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EXPERIMENTAL THERAPEUTICS

Investigating Sulfoxide-to-Sulfone Conversion as a Prodrug Strategy for a Phosphatidylinositol 4-Kinase Inhibitor in a Humanized Mouse Model of Malaria

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ABSTRACT The *in vivo* antimalarial efficacies of two phosphatidylinositol 4-kinase (PI4K) inhibitors, a 3,5-diaryl-2-aminopyrazine sulfoxide and its corresponding sulfone metabolite, were evaluated in the NOD-scid IL2Rγ^{null} (NSG) murine malaria disease model of *Plasmodium falciparum* infection. We hypothesized that the sulfoxide would serve as a more soluble prodrug for the sulfone, which would lead to improved drug exposure with oral dosing. Both compounds had similar efficacy (90% effective dose [ED_{90}], 0.1 mg kg^{-1} of body weight) across a quadruple-dose regimen. Pharmacokinetic profiling revealed rapid sulfoxide clearance via conversion to sulfone, with sulfone identified as the major active metabolite. When the sulfoxide was dosed, the exposure of the sulfone achieved was as much as 2.9-fold higher than when the sulfone was directly dosed, thereby demonstrating that the sulfoxide served as an effective prodrug for the treatment of malaria.

KEYWORDS NSG mouse model, malaria, prodrug, sulfoxide

Malaria, a mosquito-borne disease caused by infection of *Plasmodium* parasites, is one of the leading causes of death in sub-Saharan Africa. The WHO estimates that there were approximately 216 million cases of malaria worldwide, with 445,000 deaths, in 2016 (1). Public health measures and combination chemotherapy with artemisinin derivatives and partner compounds have largely succeeded in reducing morbidity and mortality over the last decade, but clearly, more progress needs to be made. Worrying reports are emerging around artemisinin-resistant parasites, which has become particularly troublesome since artemisinin-based combination therapy (ACT) regimens are currently considered the optimal standard of care (2–4). An urgent need therefore exists for treatment modalities that will overcome resistance, such as the development of novel and more efficacious antimalarial drugs and new drug combinations.

We have previously reported orally active antimalarial agents based on the 2-aminopyridine scaffold (5, 6). This work led to the identification of MMV390048, which was potent across the plasmodial life cycle except against hypnozoites, and which is currently in phase 2 clinical development (7). MMV390048 operates by inhibition of

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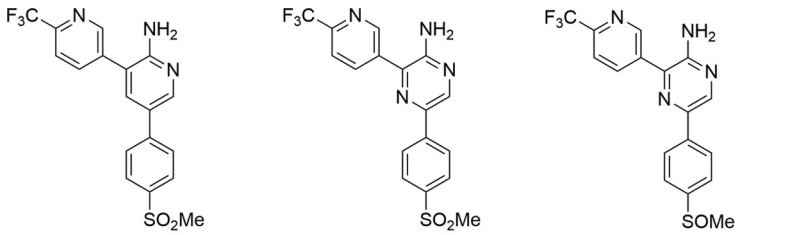

FIG 1 Structures of MMV390048, the aminopyrazine sulfone, and its corresponding sulfoxide.

Plasmodium phosphatidylinositol 4-kinase (PI4K), a novel mode of action relative to all other clinical antimalarial agents. As MMV390048 offers many advantages to current antimalarial therapies, including high activity against all resistant phenotypes (50% inhibitory concentration [IC_{50}], 17 to 26 nM) studied so far, and given the inherent risk of failure during clinical development, the identification of back-up or newer-generation PI4K inhibitors thus becomes imperative (7). Further structure-activity relationship (SAR) exploration around MMV390048 led to the identification of closely related 2-aminopyrazines that maintain potent antimalarial activity and other favorable drug metabolism and pharmacokinetic (DMPK) properties, including half-lives greater than 4 h in rats (8). The morphological effects of the aminopyrazines on *Plasmodium* spp. are likely similar to those of MMV390048 (7), though this has not been investigated. The lead aminopyrazine, a sulfone (Fig. 1), illustrated promising *in vitro* antiplasmodial and remarkable *in vivo* antimalarial activity with upwards of 99% reduction of parasitemia on 4-day quadruple dosing at 1 mg kg^{-1} of body weight in a *Plasmodium berghei* mouse model of infection (8). However, its poor solubility was identified as a key risk associated with its progression as a preclinical development candidate (8). Hence, an analogue program commenced wherein highly active more-soluble analogues with favorable pharmacokinetic attributes were identified (9). As another approach, we recognized that the corresponding sulfoxide analogue was more soluble and had similar *in vitro* activity, with an IC_{50} of 15.9 nM against NF54 *Plasmodium falciparum* compared to the sulfone (IC_{50}, 8.4 nM). Furthermore, we hypothesized that the sulfoxide would serve as a prodrug for the sulfone, which would lead to improved drug exposure on oral dosing due to the higher solubility, as has been described for other sulfoxides (10, 11). In this paper, we extend the work in this series by testing the aforementioned hypotheses in an *in vivo* humanized model of malaria.

A promising approach to test efficacy against human malaria is the use of a murine disease model utilizing immunocompromised NSG mice developed at GlaxoSmithKline (GSK) Tres Cantos (12, 13) and now implemented at the University of Cape Town's Drug Discovery and Development Centre (H3D). NSG mice are able to be intraperitoneally engrafted with human red blood cells, resulting in a gradual increase in human erythrocytes in peripheral circulation, likely via the uptake by the lymphatic system. The mice are then intravenously infected with *P. falciparum* that can specifically infect the available human erythrocytes (14). Relative to *P. berghei* infection models, being able to model *P. falciparum* infections using human erythrocytes *in vivo* offers the clear advantage that efficacy would better characterize the human disease condition.

In this paper, we use the NSG mouse model to achieve two main objectives, as follows: first, to determine whether administration of a more-soluble prodrug sulfoxide leads to a higher exposure of the corresponding sulfone relative to dosing the sulfone itself, and second, to determine whether this increase in exposure leads to better antimalarial efficacy.

RESULTS

***In vitro* antiplasmodial activity, physicochemical properties, and *in vitro* microsomal metabolism.** The activities of the sulfoxide were comparable between the

TABLE 1 *In vitro* antiplasmodial activity, physicochemical properties, and *in vitro* microsomal metabolism data of sulfoxide and sulfone

Parameter[a]	Sulfoxide	Sulfone
*Pf*NF54 IC_{50} (nM)	15.9 ± 1.0	8.4 ± 1.7
*Pf*K1 IC_{50} (nM)	14.0 ± 2.3	10 ± 1.9
*Pf*3D7$^{0087/N9}$ IC_{50} (nM)	11.8 ± 1.1	9.6 ± 0.8
Solubility (pH 7.4) (μM)	70	25
LogD	2.1	2.5
PAMPA	−4.1	−4.2
MLM E_H	0.3	<0.1
HLM E_H	0.2	<0.1

[a]LogD, the log of the distribution coefficient (D) for molecules partitioned between octanol and water at pH 7.4; PAMPA, parallel artificial membrane permeability assay; MLM E_H, mouse liver microsome extraction coefficient; HLM E_H, human liver microsome extraction coefficient.

adapted *Pf*3D7$^{0087/N9}$ and *Pf*NF54 strains (Table 1), and they were only marginally lower than those of the sulfone.

As expected, the sulfoxide was more soluble and less lipophilic than the sulfone. However, the passive permeabilities of compounds were comparable. The sulfoxide was moderately stable in human (HLM) and mouse liver microsomes (MLM), while the sulfone was considerably more stable.

Pharmacokinetics of sulfoxide and sulfone. The *in vivo* PK of the sulfoxide and sulfone was determined in healthy male C57BL/6 mice (Fig. 2) upon intravenous (i.v.) dosing at 1.5 mg kg^{-1}. The sulfoxide showed a short half-life ($t^1/_2$) (<1 h) and was rapidly cleared with high conversion to the sulfone as the main metabolite. The exposure (area under the concentration-time curve [AUC]) of the sulfone in whole blood over the course of the experiments was more than 15-fold higher than that of the parent sulfoxide (see Table S3 in the supplemental material).

Metabolite identification. The main metabolite generated from the sulfoxide both *in vitro* and *in vivo* was the corresponding sulfone, with the only other metabolite being a minor dioxidation metabolite (see the supplemental material). The dioxidation metabolite was also detected at low levels from the sulfone both *in vitro* and *in vivo*. Microsomal incubations confirmed that metabolism of the sulfoxide and sulfone was enzyme and NADPH dependent.

Therapeutic efficacy of sulfoxide and sulfone. (i) Quadruple-dose regimen of sulfoxide and sulfone. Fig. 3 shows the parasitemia observed for the sulfoxide and sulfone following a quadruple-dose regimen against *P. falciparum*. In both cases, four consecutive doses of 0.5 mg kg^{-1} and 1.5 mg kg^{-1} translated into the highest efficacy, with 0.1 mg kg^{-1} being less effective and 0.05 mg kg^{-1} being ineffective, rendering results similar to those of the untreated control group. Interestingly, the highest dose of 10 mg kg^{-1} did not translate into the highest efficacy, though the exposures (peak concentration of drug in whole blood [C_{max}] and AUC) were clearly higher (Table S4).

Blood samples collected on the first day of treatment were used to determine the PK parameters for both the sulfoxide and sulfone in the infected NSG mice. The

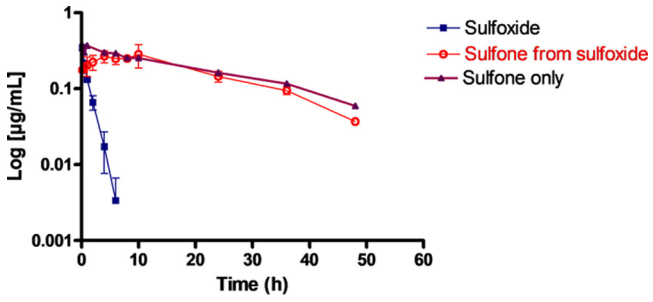

FIG 2 Mean log circulating levels of the sulfoxide (bold lines), its sulfone metabolite (dotted lines), and sulfone only following i.v. administration of either sulfoxide or sulfone.

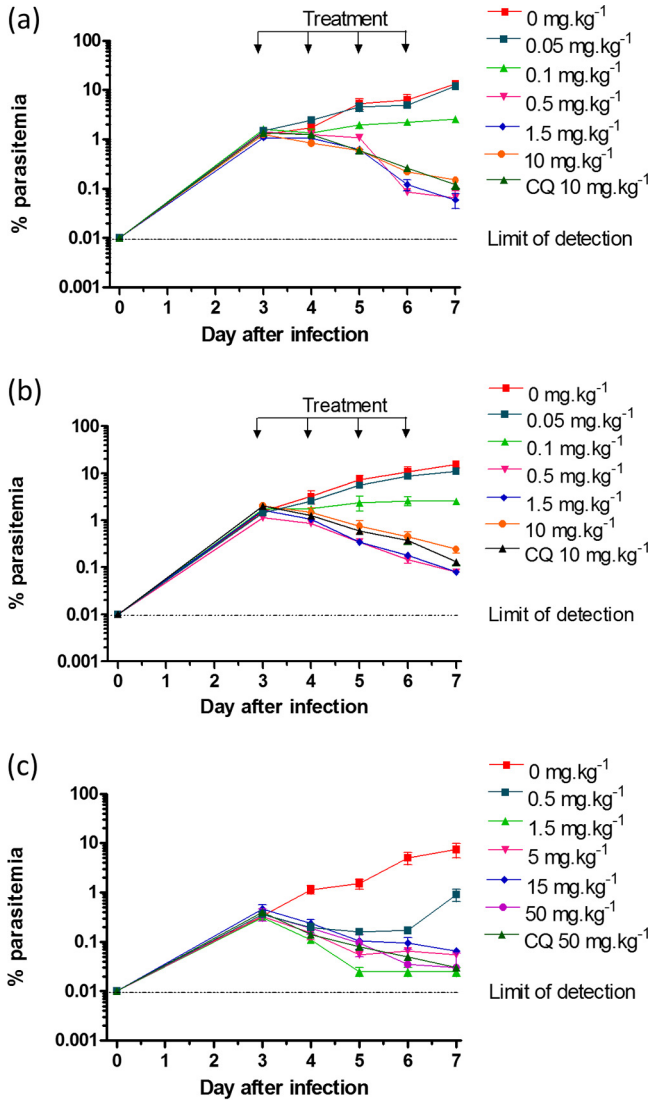

FIG 3 Percent parasitemia observed in the NSG model of *P. falciparum* infection. (a) Daily quadrupole oral dosing of sulfoxide. (b) Daily quadruple oral dosing of sulfone. (c) Single oral dose of sulfoxide. The result for chloroquine (CQ) is included in each graph.

sulfoxide was rapidly absorbed (time to C_{max} [T_{max}], 0.375 to 2 h) at all doses, with the sulfone being detected as the main metabolite. The sulfone had a much higher exposure (Table S4), suggesting that the efficacy observed for the sulfoxide was mainly due to this conversion. The dioxidation metabolite of the sulfoxide (mono-oxidation of the sulfone) is considered too low level to be consequential for the observed efficacy. The absorption of the sulfone was considerably slower (T_{max}, 4 to 7 h), and its exposure (AUC) was higher following oral administration of the sulfoxide, demonstrating the benefits of the prodrug approach. As the dose escalated, the ratio of sulfone to sulfoxide decreased; for example, at 1.5 mg kg^{-1}, the ratio was 30, while at 10 mg kg^{-1}, it was 16.

(ii) Single-dose regimen of the sulfoxide. The PK profiles of both the sulfoxide and its metabolite showed that significant levels of the sulfone were still in circulation 24 h after dosing. This would suggest that the sulfone metabolite accumulates during a quadruple-dose regimen of the sulfoxide in NSG mice. A single-dose study was therefore performed to assess the efficacy and PK of the sulfoxide. The PK profile is summarized in Table S5. The *in vivo* therapeutic efficacy against *P. falciparum* following a single dose of the sulfoxide is illustrated in Fig. 3. The single dose of 1.5 mg kg^{-1}

TABLE 2 Comparison of pharmacokinetic parameters of the sulfoxide and its sulfone metabolite following oral administration of 1.5 mg kg^{-1} sulfoxide[a]

Mouse strain	C_{max} ($\mu g\ ml^{-1}$)	T_{max}(h)	$AUC_{(0-48)}$ ($\mu g\ h\ ml^{-1}$) Sulfoxide	$AUC_{(0-48)}$ ($\mu g\ h\ ml^{-1}$) Sulfone	Ratio $AUC_{(0-t)}$ Sulfone/sulfoxide
C57BL/6	0.104	1	0.221	7.08	32.0
Healthy NSG	0.0537	1	0.153	7.00	45.8
Infected NSG	0.0321	1	0.165	6.64	40.0

[a]$AUC_{(0-48)}$, AUC from time 0 to 48h; $AUC_{(0-t)}$, AUC from time 0 to time *t*.

translated to the highest efficacy, with higher doses leading to a lower drop in parasitemia, despite increases in exposure as the dose escalated. Similar reductions in parasitemia were seen for the single dose of 15 mg kg^{-1} compared to the four consecutive doses of 10 mg kg^{-1} (Fig. 3).

Exposure in healthy versus infected mice. The oral PK parameters of the sulfoxide were determined in healthy NSG and infected NSG mice, as well as in healthy C57BL/6 mice, at 1.5 mg/kg (Table 2). The combined AUCs of the sulfoxide and sulfone were similar in healthy C57BL/6 and healthy NSG mice, suggesting that absorption of the sulfoxide was comparable in the two strains. However, the sulfoxide-to-sulfone conversion was faster in the NSG mice, as evidenced by the lower sulfoxide C_{max} and the higher sulfone-to-sulfoxide ratio. Exposures of the sulfoxide and sulfone were marginally lower in infected NSG mice, suggesting an effect of the infection on the pharmacokinetics of the compounds.

Dose-response relationship in the NSG malaria mouse model. To evaluate the ED_{90} (on day 7 following infection) of the compounds, dose-response experiments were conducted in the NSG mouse model of malaria at a quadruple-dose regimen of 0.05, 0.1, 0.5, 1.5, and 10 mg kg^{-1} administered daily for four consecutive days. An additional single-oral-dose experiment was carried out with the sulfoxide, at 0.5, 1.5, 5, 15, and 50 mg kg^{-1}. In both series of experiments, dosing was initiated on day 3 following infection, and the percent parasitemia was evaluated on days 3 to 7 daily as the primary efficacy readout. The relationship between parasitemia and dose was described by a sigmoid dose-response model and was used to determine the ED_{90} and AUC_{ED90} for the compounds (Table 3). Sigmoidal plots of parasitemia relative to dose and AUC are illustrated in Fig.S7 and S8 in the supplemental material. The ED_{90} value of 2.80 mg kg^{-1} obtained for chloroquine compared well with those found in previous publications (14). Both parent compounds displayed a maximum reduction in para-

TABLE 3 Efficacy parameters for both compounds with log fit against *P. falciparum* *Pf*3D7$^{0087/N9}$

Parameter by method of estimation (dosing regimen)	Goodness of fit	Mean
Sulfoxide (quadruple dose)		
ED_{90} (mg kg^{-1})	0.9682	0.12
AUC_{ED90} ($\mu g\ h\ ml^{-1}$)	0.9681	0.39
$C_{maxED90}$ ($\mu g\ ml^{-1}$)	0.9681	0.02
Sulfone (quadruple dose)		
ED_{90} (mg kg^{-1})	0.9627	0.12
AUC_{ED90} ($\mu g\ h\ ml^{-1}\ day^{-1}$)	0.9621	0.20
$C_{maxED90}$ ($\mu g\ ml^{-1}$)	0.9620	0.03
Sulfoxide (single dose)		
ED_{90} (mg kg^{-1})	0.9538	0.51
AUC_{ED90} ($\mu g\ h\ ml^{-1}$)	0.9544	1.33
$C_{maxED90}$ ($\mu g\ ml^{-1}$)	0.9538	0.09
Chloroquine		
ED_{90} (mg kg^{-1})	0.9994	2.80
AUC_{ED90} ($\mu g\ h\ ml^{-1}$)	0.9465	1.79

sitemia compared to the untreated control groups, with ED_{90} values of 0.12 mg kg^{-1} for both the sulfoxide and sulfone following the quadruple-dose regimen. The average estimated daily exposures (AUC_{ED90}) required to reduce parasitemia by 90% compared to the untreated control group were 0.39 and 0.20 μg h ml^{-1} day^{-1} for the sulfoxide and sulfone, respectively. The single-dose study resulted in higher ED_{90} and AUC_{ED90} values of 0.51 mg kg^{-1} and 1.33 μg h ml^{-1} over the 96-h period, respectively (Table 3 and Fig. S8). Single dosing of the sulfoxide therefore was demonstrated to be highly efficacious against *P. falciparum* in the model, though it was less efficacious than multiday dosing, as would be expected. In order to determine a pharmacokinetic/pharmacodynamic (PK/PD) driver responsible for the observed efficacy, we determined the goodness-of-fit profiles in the form of R^2 values derived from the sigmoid dose-response model used to describe the data. However, no concise conclusion could be made from the R^2 values due to the steepness of the slope.

DISCUSSION

The NSG murine malaria model allows *in vivo* evaluation of the antimalarial efficacy of promising compounds. The use of *P. falciparum*, a human pathogen, allows a determination of pharmacodynamic parameters that can be used to guide the prediction of such parameters in humans. We used this model to investigate the efficacy of a sulfoxide, its sulfone metabolite, and the contribution of this conversion to antimalarial activity. The ED_{90} values of 0.12 mg kg^{-1} in the model for both the sulfoxide and sulfone were lower than those for published compounds in this series, namely MMV390048 (ED_{90}, 0.57 mg kg^{-1}) and UCT943 (ED_{90}, 0.25 mg kg^{-1}) (7, 24). Clearly, the higher activity in the model is due to the combination of multiple factors, including *in vitro* efficacy, protein binding, partitioning into red blood cells, and pharmacokinetics.

Metabolism of sulfoxides typically produces sulfones as metabolites, though several enzymes, including cytochrome P450s, also catalyze the reductive reaction to sulfides (25–27). Sulfoxides are typically more soluble than the corresponding sulfones and can therefore be exploited as prodrugs for the delivery of sulfones (10). As part of our efforts to develop new antimalarial compounds, we sought to investigate whether this prodrug approach could be used to deliver higher exposures of the sulfone than might be achieved by dosing the sulfone itself and to investigate the contribution of the sulfoxide and sulfone to the *in vivo* antimalarial efficacy.

There were no significant *in vitro* differences between the IC_{50}s of the sulfoxide against the strains of *P. falciparum* tested, including against the *Pf*3D7$^{0087/N9}$ strain adapted for use in the NSG infection model. Furthermore, the main metabolite detected both *in vitro* and *in vivo* was the sulfone metabolite. As both the sulfoxide and sulfone are similarly active against *P. falciparum in vitro*, activity in an *in vivo* model of infection is likely to reflect the exposure of both compounds combined. However, the conversion kinetics of the sulfoxide into the sulfone was sufficiently rapid (AUC ratios of sulfone to sulfoxide were greater than 30, as seen in the i.v. PK with the C57BL/6 mice), such that the demonstrated *in vivo* activity was largely due to the sulfone. This clearly translated in a similar manner to oral dosing in the NSG mouse, though there was a measure of saturation for the conversion of the sulfoxide to the sulfone, with the sulfone-to-sulfoxide ratios decreasing as the dose escalated. The sulfone was also the main metabolite in human liver microsomes, suggesting that metabolism should be qualitatively similar in mice and humans. Overall, oral administration of the sulfoxide at all the doses utilized resulted in higher exposures of the sulfone than with administration of the sulfone itself (Fig. 4).

For example, close to the ED_{90} of 0.12 mg kg^{-1} (that is, the 0.1 mg kg^{-1} dose), the AUC of the sulfone due to sulfoxide dosing was 1.8-fold higher than that seen with dosing the sulfone itself. At the highest 10-mg kg^{-1} doses, the differential was 2.9-fold. These results show that dose escalation of the sulfoxide led to a superscaling effect, whereby the higher doses delivered disproportionately higher exposures of the sulfone, while higher doses of the sulfone led to flat if not lower relative exposures. Hence, the

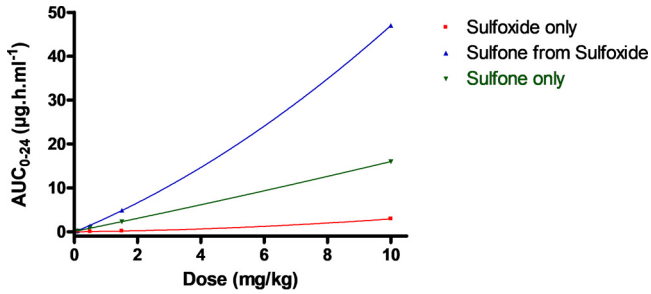

FIG 4 Exposure (AUC_{0-24}) of the sulfoxide, sulfone metabolite, and sulfone when either the sulfoxide or the sulfone is administered orally to infected NSG mice.

sulfoxide indeed served well as a prodrug to deliver higher drug exposures. The lower solubility of the sulfone made it difficult to achieve higher exposures. The superscaling seen with dose escalation of the sulfoxide indicates that at least one important clearance mechanism for the sulfone was saturated. Since the sulfoxide and sulfone are similarly active against *P. falciparum in vitro*, it is well understood for precision of PK/PD analyses that total concentrations of sulfoxide plus sulfone would be slightly better to use for studies with the sulfoxide. *In vivo*, the sulfone illustrated a long half-life of 16 h. This suggests that appropriate dosing would allow for a sustained exposure, which is important to a potential single-dose eradication of the parasite.

The sulfoxide showed potent *in vivo* activity, with an ED_{90} of 0.12 mg kg^{-1} in the 4-day dose NSG model of infection. It was therefore equipotent to the sulfone, which displayed a similar ED_{90}. The PK profile of the sulfoxide showed that it was rapidly cleared, with no levels detectable past 10 h at all doses. The AUC of the sulfone metabolite was 2- to 3-fold higher than the AUC of the parent compound and remained detectable for more than 24 h after dosing. The reason why the higher exposure resulting from dosing the sulfoxide relative to the sulfone did not lead to a measurable improvement in ED_{90} remains unclear. This could be due to the imprecise determination of the ED_{90} resulting from the sharp inflection of the dose-response and exposure-response curves. Alternatively, a hint to understanding PK/PD comes from the observation that though the 0.05-mg kg^{-1} dose of the sulfoxide and the 0.1-mg kg^{-1} dose of the sulfone had about equal AUC values, the sulfoxide performed less well, with no drop in parasitemia, relative to the sulfone with a 90% drop in parasitemia (Fig. 3). The C_{max} was lower for the sulfoxide (9.6 μg ml^{-1}) than for the sulfone (14.2 μM), and the T_{max} was later (Table S4). It is possible that C_{max} influences efficacy more strongly than AUC, accounting for the higher parasitemia of the 0.05-mg kg^{-1} dose than that of the 0.1-mg kg^{-1} dose. Clearly, a more extended dose fractionation study would help elucidate the primary driver of efficacy (28, 29).

Interestingly, we also observed that the highest dose in the quadruple-dosing experiments for both the sulfoxide and sulfone increased rather than reduced parasitemia relative to the optimal 0.5- to 1.5-mg kg^{-1} doses. The sulfoxide was also effective as a single dose, with an ED_{90} of 0.51 mg kg^{-1}, and similarly showed an (albeit less pronounced) optimal dose of 1.5 mg kg^{-1}. There is considerable interest in identifying drug regimens that would deliver single-dose cures (30), hence the value of comparing single- versus multiple-day dosing. Varying the doses and dosing intervals will further help delineate PK/PD relationships that are important toward maximizing ultimate efficacy in the clinical setting. However, the cause is unclear for the seemingly aberrant dose and exposure responses, a PK/PD discrepancy that has not been seen before with other compound classes of antimalarials (31–33). The expectation might be that efficacy would plateau with achievement of a maximum effect and there might be a limit to the speed of parasite clearance. Experimental variability once this plateau is reached does not explain this decreasing parasitemia result, as similar results were seen for both the sulfone and sulfoxide, with quadruple dosing versus single dosing, and for other PI4K-inhibiting aminopyridines and aminopyrazines. We speculate that there is

involvement of a host factor that is inhibited by these PI4K inhibitors in the malaria parasite. This class of compounds is selective for *Plasmodium* over human PI4K as well as most other human lipid and protein kinases that are believed to be associated with intracellular signaling and trafficking and host defense (20). Binding to human lipid kinase PIP4K2C (phosphatidylinositol-5-phosphate 4-kinase, type II, gamma) has been demonstrated, but its expression in red blood cells is unknown, as is the consequence of its inhibition (7).

Conclusion. Following a quadruple-dose regimen and a single-dose regimen, the aminopyrazine sulfoxide was demonstrated to be highly active against *P. falciparum* in the NSG mouse model of malaria infection. PK profiling showed that the sulfoxide was converted to the corresponding equally active aminopyrazine sulfone. The higher levels of the sulfone relative to the sulfoxide, when sulfoxide is dosed, and its longer half-life suggest that the sulfone metabolite is responsible for the efficacy observed with the sulfoxide. Furthermore, dosing with the sulfoxide generated higher exposures of the sulfone metabolite than dosing with the sulfone, which demonstrates the pharmacokinetic effectiveness of the prodrug approach. This effectiveness is predicted to translate to humans based on the qualitative similarity in microsomal metabolisms between mouse and human liver microsomes. However, the prodrug-mediated increase in exposure did not lead to an improvement *in vivo* efficacy, as there are as-yet-unexplained limiting factors associated with the pharmacodynamic response observed with the levels of parasitemia. Within this context, it is acknowledged that the NSG model does have limitations compared to the human condition, not the least of which is the absence of an immune system. Hence, more investigations are needed to understand the PK/PD relationships associated with the sulfoxide, sulfone, and other similarly active PI4K-inhibiting antimalarial agents.

MATERIALS AND METHODS

Chemistry. The aminopyrazine sulfoxide and sulfone were synthesized from commercially available 2-aminopyrazine, as previously described (8).

***In vitro* efficacy.** Compounds were screened against multidrug-resistant (*Pf*K1) and -sensitive (*Pf*NF54) strains of *P. falciparum in vitro* using the modified [^{3}H]hypoxanthine incorporation assay (15). Additionally, the compounds were evaluated against the adapted strain of *P. falciparum* 3D7 harvested from the NSG model of infection as a comparator; this was done using the *Plasmodium* lactate dehydrogenase (pLDH) assay (16). All assays were performed in triplicate in two independent experiments.

***In vitro* absorption, distribution, metabolism, and excretion assay.** Kinetic solubility, lipophilicity, metabolic stability, and parallel artificial membrane permeability assay (PAMPA) of the compounds were measured using standard protocols, as previously described (17).

Ethics statement. All studies and procedures were conducted with prior approval of the animal ethics committee of the University of Cape Town (approval numbers 013/028 and 014/028) in accordance with the South African National Standard (SANS 10386:008) for the Care and Use of Animals for Scientific Purposes (18), and guidelines from the Department of Health (19).

***In vivo* pharmacokinetics in healthy C57BL/6 mice.** Before designing the NSG experiments, it was necessary to establish the pharmacokinetic profiles of both compounds in a generic mouse strain. These experiments were performed in C57BL/6 mice, which have been routinely used for other compounds (20–22). The sulfoxide and sulfone were administered orally and intravenously at a dose of 1.5 mg kg^{-1} ($n = 3$). For the oral administration, the compounds were formulated in 0.5% hydroxypropylmethyl cellulose (HPMC) containing 0.2% Tween 80. The intravenous doses were administered in dimethylacetamide-polyethylene glycol 400-polypropylene glycol (DMA-PEG400-PPG) (10:30:60). Blood samples were collected via tail bleeding at predetermined time points and stored at −80°C until liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

***In vivo* efficacy and pharmacokinetics. (i) *In vivo* antimalarial efficacy studies.** The therapeutic efficacy of both compounds was evaluated using the NSG mouse model for malaria previously described (13, 14). Male NSG mice engrafted with human erythrocytes (approximately 60%) were infected with 20×10^6 *P. falciparum Pf*3D7$^{0087/N9}$ cells, a strain developed at GSK for proliferation in engrafted mice. Infections were conducted via intravenous injection (day 0). Treatment commenced on day 3 and ended on day 7 following infection.

The efficacy of the sulfoxide and sulfone was evaluated following a quadruple-dose regimen, administered as a single oral dose for four consecutive days. Additionally, the sulfoxide was evaluated following a single-dose regimen.

For the quadruple-dose regimen, the compounds were dosed as single doses for four consecutive days at 0.05, 0.1, 0.5, 1.5, and 10 mg kg^{-1}. The experimental group for each dose consisted of 2 mice per group. Both compounds were formulated in 0.5% HPMC containing 0.2% Tween 80 and were administered via oral gavage in an administration volume ranging from 200 to 300 μl, to maintain an equal

compound dose relative to the weight of each mouse. Chloroquine was included in experiments as a positive control and dosed at 2.5, 5, and 10 mg kg^{-1}. Chloroquine was formulated in saline solution. Additionally, two negative-control groups were included, one receiving 0.5% HPMC containing 0.2% Tween 80 only and the other containing saline only. Blood samples (2 μl) were collected on days 3, 4, 5, 6, and 7 to determine parasitemia levels by flow cytometry using a bidimensional staining technique, as previously described (23). Additionally, to determine the pharmacokinetic (PK) parameters, blood samples (10 μl) were collected via tail bleeding predose and at 0.25, 0.5, 1, 3, 5, 8, and 24 h postdose in 1.5-ml lithium heparin Eppendorf tubes to prevent blood coagulation. Samples were stored at −80°C prior to LC-MS/MS analysis of the sulfoxide and sulfone.

For the single-dose regimen of the sulfoxide, an experimental procedure similar to the one described for the quadruple-dose regimen was used, with the modification that each experimental group received a single dose of 0.1, 0.5, 1.5, 5, 15, and 50 mg kg^{-1} on day 3 following infection. Chloroquine was included in the experiment as a positive control and dosed at 50 mg kg^{-1}. Blood samples (10 μl) were collected predose and at 0.25, 0.5, 1, 2, 4, 6, 8, 10, 24, 48, 72, and 96 h postdose for PK evaluation. The samples were stored at −80°C prior to LC-MS/MS analysis of the sulfoxide and its sulfone metabolite.

(ii) Dose-response relationship analysis. Data analysis was performed using nonlinear fitting to a logistic equation of $\log_{10}$(% parasitemia on day 7 after infection) compared to the untreated control group using GraphPad Prism 4 software. This analysis determines the pharmacodynamic readout in the form of ED_{90} and AUC_{ED90} values.

The 90% effective dose (ED_{90}) is defined as the dose in milligrams per kilogram that reduces parasitemia on day 7 following infection by 90% compared to untreated control group. AUC_{ED90} is defined as the average estimated daily exposure required to reduce parasitemia from peripheral blood on day 7 following infection by 90% compared to the untreated control group. The equation used to fit the data is y = bottom + (top − bottom)/[1 + 10([$\log_{ED50}$ − x] × Hill Slope)]. This equation is a four-parameter logistic equation where "bottom" is the y value at the bottom plateau and "top" is the y value at the top plateau. Log_{ED50} is the x value when the response is halfway between the bottom and top. "Hill Slope" describes the steepness of the curve. This variable is also called the slope factor or the Hill coefficient. If it is positive, the curve increases as x increases. If it is negative, the curve decreases as x increases. A standard sigmoid dose-response curve (previous equation) has a Hill Slope of 1.0. When the Hill Slope is less than 1.0, the curve is shallower. When the Hill Slope is greater than 1.0, the curve is steeper.

The ED_{90} is calculated by fitting the variable $y = \log_{10}$(parasitemia at day 7 after infection) and the variable $x = \log_{10}$(dose level in mg kg^{-1}), defined as an ordered pair for each individual of the study. The AUC_{ED90} is calculated by fitting the variable $y = \log_{10}$(parasitemia at day 7 after infection) and the variable $x = \log_{10}$(AUC of compound during the first 24 h after the first drug administration, in μg h ml^{-1}), defined as an ordered pair for each individual of the study. The ED_{90} and AUC_{ED90} are calculated by interpolation of the x value that corresponds to $\text{antilog}_{10}(y$ = top − 1) in each respective best-fitted curve.

(iii) Pharmacokinetic analysis. The whole-blood concentrations of the sulfoxide and sulfone were determined using a quantitative LC-MS/MS method. Sample preparation was achieved with a protein precipitation extraction method, using 5 μl whole blood and 195 μl acetonitrile-methanol (ACN-MeOH) (80:20) for the NSG samples and 20 μl whole blood with 180 μl ACN-MeOH (80:20) for the C57BL/6 samples. After centrifugation, the supernatant was transferred to 96-well plates for analysis. LC-MS/MS was carried out on an AB Sciex QTrap 5500 instrument coupled to an Agilent 1260 high-performance liquid chromatograph (HPLC). Separation was achieved on a Poroshell EC-C_{18} column, using 0.1% formic acid in water as the aqueous mobile phase and acetonitrile as the organic mobile phase. The analytical limit of quantitation (LOQ) was 0.5 ng/ml. The combined accuracy and precision (% nom) statistics were ±15% for the standards and quality controls of both compounds. Concentration-versus-time data were used to derive the PK parameters using noncompartmental analysis on PK Solutions 2.0 (Summit Research Services, Montrose, CO, USA).

(iv) Metabolite analysis. Metabolites of the sulfoxide and sulfone were identified in healthy mice using PK samples as well as in mouse liver microsomes (see the supplemental material).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00261-18.

TEXT S1, PDF file, 0.5 MB.

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