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Scalable Preparation and Differential Pharmacologic and Toxicologic Profiles of Primaquine Enantiomers

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Hematotoxicity in individuals genetically deficient in glucose-6-phosphate dehydrogenase (G6PD) activity is the major limitation of primaquine (PQ), the only antimalarial drug in clinical use for treatment of relapsing *Plasmodium vivax* malaria. PQ is currently clinically used in its racemic form. A scalable procedure was developed to resolve racemic PQ, thus providing pure enantiomers for the first time for detailed preclinical evaluation and potentially for clinical use. These enantiomers were compared for antiparasitic activity using several mouse models and also for general and hematological toxicities in mice and dogs. (+)-(*S*)-PQ showed better suppressive and causal prophylactic activity than (−)-(*R*)-PQ in mice infected with *Plasmodium berghei*. Similarly, (+)-(*S*)-PQ was a more potent suppressive agent than (−)-(*R*)-PQ in a mouse model of *Pneumocystis carinii* pneumonia. However, at higher doses, (+)-(*S*)-PQ also showed more systemic toxicity for mice. In beagle dogs, (+)-(*S*)-PQ caused more methemoglobinemia and was toxic at 5 mg/kg of body weight/day given orally for 3 days, while (−)-(*R*)-PQ was well tolerated. In a novel mouse model of hemolytic anemia associated with human G6PD deficiency, it was also demonstrated that (−)-(*R*)-PQ was less hemolytic than (+)-(*S*)-PQ for the G6PD-deficient human red cells engrafted in the NOD-SCID mice. All these data suggest that while (+)-(*S*)-PQ shows greater potency in terms of antiparasitic efficacy in rodents, it is also more hematotoxic than (−)-(*R*)-PQ in mice and dogs. Activity and toxicity differences of PQ enantiomers in different species can be attributed to their different pharmacokinetic and metabolic profiles. Taken together, these studies suggest that (−)-(*R*)-PQ may have a better safety margin than the racemate in human.

The 8-aminoquinolines (8-AQs) are a drug class showing broad and potent antiparasitic activity (1). They are the only drugs known to kill the tissue schizont form (hypnozoite) of the parasites causing relapsing malaria and further have the capacity to block infection by preventing mosquito-injected sporozoites from establishing infection in the liver. They also have gametocidal activity and can interrupt disease transmission from mosquitos feeding on an infected patient and subsequently feeding on noninfected persons. Consequently, any strategy for eradication of malaria will likely need to incorporate utilization of an 8-AQ (1). Primaquine (PQ), the only clinically approved 8-AQ, is currently used for the treatment of relapsing malaria (2, 3) and as a prophylactic agent against all major forms of human malaria (4). This drug is ineffective against blood-stage malaria parasites at the effective antirelapse doses (3). In addition, PQ is effective in combination with clindamycin for the treatment and prophylaxis of *Pneumocystis jirovecii* (formerly *Pneumocystis carinii*) pneumonia in immunosuppressed patients (5). PQ has also shown significant activity against other disease-causing parasites, such as *Trypanosoma* (6) and *Leishmania* (7). The major limitation to broad clinical use of PQ and other 8-AQ antiparasitic agents is that they cause methemoglobinemia (8, 9) and hemolytic anemia (9, 10) in individuals genetically deficient in glucose-6-phosphate dehydrogenase (G6PD) activity.

PQ is clinically used in its racemic form, a mixture of two enantiomers. We previously reported (11) different therapeutic indices for individual enantiomers of another member of this class, 8-[(4-amino-1-methylbutyl)amino]-6-methoxy-4-methyl-5-[3,4-dichlorophenoxy]quinoline. With this 8-AQ analog, the (−)-*R* enantiomer, NPC1161B, had a better therapeutic index than the (+)-*S* enantiomer, NPC1161A, or the racemate, NPC1161C (Fig. 1). Limited previous results comparing the individual enantiomers of PQ, made available by a challenging and expensive resolution of racemic PQ (12), also suggested different therapeutic indices and rate of metabolism. Schmidt et al. (13) reported that (+)-(*S*)-PQ, (−)-(*R*)-PQ, and racemic PQ (Fig. 1) were equally curative against sporozoite-induced *Plasmodium cynomolgi* infections in rhesus monkeys, the only experimental model for relapsing malaria. They also reported that (+)-(*S*)-PQ was approximately 4 times as toxic (acute lethality) as the (−) form in mice but, unexpectedly, the (−) form was 3 to 5 times as toxic as the (+) form and at least twice as toxic as the racemate in

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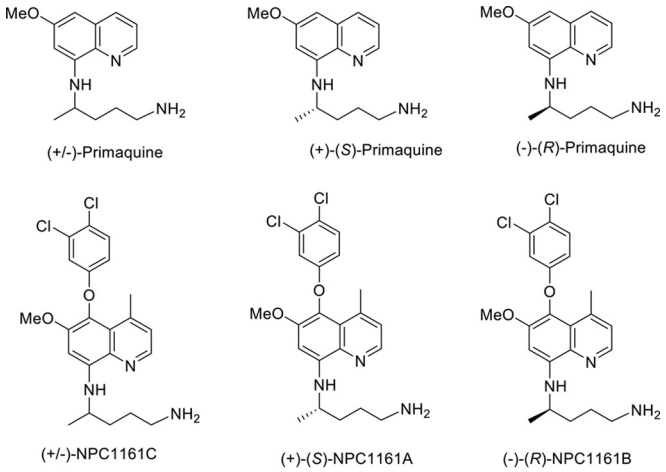

FIG 1 Structures of primaquine, NPC1161C, and their enantiomers.

rhesus monkeys (13). In an *in vitro* tissue schizontocidal assay with mouse hepatocytes, the (+) isomer was found to be equally active and slightly less cytotoxic than (±)-PQ (14). On the other hand (−)-PQ was less active and less cytotoxic than (±)-PQ. In another study, (−)-PQ produced more methemoglobin and caused less membrane leakiness in human erythrocytes, whereas with the (+) isomer, the opposite was observed (15). Nicholl et al. (16) reported similar rates of clearance for individual enantiomers of PQ in an isolated perfused rat liver preparation but a more facile conversion of the (−)-PQ to the carboxy metabolite, which was thought to be inactive. In previous studies, we observed no selectivity for metabolism of the (+) and (−) isomers in a rat liver microsomal preparation, but microsomes with the mitochondrial fraction showed a marked preference for the conversion of the (−) isomer to the carboxyprimaquine metabolite (17). When racemic PQ was administered to laboratory rats, a majority of residual PQ excreted in the urine was the (+) isomer (17). In mice treated with racemic PQ, the two enantiomers exhibited similar plasma PK profiles (maximum concentration in serum [C_{max}] and time to maximum concentration in serum [T_{max}]) (18). However, plasma (−)-PQ level declined faster than (+)-PQ. A pronounced difference was noted in the plasma PK profile of the enantiomers of carboxy-PQ (cPQ), the major PQ metabolite. The C_{max} for (−)-cPQ was more than 17-fold higher than that for (+)-cPQ (18).

With the disparate toxicity profiles of PQ enantiomers in different species and given the steep dose response curve for PQ in humans, Schmidt and coworkers (13) concluded, 35 years ago, that the available data warranted a separate clinical evaluation of PQ enantiomers. However, so far no work has been done to evaluate individual PQ enantiomers in humans. A major impediment to such clinical evaluation was the lack of availability of PQ enantiomers of cGMP quality. The cumbersome nature of the method previously used to resolve PQ (12) would not provide the enantiomers economically nor scale appropriately for cGMP preparation. To overcome this limitation and to provide PQ enantiomers economically, we have developed a simple procedure to resolve racemic PQ on a large scale. With quantities of the individual enantiomers in hand, we evaluated more extensively the comparative antiparasitic activities and toxicities of the enantiomers in mice and dogs. Evaluations of antimalarial efficacy, pharmacokinetics, and toxicity in nonhuman primates also have been completed (D. Saunders, P. Vanachayangkul, P. Khemawoot, R. Imerbsin, R. Siripokasupkul, B. L. Tekwani, A. Sampath, N. P. D. Nanayakkara, C. Ohrt, P. Teja-Isavadharm, and L. A. Walker, unpublished data). In this article, we extend earlier evidence suggesting marked species differences in PQ enantiomer profiles and emphasize that there is warrant for comparing PQ enantiomers in humans with respect to hematological liability in G6PD-deficient subjects and antimalarial efficacy.

MATERIALS AND METHODS

Materials and chemicals. Primaquine diphosphate and other reagents were purchased from Sigma-Aldrich (St. Louis, MO). Solvents (certified American Chemical Society [ACS] and high-pressure liquid chromatography [HPLC] grade) were purchased from Fisher Scientific (Atlanta, GA).

Resolution of racemic primaquine. Experimental details for the resolution of racemic primaquine are provided in the supplemental material.

Crystallographic data for NPC1161A (*R*)-(+)-phenylethylurea derivative. Experimental details, crystallographic data, and the ORTEP (Oak Ridge thermal ellipsoid plot) diagram for this compound are provided in the supplemental material.

Antimalarial blood schizontocidal activity in mice. A suppressive-curative *Plasmodium berghei* mouse model was employed for *in vivo* blood schizonticidal antimalarial efficacy evaluation. The *in vivo* antimalarial activity was determined for mice infected with *Plasmodium berghei* (NK-65 strain) according to Peters' 4-day suppressive test, which has been modified to a 3-day treatment schedule. Male mice (Swiss Webster strain) weighing 18 to 20 g were intraperitoneally inoculated with 2×10^7 parasitized red blood cells obtained from a highly infected donor mouse. Mice were divided into different groups, with 5 mice in each group. The solutions of PQ (racemate and the pure enantiomers) were prepared in Nanopure sterile water and administered orally (p.o.) through gavage to the mice about 2 h after the infection (day 0). The mice were treated once daily for three consecutive days (days 0 to 2). A control group of mice was treated with an equal volume of vehicle, while another control group was treated with the standard antimalarial drug chloroquine. The mice were closely observed after every dose for any apparent signs of toxicity, and the body weights were recorded once daily. Blood smears were prepared on different days (till day 28 postinfection) by tail snip, stained with Giemsa stain, and evaluated under a microscope for determination of parasitemia. Mice without parasitemia after day 28 postinfection were considered cured. These groups of animals were also tested to determine levels of hemoglobin and methemoglobin. For determination of hemoglobin and methemoglobin, about 50 μl of blood was collected with tail snip. The blood was diluted 1:5 with phosphate-buffered saline supplemented with glucose containing EDTA. The levels of hemoglobin and methemoglobin were evaluated using an IL-682 co-oximeter precalibrated with rodent blood.

Antimalarial causal prophylaxis in mice. ICR female mice were each inoculated with 80,000 to 100,000 sporozoites (in 0.1 ml of phosphate-buffered saline and 5% bovine serum albumin) of the *Plasmodium berghei* ANKA strain. The sporozoites were isolated by dissection from *Anopheles dirus* mosquitoes fed on donor mice. On days −1, 0, and 1, (+)- and (−)-PQ were administered at doses ranging from 5 to 40 and 10 to 160 mg/kg/day, respectively ($n = 5$ for each dose level). Racemic primaquine was administered at 25 mg/kg/day. Drug administration was performed once daily for 3 days using a vehicle of 0.5% hydroxyethylcellulose–0.1% Tween 80 (HECT) and delivered orally via a 20-gauge plastic oral feeding tube. Once inoculated with active sporozoites, mice develop parasitemia (microscopic examination) on the 4th day; all untreated mice die (or reach the study endpoint of 5% parasitemia, requiring euthanasia) on days 6 to 8. Administration of antimalarial drugs with causal prophylactic activity will delay (at low doses) or prevent altogether (at effective doses)

TABLE 1 Suppressive antimalarial activities of primaquine enantiomers in mice

Compound	Dose, mg/kg/day (×3 days)	% parasitemia suppression[a]		Survival[b]; day(s) of death (MST)[c]	No. cured/no. treated[d]
		Day 5	Day 7		
Primaquine	11.1	97.47 ± 4.47	54.91 ± 39.25	3/5; 21, 21, 28, 28, 28 (25.2 ± 3.8)	0/5
	33.3	100	100	5/5; (>28)	0/5
	100	100	100	5/5; (>28)	4/5
(+)-Primaquine	11.1	79.85 ± 20.65	70.64 ± 13.96	0/5; 15, 18, 18, 21, 21 (18.6 ± 2.5)	0/5
	33.3	100	100	4/5; 24, 28, 28, 28, 28 (27.2 ± 1.8)	0/5
	100	100	100	5/5; (>28)	2/5
(−)-Primaquine	11.1	13.34 ± 39.01	32.30 ± 23.35	0/5; 12, 14, 14, 18, 18 (15.2 ± 2.7)	0/5
	33.3	90.35 ± 13.89	46.04 ± 37.02	4/5; 8,28,28,28,28 (24.0 ± 8.9)	0/5
	100	100	100	5/5; (>28)	2/5
Vehicle				0/5; 5/14/17/17/17 (14.0 ± 5.2)	0/5

[a] Percent suppression of parasitemia was calculated by considering the mean parasitemia in the vehicle control to be 100%. Values are means ± SD of results for five animals.
[b] Number of animals that survived day 28/total animals in group (day of death postinfection).
[c] MST, mean survival time (days). Values are means ± SD of results for five animals.
[d] Number of mice without parasitemia (cured) till day 28 postinfection.

the rise in parasitemia. Successful causal prophylaxis is determined by survival to day 31 postinoculation.

Activity against *Pneumocystis carinii* infection in mice (19). Female BALB/c mice free of *Pneumocystis*, 6 to 8 weeks of age (Harlan Sprague Dawley) were immunosuppressed by the administration in drinking water of 1.2 mg/ml dexamethasone. After 4 days, animals were transtracheally inoculated with 10^6 *P. carinii* organisms and were continued on immunosuppressive agents. At 4 weeks postinoculation, treatment was begun and continued for 3 weeks. There were 10 mice in each group. Test compounds [(+)-PQ, (−)-PQ, or racemic PQ] were administered in drinking water to deliver doses approximating 2, 5, or 10 mg/kg/day. The drugs were prepared fresh daily, and consumption for each group was monitored and adjusted as needed to ensure proper dosing. A group of untreated animals served as a negative control, and positive-control groups included trimethoprim (TMP)-sulfamethoxazole (SMX) (50 and 250 mg/kg/day) and NPC1161B (10) (0.25-mg/kg/day) treatments. At the end of 3 weeks of therapy, animals were anesthetized and exsanguinated by cardiac puncture. Lungs were removed, and representative portions of lower lobes were used to make impression smears. Four impression smears, fixed in methanol, were evaluated for the presence of *P. carinii* by staining with Giemsa stain. Slides were blinded as to treatment and examined microscopically by two experienced individuals. The ratios of the number of animals cured (no detectable infection) to the total number treated were recorded.

Hematological toxicity for G6PD-deficient human red cells in NOD-SCID mice. Eight- to nine-week-old female NOD.CB17-Prkdc^{scid}/J mice (NOD-SCID mice) (Jackson Laboratories, Bar Harbor, ME) were transfused intraperitoneally (i.p.) with African variant G6PD-deficient human red blood cells (huRBC) for 14 days as described previously (20). Approximately 5 μl of blood was analyzed for the presence of huRBC using phycoerythrin (PE)-conjugated anti-human CD235a antibody (Abcam, Cambridge, MA), and cells were acquired on a Guava EasyCyte Plus flow cytometer (Millipore, Billerica, MA). Analysis of the flow data was done using the FloJo software program (TreeStar, Inc., Ashland, OR). Mice with peripheral huRBC levels greater than 60% were randomized for drug treatment, with 4 to 5 mice per group assigned. PQ or enantiomers were resuspended in PBS and given i.p. twice daily for 7 days.

Comparison of PQ enantiomer toxicity in beagle dogs. Six female beagle dogs (3/group) were assigned to the study and were dosed once daily for up to 3 days with 4.86 mg/kg/day of either (+)-PQ diphosphate or (−)-PQ diphosphate; dosage was calculated as the free base. The final (4th) day of dosing was not performed due to the deteriorating health of the dogs dosed with (+)-PQ. Parameters evaluated included mortality, clinical observations, body weights, serum chemistry, and hematology. Gross necropsies were also performed on animals sacrificed in a moribund state; animals surviving to the end of the study were returned to the stock colony.

RESULTS

Preparation and spectral characterization of PQ enantiomers. The preparation of PQ enantiomers and their characterization are summarized in Fig. S1 in the supplemental material. PQ phthalimide, which is an intermediate in the synthesis of PQ (or can be readily prepared by treating commercially available PQ with phthalic anhydride), was resolved by fractional crystallization with (+)- and (−)-tartaric acid. This procedure can be implemented on a large scale to generate large amounts of PQ enantiomers economically and is amenable for incorporation into a cGMP manufacturing process.

Comparative antimalarial blood schizonticidal activity in mice. Though at clinically used doses PQ has little or no blood schizonticidal activity in humans, with higher doses in rodent models, suppression of parasitemia was readily observable. Evaluation of racemic PQ and its enantiomers against *Plasmodium berghei* in a rodent model showed that they were partially curative at 100 mg/kg/day (Table 1). PQ racemate and the enantiomers were not curative at doses of 33.3 and 11.1 mg/kg/day. Even though no conclusions could be made on relative potencies of the racemate and (+)-PQ based on the parasitemia suppression data at the dose of 11.1 mg/kg/day, comparison of these data at this dose for day 5 and day 7 indicated that (−)-PQ was less suppressive than (+)-PQ (*P* values 0.0098 and 0.0136, respectively) or the racemate (*P* values 0.0014 and 0.3005, respectively). At the dose of 33.3 mg/kg/day, both the racemate and (+)-PQ had total suppression of parasitemia on days 5 and 7, whereas (−)-PQ showed only partial suppression. Racemic and (+)-PQ showed some signs of toxicity at the 100-mg/kg dose. The mice treated with both racemic and (+)-PQ showed less mobility and grooming than the animals in the control, chloroquine-, and (−)-PQ-treated groups. The mice treated with (+)-PQ also underwent a reversible loss in body weight on days 4 to 6 posttreatment (Fig. 2). The hemato-

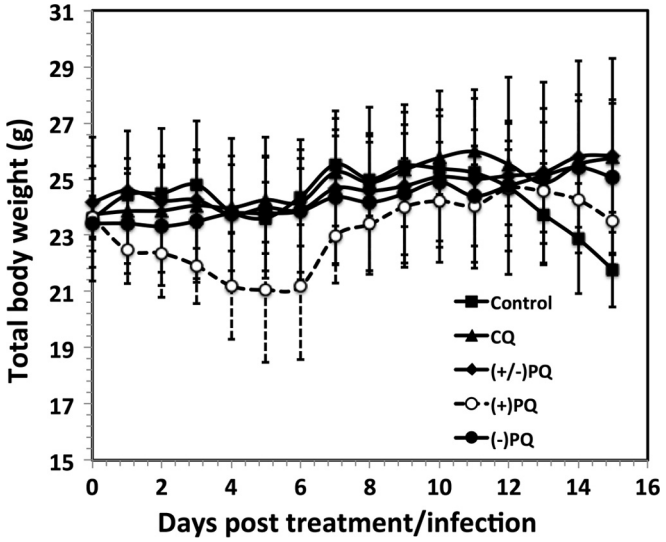

FIG 2 Effect of treatment with PQ enantiomers on total body weight of *Plasmodium berghei*-infected mice. The *P. berghei*-infected mice were treated once daily for 3 days (days 0, 1, and 2) (2 h after infection) with racemic PQ and PQ enantiomers. Chloroquine was tested as a control drug. The results shown here are for the 100-mg/kg dose. The lower doses did not show any effect on total body weights. Each point shows mean ± SD values from 5 animals in each group.

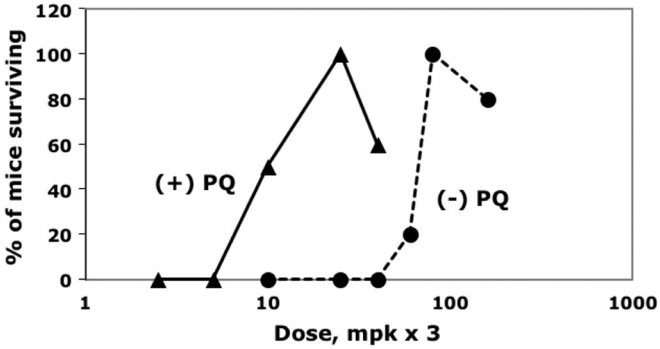

FIG 3 Prophylactic antimalarial activities of (+)- and (−)-primaquine against *P. berghei* in mice. Mice were inoculated with sporozoites on day 0. Treatment with the drugs (p.o.) was daily on days −1, 0, and 1. Mice normally succumb to infection at about 1 week postinfection. The graph represents the percentage of mice surviving to day 31 (study end). mpk, mg/kg.

logical parameters, namely, total hemoglobin and methemoglobin levels, were not significantly altered in the mice following treatment with (±)-PQ or either of the PQ enantiomers (Table 2). The hematological toxicities of racemic PQ and the enantiomers were evaluated separately in beagle dogs and also using the humanized SCID mouse model.

Comparative antimalarial causal prophylaxis in mice. The difference in antimalarial potency between the two enantiomers was much more prominent for the developing liver stages in the causal prophylaxis model. In this assay, (+)-PQ protected 50% of animals at a dose of 10 mg/kg/day for 3 days and 100% of animals at a dose of 25 mg/kg/day, whereas (−)-PQ conferred 100% protection only at 80 mg/kg/day (Fig. 3). Two animals treated with (+)-PQ at dose of 40 mg/kg/day and one animal treated with (−)-PQ at a dose of 160 mg/kg/day died due to toxicity, indicating that the acute systemic toxicity of (+)-PQ is also greater than that of (−)-PQ for this species. At a dose of 25 mg/kg/day, racemic PQ conferred 100% protection. The data were analyzed in the GraphPad Prism software program by sigmoidal emax nonlinear regression, and the 80% effective doses (ED_{80}s) are as follows: (+)-PQ ED_{80} = 10.5 mg/kg/day for 3 days; (−)-PQ ED_{80} = 63.7 mg/kg/day for 3 days. The racemic PQ dose response was not determined in this study, but in a previous study in our lab (AFRIMS), 20 mg/kg/day for 3 days provided effective prophylaxis for 80% of the mice (M. Gettayacamin and A. Tungtaeng, unpublished data). Also, in 19 different experiments using 25 mg/kg/day for 3 days for racemic PQ as a positive control (n = 5 in each experiment), the 25-mg/kg dose was at or above the ED_{80} (80% survival in 3/19 experiments and 100% survival in 16/19 experiments) (Gettayacamin and Tungtaeng, unpublished). Thus, the ED_{80} for racemic PQ is estimated at between 20 and 25 mg/kg, likely closer to 20; since half of this dose is an ED_{80} for (+)-PQ, these data nicely reconcile and suggest that all of the activity of the racemate in standard effective rodent doses of racemic PQ resides in the (+) enantiomer.

Comparative antipneumocystis activity in mice. When the enantiomers were evaluated using a mouse model for suppression of *Pneumocystis* pneumonia infection, it was also observed that the (+)-PQ enantiomer is more active (Table 3). At a dose of 10

TABLE 2 Effects of treatment of *Plasmodium berghei*-infected mice with primaquine enantiomers on methemoglobin levels[a]

Group	% Methemoglobin	*P* value
Vehicle control	0.82 ± 0.65	
Chloroquine	0.78 ± 0.22	0.9 (NS)
(±)-Primaquine	1.22 ± 0.31	0.25 (NS)
(+)-Primaquine	1.12 ± 0.25	0.36 (NS)
(−)-Primaquine	0.66 ± 0.51	0.68 (NS)

[a] The *P. berghei*-infected mice were treated once daily for 3 days (days 0, 1, and 2) (2 h after infection) with racemic PQ and PQ enantiomers. Chloroquine was tested as a control drug. The results shown here are for the 100-mg/kg dose. Blood was collected 20 h after the last dose by tail snip, and methemoglobin levels were estimated by using a co-oximeter. Values are means ± SD of results for 5 animals in each group. NS, not significantly different from results with the vehicle control.

TABLE 3 Oral efficacies of primaquine enantiomers and racemate against pneumocystis infection in mice

Compound	Dose, mg/kg/day (×21 days)	Activity, no. cured/no. treated (Giemsa stain)
Primaquine	10	5/10
	5	0/10
	2	0/10
(+)-Primaquine	10	9/10
	5	3/10
	2	0/10
(−)-Primaquine	10	0/10
	5	0/10
	2	0/10
TMP-SMX	50/250	9/10
NPC1161B	0.25	10/10
Control		0/10

mg/kg/day, administered over 21 days, (+)-PQ was as active as the TMP-SMX positive control at 50/250 mg/kg/day, with 9/10 mice cleared of parasites. However, (−)-PQ was without any effect at the same dose level. The racemic PQ showed intermediate potency, as expected, with 5/10 mice cured at the 10-mg/kg/day dose level.

Comparative general toxicity and methemoglobinemia in beagle dogs. There was a striking difference in tolerability of PQ enantiomers in beagle dogs. A previous preliminary study by our group (N. P. D. Nanayakkara, J. D. McChesney, and A. M. Clark, unpublished data) had shown that racemic PQ administered to beagle dogs was well tolerated at 5 mg/kg/day for 4 days but elicited modest methemoglobinemia, with females responding somewhat more than males. In the current study, treatment of 3 female dogs with 4.86 mg/kg (+)-PQ daily for 2 or 3 days resulted in unexpected serious morbidity, with rises in aspartate aminotransferase, alanine aminotransferase, creatine kinase, and total bilirubin evident as early as day 2 and requiring suspension of dosing at day 3 and termination of all animals by day 6. Methemoglobin values moderately increased in a time-dependent manner (from 5.9% ± 1.7% pretreatment to 11.1% ± 0.5% at day 4) and then returned toward the baseline level once dosing stopped. Decreases in eosinophils and lymphocytes and increases in leukocytes, neutrophils, monocytes, and reticulocytes were also observed, but hemoglobin/hematocrit values remained unchanged. Animals treated with (+)-PQ consistently lost body weight (16 to 17% over 6 days) until the time of moribund termination. These animals also displayed lung damage at necropsy (discoloration and/or firm/heavy lobes in 2/3 animals).

In contrast to the (+)-PQ findings, all animals treated with (−)-PQ at the same dose level ($n = 3$) survived through the scheduled study period, with only incidental findings noted. The (−)-PQ group showed only moderate weight loss over the course of study days 1 to 8; the overall weight loss in the (−)-PQ group ranged from approximately 3 to 8%. All clinical chemistry and hematological parameters for the (−)-PQ-treated animals remained comparable to baseline values through the end of the study, although platelet counts appeared to be slightly decreased by day 8.

Comparative hemolytic responses on G6PD-deficient human RBCs in SCID mice. To assess if there were differences in hemolytic toxicities of the PQ enantiomers, NOD/SCID mice engrafted with huRBC from a G6PD-deficient donor were treated with racemic PQ at a dose previously shown to induce hemolytic toxicity in this model (12.5 mg/kg/day) (20) and with PQ enantiomers at the same dose and at 6.25 mg/kg/day (Fig. 4). Treatment with the (+)-PQ enantiomer resulted in hemolytic toxicity similar to that of racemic PQ, as indicated by the degree of loss of huRBC after day 7 of treatment. In contrast, the levels of huRBC for mice treated with the (−)-PQ enantiomer were not significantly different from those for the mice given phosphate-buffered saline (PBS) alone. The hemolytic response of (+)-PQ was significantly higher than that of (−)-PQ.

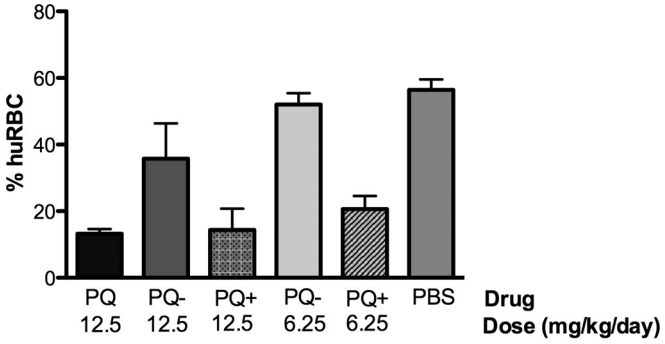

FIG 4 Hemolytic toxicities of PQ enantiomers in NOD-SCID mice engrafted with G6PD-deficient human erythrocytes. The data represent the loss of human erythrocytes on day 7 of treatment. Each bar represents the mean values ± SD from four animals. The data were analyzed by using the Student *t* test. *P* values, PBS versus PQ (12.5), <0.0001 (statistical significant difference [S]); PBS versus (+)-PQ (12.5), 0.003 (S); PBS versus (−)-PQ (12.5), 0.05 (S); PBS versus (+)-PQ (6.25), 0.0004(S); PBS versus (−)-PQ (6.25), 0.38 (difference statistically not significant [NS]); PQ (12.5) versus (+)-PQ 12.5, 0.35 (NS); PQ (12.5) versus (−)-PQ (12.5), 0.0002 (S); (+)-PQ (12.5) versus (−)-PQ (12.5), 0.007 (S).

DISCUSSION

The importance of stereoisomerism in modern drug development is receiving increased attention, and considerations of differential metabolism, pharmacokinetics, and/or pharmacodynamics of pairs of stereoisomers are the general rule, not the exception. With regard to antimalarial drugs, a number of prominent examples are known (21). PQ is one such drug, developed more than 60 years ago, when analytical and preparative methodologies were limiting and when the impact of enantiomeric differences was less well recognized. Certainly in the modern era, it would be very difficult to obtain regulatory approvals for a racemic drug candidate without verification of similar metabolic fates and safety profiles of the enantiomers.

Much effort has been devoted over the years to the study of PQ in efforts to find approaches to delay its clearance and to understand better the role of reactive metabolites in efficacy and toxicity. In the course of these studies, a few laboratories, including ours, have evaluated individual PQ enantiomers, though availability of these was somewhat limited (13–18). The metabolism studies in rats revealed a greater stability of (+)-PQ and more rapid conversion of (−)-PQ to the carboxyprimaquine metabolite (16–18). In an *in vitro* plasmodial tissue schizonticidal assay, the (+) isomer was found to be equally active and slightly less cytotoxic than racemic PQ (14). In the same assay, (−)-PQ was less cytotoxic and less active than racemic PQ (14). Agarwal et al. (15) reported more methemoglobin production and less membrane leakiness for (−)-PQ in human erythrocytes and the opposite for the (+) isomer. However, the concentrations of PQ tested in these studies (14, 15) were quite high, and the relevance of the findings to the active metabolite species is unknown.

In the current study, we developed an efficient large-scale method for preparation of the pure PQ enantiomers. Using these two enantiomers, we confirmed and extended the findings on the effects of PQ enantiomers in mice. Using two mouse models of malaria (blood and exoerythrocytic stages), we found that (+)-PQ was more potent than (−)-PQ. Similar findings were observed for *Pneumocystis* pneumonia in mice. The improved activities we observed in mice for (+)-(*S*)-PQ over those for the (−)-(*R*)-form contrasted with that for the enantiomers of the 8-AQ with the 5-aryloxy substituent, NPC1161C (11). With these enantiomers, (−)-(*R*)-NPC1161B had better activity than (+)-(*S*)-NPC1161A for malaria and *Pneumocystis* pneumonia in mouse models (11). These findings raised the question of whether

the absolute configurations of (−)-PQ and (−)-NPC1161B are both *R*. Previously, we determined the absolute configuration of NPC1161A and NPC1161B by comparing their circular dichroism (CD) spectra with those of (+)-(*S*)-PQ and (−)-(*R*)-PQ (see Fig. S2a in the supplemental material). We confirmed the absolute configuration of (+)-NPC1161A to be *S* by X-ray crystallography of its (*R*)-(+)-phenylethylurea derivative (see Fig. S2b). This observation indicates that the absolute configurations of the respective (+)-enantiomers of PQ and the analog are not different, but some other structural feature dictates the divergence in the potency profiles of their enantiomer pairs.

Even though the enantioselectivity for the antiparasitic activity profile of primaquine enantiomers was opposite to that for NPC1161C enantiomers, systemic toxicity profiles with mice for enantiomers of PQ and NPC1161C were similar, where the (+)-(*S*)-enantiomer was more toxic. The systemic toxicity of (+)-PQ was 3- to 5-fold greater than that of (−)-PQ. This was consistent with what Schmidt reported in 1977 (13). In the same study, Schmidt also reported that racemic PQ and enantiomers had equivalent tissue schizonticidal activities against *Plasmodium cynomolgi* in rhesus monkeys and that (−)-PQ caused liver injury at high doses. At the same dose, liver toxicity was much less prominent with (+)-PQ in monkeys. Schmidt proposed, based on the primate findings, that (+)-PQ might afford a safer alternative to racemic PQ in humans (13).

An important consideration for the human application, however, is that the dose-limiting toxicity for PQ at dose levels currently employed is the hemolytic anemia elicited in G6PD-deficient subjects (9, 10). Hepatotoxicity has not been reported as a serious limitation for clinical use of PQ (2, 3). It is difficult to study hemolytic effects of PQ in rodents and monkeys since they are relatively insensitive to this toxicity. Three avenues of exploration shed additional light on this. In the beagle dog, traditionally used to study methemoglobin toxicity of 8-aminoquinolines, (+)-PQ showed severe systemic toxicity at the 5-mg/kg dose level, requiring suspension of dosing and termination of administration after the second daily dose. At this dose, (+)-PQ caused a clear but modest increase in methemoglobin by the second day, while (−)-PQ had no effect even after 3 days of dosing.

We have recently developed a mouse model to study drug-induced hemolysis of human G6PD-deficient RBCs (20). Using NOD-SCID mice, it was demonstrated that PQ selectively destroys erythrocytes engrafted from a G6PD-deficient but not G6PD-normal human donors. In the present work, we used this model to evaluate the differences in sensitivity to PQ enantiomers and found that (+)-PQ is about 3 times more hemolytic to human G6PD-deficient RBCs in SCID mice. In other work (Saunders et al., unpublished), our groups have confirmed Schmidt's findings that the two enantiomers were equally efficacious and (−)-PQ caused liver toxicity at higher doses in rhesus monkeys but also extended these to show that (+)-PQ more consistently generated methemoglobinemia in this nonhuman primate. To the extent that this reflects potential hemolytic toxicity, it would suggest that (−)-PQ may have a therapeutic index advantage over the racemate in treatment of malaria in humans. Though (−)-PQ gave evidence of greater liver toxicity in the monkeys, this occurred only at doses well above the therapeutic dose levels.

Activity and toxicity differences of PQ enantiomers can be attributed to their different pharmacokinetic and metabolic profiles. PQ requires metabolic activation for its antiparasitic activity and toxic effects (22). Deamination of the primary amino group of the side chain by monoamine oxidase (MOA) and hydroxylation of the quinoline ring by CYP enzymes have been identified as the two major metabolic pathways of PQ in *in vitro* (22, 23) and *in vivo* (24) systems. Even though the aldehyde, the deamination product of PQ by MOA, has not been detected, its oxidized product carboxyprimaquine has been identified as the major circulating metabolite of racemic PQ in humans (25) and animals (26, 27). Biological studies have shown that carboxyprimaquine was inactive (28) and nonhematotoxic (29). In contrast, ring-hydroxylated metabolites of PQ generated by CYP enzymes (22, 23) can undergo redox cycling, generating reactive oxygen species (ROS) (30), which have been suggested to be responsible for antimalarial activity (31) and hematotoxicity (29, 32). Various PQ ring-hydroxylated metabolites and their oxidized products produced by CYP enzymes have been identified (22), and some of them have been detected as minor metabolites in human liver microsomes (33). Some of the hydroxylated PQ metabolites have been shown to be active *in vitro* (34) and *in vivo* (28) and more hematotoxic than the parent compound in *in vitro* assays (32, 34–36). Their activity and toxicity have been linked to their superoxide generation capacity (34). If ROS are responsible for both activity and hematotoxicity, the question remains whether it is possible for one enantiomer to have a better therapeutic index than the racemate. Ring-hydroxy primaquine metabolites are generated by CYP enzymes in the liver, where sporozoites (tissue schizonts) and hypnozoites reside. For hematotoxicity to occur, they need to be transported into erythrocytes. Of ring-hydroxylated metabolites, 5-hydroxyprimaquine has been suggested to be the major CYP-mediated metabolite and has been shown to cause hematotoxicity through generation of ROS (32, 35, 36). Synthetic 5-hydroxyprimaquine was found to be unstable (35) and underwent spontaneous oxidation, leading to quinone-imine and its 6-demethyl analog. Recovery studies we carried out have shown that 5-hydroxyprimaquine and its oxidized products irreversibly bound to proteins (or other macromolecules), with resulting very low recovery. Of other ring-hydroxylated metabolites which are capable of undergoing redox cycling, 7-hydroxy-primaquine is also expected to be unstable, whereas synthetic 2- and 4-hydroxyprimaquine were found to be stable (37, 38). Even though ring-hydroxylated primaquines have been shown to cause hematotoxicity *in vitro*, their relative distribution in the liver and erythrocytes has not been determined. There is also a possibility that primaquine enters erythrocytes by diffusion and gets oxidized to quinone imines or other redox-active species by ROS present therein.

Metabolic studies have shown different metabolic rates and profiles for primaquine enantiomers. The differential activity profiles we observed for PQ enantiomers in different animal models may be due to their pharmacokinetic differences. Our studies (18) in mice have shown that with administration of racemic PQ in mice, (−)-PQ had a shorter half-life (45 min) than (+)-PQ (78 min). The inactive metabolite, carboxyprimaquine, rapidly appears in serum and is predominantly the (−) form. Thus, a significant portion of (−)-PQ was rapidly converted to an inactive metabolite, carboxyprimaquine, whereas (+)-PQ was presumably preferentially converted to ROS-generating ring-hydroxylated products, although the fate of a majority of PQ was unaccounted for (18). In other work (Saunders et al., unpublished), we observed that in rhesus monkeys, (+)-PQ had a somewhat longer

half-life and much higher C_{max} and area under the concentration-time curve (AUC) than (−)-PQ; though (−)-PQ was preferentially converted to the carboxy metabolite compared to (+)-PQ, the differential was not as pronounced as that observed with mice.

After more than 60 years of clinical use, uncertainties about PQ metabolism and toxicity in humans remain. A key question is whether the two enantiomers show differential therapeutic indices in humans. We have developed preliminary evidence (39) that when the racemate was administered to healthy volunteers, there was a dramatic difference in the conversion of the two enantiomers to carboxyprimaquine, the major circulating metabolite in humans (25), even though the serum concentrations of the enantiomers were similar. As observed in other species, (−)-PQ is much more readily converted to the carboxylic acid form. However, since the profile and distribution of ring-hydroxylated metabolites emanating from each enantiomer in the liver and erythrocytes is not known, the relative toxicities and efficacies of the two enantiomers in humans remain a mystery. Currently PQ is administered at lower doses (15 mg/day) for long duration (14 days) as a precautionary measure to prevent hematotoxicity in G6PD-deficient individuals (2, 3). Most of the treatment failures of PQ have been attributed to lower dosage and poor compliance (3). Even a modest improvement of the therapeutic index of PQ by selecting the enantiomer with better activity and toxicity profiles would greatly enhance the therapeutic utility of this drug. A "phase I"-type human study comparing the pharmacokinetics and tolerability of PQ enantiomers would entail minimal risk and potentially be a very informative study. From an ethical perspective, since individuals using PQ (in its currently available racemic form) are routinely exposed to both enantiomers, if dosing is initiated at half of the clinically employed dose (30 or 45 mg of PQ base), there would be little expectation of any untoward risk in such a study. This would possibly afford a rapid path to an improved liver-stage and gametocidal antimalarial drug (40) and would in addition give guidance for future 8-aminoquinoline drug development with respect to stereochemistry issues in humans.

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