafilm may allow successful completion of the experiment. However, in most cases, it is usually more efficient to abort the run and restart after fixing the problems listed above.

Unexpected results/variability—Once Basic Protocols 1–3 have been satisfactorily completed, the definitive PK/PD experiments of Basic Protocol 4 have proven quite reproducible. Nevertheless, unexpected or variable results may arise. In addition to microbial contamination and mechanical failures described above, malaria parasites are exquisitely sensitive to some plastics. Even different lots of the same product may cause toxicity. Materials listed throughout this unit have been carefully tested for compatibility with malaria parasites, and in general they represent the very few options that have proven nontoxic. However, when a new lot of tubing (in particular) is purchased or if the composition of a system component changes, we advise testing for any effect on parasite proliferation before proceeding with PK/PD experimentation. Parallel testing of drug response in flasks in Basic Protocol 4 steps 21–22 provides a basis for detecting and analyzing any unexpected PK/PD results.

Anticipated Results

In Basic Protocol 1, a dose-response curve is generated based on [³H]-hypoxanthine incorporation as a measure of antimalarial activity. In our hands, EC₅₀ values from independent determinations generally fall within 30% of one another (Posner et al., 1997). Basic Protocol 2 evaluates interaction of the drug with the hollow fiber system components, while Basic Protocol 3 provides a basis for making (and testing) adjustments that compensate for this common effect. Ultimate validation of this method stems from assay of drug concentrations in timed PK samples. PK/PD governance is ascertained in Basic Protocol 4. The relatively simple PD endpoint we use – cell count – can be measured directly by microscopy, as described (tedious, time-consuming, somewhat subjective) or by surrogate assays *e.g.* [³H]-hypoxanthine incorporation or SYBR Green (Sherlach and Roepe, 2014 and references therein). In our hands and for triplicate determinations, the standard deviations in percent efficacy obtained in Basic Protocol 4 are typically within 10% of the mean.

Time Considerations

This work is labor- and time-intensive. *P. falciparum*'s 48 hr life cycle mandates that these experiments, which may be completed within hours for most bacteria, require several days for malaria parasites. Assuming availability of radiolabeled tracer, no unanticipated problems, straightforward results in the drug-binding and -release studies, and support staff

to prepare and breakdown the apparatus, at least six weeks of continuous experimental time are required to run a drug through all four sequential protocols, including three independent runs of Basic Protocol 4.

The 96-well microtiter plate cytotoxicity assay in Basic Protocol 1 spans five days; however, only about 6 hr of hands-on work is required. From setup to data analysis, Basic Protocol 2 and 3 can require 7–14 days, depending on the extent of drug binding and whether iterative adjustments and validations are necessary. Basic Protocol 4 requires five days to set up and run one experiment, plus time for data analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Pharmacokinetics in vivo. Upon absorption of an orally administered drug, concentration in blood rises, and reaches a maximum (C_{MAX}) as distribution, metabolism, and elimination prevail. Concentration then falls at a characteristic rate ($t_{1/2}$) until the drug is eliminated from the blood. During this period, drug concentrations persist above a Minimum Inhibitory Concentration (MIC) for a defined amount of time (T_{MIC}). The area under the concentration-time curve (AUC) is a measure of drug dose and exposure. (B) Determining the efficacy-driving PK parameter. Identical AUCs can be achieved using two extreme dosing regimens that emphasize different PK parameters. A single peak (blue) will achieve high C_{MAX} with relatively low T_{MIC} . For the same AUC, a constant infusion (C_{INF} , red) provides relatively low concentration with long T_{MIC} . The difference in outcome of these regimens reveals the efficacy-driving PK parameter.

Figure 2.

Workflow for identifying the PK driver of drug efficacy involves a series of experiments. At the start, conventional dose-response assays (blue) provide an EC_{40} value to be used for the T_{MIC} infusion regimen. Drug solutions in medium are then circulated through the hollow fiber apparatus to ascertain whether non-specific binding to the inner surfaces, and subsequent release from these surfaces, will significantly impact the programmed PKs (yellow). In most cases interference can be corrected or accommodated by a pre-incubation step and/or change in target half-life (orange). Finally, with parasitized RBCs in the system, PK/PD is assayed in triplicate (red) for robust identification of the PK driver of antimalarial efficacy.

Figure 3.

The cartridge for PK/PD studies with *P. falciparum* comprises a custom made glass cylinder with inlet, outlet, and two sampling ports, traversed by single-use dialysis tubing. Inlet and outlet ports direct medium flow through the dialysis tubing. Sampling ports permit sterile access to the extra-tubing PD compartment. The main body of the cartridge has an outer diameter of 2 cm, length of 7 cm and volume of 15 ml. Each sampling ports is 2 cm long, 0.4 cm inner diameter, and 0.6 cm outer diameter. See Basic Protocol 2 and Video 2 for cartridge construction.

Figure 4.

The primary loop (coded with purple tape) includes a single cartridge linked to its primary loop bottle by tubing. Medium is pumped from the cartridge to the bottle as indicated by the arrow on the pump cassette. Rapid flow in the primary loop is achieved by the large diameter purple-white 2-stop tubing that traverses the pump cassette. A sterile vent at the center-top of the primary loop bottle permits gas exchange in the incubator.

Figure 5.

Schematic of the complete PK/PD system for a single cartridge includes a primary loop (purple) and the modules that feed into (green), and out from (yellow), the primary loop bottle. As medium circulates in the primary loop, its composition is dynamically changed by medium entering from the input bottle. Simultaneous removal of fluid via the output line maintains a constant volume in the primary loop. A single pump serves all three routes.

Figure 6.

One cartridge PK/PD system includes the pump, primary loop with its cartridge and bottle (purple), and the associated input module (green) and output module (yellow) that link to the primary loop bottle and permit dynamic variation in drug concentrations. Flow rate in the input and output modules, governed by the orange-white and red-red 2-stop tubing (respectively), is substantially less than that of the primary loop. Direction of flow is denoted by black arrows on pump cassettes. See text for details.

Figure 7.

Commercially-available components are assembled as indicated. Key organizing points for fluid trafficking are the hand-drilled bottle caps traversed by connectors that are secured with marine glue. **(A)** Input line is prepared by combining A: orange-white 2-stop tubing; B: 1/16" straight connectors; C: 30 cm standard connecting tubings; D: 3/32" male luers; and E: slide clips. Connection to input bottle and primary loop bottle port are via luers at D. **(B)** Cartridge sampling ports and inlet/outlet are assembled from A: 3/16" female luers; B: 3.5 cm cartridge tubings; C: 5.5 cm cartridge tubings; and D: 3/16" male luers. **(C)** Input/Output bottle cap (bottom view) comprises the cap with its connectors (see Panel E) and A: standard connecting tubings (5cm for output, 20.5 cm for input). **(D)** Output line is assembled from A: red-red 2-stop tubing; B: 1/16" straight connectors; C: 60 cm standard connecting tubings; and D: 3/32" male luers. Connection to output bottle and primary loop bottle port are via luers at D. **(E)** Input/Output bottle cap (top view) is made from A: 3/32" elbow connectors; B: 1/16" straight connector (link this to 2.5 cm standard connecting tubing and add 3/32" male luer to make the venting port, not depicted); C: 5 cm standard connecting tubings; and D: 3/32" female luers. **(F)** Primary loop is a combination of A: 3/32" female luers; B: purple-white 2-stop tubing; C: 3/32" male luers; D: 45 cm standard connecting tubing; E: slide clips; F: 22 cm standard connecting tubing; and G: 1/16" straight connector. Primary loop bottle is connected at left A of 2-stop tubing and G. **(G)** Primary loop bottle cap (bottom view) comprises the cap with its connectors (see Panel H) and A: 7.5 cm

standard connecting tubing; *B*: 3.1 cm standard connecting tubing; *C*: 3.0 cm standard connecting tubing; *D*: 3/32" elbow connector; *E*: 3.0 cm standard connecting tubing; and *F*: 4.4 cm standard connecting tubing. Lengths of *B*, *C* and *F* must be exact to achieve the 50 mL V_d specified in the text, and elbow connector *D* must be situated directly under the orifice of tubing *B* for correct mixing of solutions. **(H)** Primary loop bottle cap (top view) consists of *A*: 3/32" elbow connectors; *B*: 1/16" straight connector (link this to 2.5 cm standard connecting tubing and add 3/32" male luer to make the venting port, not depicted); *C*: 4 cm standard connecting tubings; *D*: 3/32" female luers; *E*: 15 cm standard connecting tubings; and *F*: 3/32" male luer. Primary loop is connected at *F* (to *A* in Panel F) and at *E* (to *G* in Panel F).

Table 1

Layout for 96-Well Plate Assay of Antimalarial Activity against *P. falciparum*

	1	2	3	4	5	6	7	8	9	10	11	12
A		Conc A ₁				Conc A ₂				Conc A ₃		
B		Conc A ₄				Conc A ₅				Conc A ₆		
C		Conc A ₇				Conc A ₈				Conc A ₉		
D		Conc A ₁₀			Vehicle A					RBC only		
E		Conc B ₁				Conc B ₂				Conc B ₃		
F		Conc B ₄				Conc B ₅				Conc B ₆		
G		Conc B ₇				Conc B ₈				Conc B ₉		
H		Conc B ₁₀			Vehicle B					Parasite only		

Two drugs (A and B) are assayed per plate, each at ten concentrations. Drug concentrations are tested in quadruplicate (depicted as blocks of four wells). *RBC only* controls (shaded D9-12) are uninfected RBCs with vehicle-free, drug-free medium; all other wells contain parasitized RBCs. *Parasite only* controls (H9-12) contain vehicle-free, drug-free medium. *Vehicle* controls (D5-8 and H5-8) contain vehicle but no drug. The remaining wells have drug, in a constant final concentration of vehicle, to match that of the cognate vehicle control.