Primaquine Enantiomers: Scalable Preparation and Differential Pharmacologic and Toxicologic Profiles


National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences and Department of Pharmacology University of Mississippi, University, Mississippi, USA; Department of Veterinary Medicine, United States Army Medical component, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand; Department of Microbiology and Immunology, SUNY Upstate Medical University, Syracuse, New York, USA; Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, Indiana, USA; Department of Chemistry, Louisiana State University, Baton Rouge, Louisiana, USA; Department of Experimental Therapeutics, Walter Reed Army Institute of Research, Silver Spring, Maryland, USA; Ironstone Separations, Inc., 147 County Road 245, Etta, Mississippi, USA

Address correspondence to N. P. Dhammika Nanayakkara, dhammika@olemiss.edu.
ABSTRACT: 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 *P. 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 in 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 to mice. In Beagle dogs (+)-(S)-PQ caused more methemoglobinemia and was toxic at 5 mg/kg/day orally for 3 days, while the (-)-(R)-PQ was well tolerated. In a novel mouse model of hemolytic anemia associated with human glucose-6-phosphate dehydrogenase (G6PD) deficiency, it was also demonstrated that (-)-(R)-PQ was less hemolytic compared to (+)-(S)-PQ to 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.

KEYWORDS: Primaquine, enantiomers, malaria, pneumocystis pneumonia, methemoglobinemia, toxicity, hemolytic anemia, glucose-6-phosphate dehydrogenase deficiency
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 relapsing malaria and further have the capacity to block infection by preventing mosquito injected sporozoites from establishing in the liver. They also have gametocidal activity and can interrupt disease transmission from mosquitoes feeding on an infected patient and subsequently feeding on non-infected 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 anti-relapse doses (3). In addition, PQ is effective in combination with clindamycin for the treatment as well as prophylaxis of *Pneumocystis jirovecii* (formerly *P. 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 and (-)-(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 four times as toxic (acute lethality) as the (-)-form in mice but, unexpectedly, the (-)-form was 3-5 times as toxic as the (+)-form and at least twice as toxic as the racemate in Rhesus monkeys (13). In an in vitro tissue schizontocidal assay in 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, both enantiomers exhibited similar plasma PK profiles (Cmax and Tmax) (18). However plasma (-)-PQ level declined faster as compared with (+)-PQ. A pronounced difference was noted in the plasma PK profile of the enantiomers of carboxy-PQ (cPQ), the major PQ metabolite. The Cmax for (-)-cPQ was more than 17 fold higher compared to (+)-cPQ (18).
With the disparate toxicity profiles of PQ enantiomers in different species, and given the steep dose response for PQ in human, 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 in 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. A companion paper describes evaluations of antimalarial efficacy, pharmacokinetics and toxicity in non-human primates (19). In these papers, we extend earlier evidence suggesting marked species differences in PQ enantiomer profiles, and emphasize that there is warrant for comparing PQ enantiomers in human 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 ACS and 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 supplementary material.
Crystallographic Data for NPC1161A (R)-(+)-phenylethylurea derivative. Experimental details, crystallographic data and the ORTEP diagram for this compound are provided in the supplementary 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 in mice infected with Plasmodium berghei (NK-65 strain) according to Peters’ 4-day suppressive test, which has been modified to a three-day treatment schedule. Male mice (Swiss Webster strain) weighing 18-20 g were intraperitoneally inoculated with 2x10^7 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 nano pure sterile water and administered orally through gavage to the mice about 2 h after the infection (Day 0). The mice were treated once daily for 3 consecutive days (Days 0-2). 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 post infection) by tail snip, stained with Giemsa and evaluated under a microscope for determination of the parasitemia. Mice without parasitemia after day 28 post infection were considered cured. These groups of animals were also tested for the level of hemoglobin and methemoglobin. For determination of hemoglobin and methemoglobin about 50 µl of the blood was collected with tail snip. The blood was diluted 1:5 with PBSG containing EDTA. The levels of hemoglobin and methemoglobin were evaluated with a Co-Oximeter IL682 pre-calibrated with rodent blood.
Antimalarial causal prophylaxis in mice. ICR female mice were each inoculated with 80,000-100,000 sporozoites (in 0.1 ml of phosphate-buffered saline, 5% bovine serum albumin) of 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 three days using a vehicle of 0.5% hydroxyethylcellulose: 0.1% Tween 80 (HECT) and delivered orally via a 20G 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 6-8. Administration of antimalarial drugs with causal prophylactic activity will delay the 4-day patency (at low doses) or prevent the parasitemia altogether (at effective doses). Successful causal prophylaxis is determined by survival to day 31 post-inoculation.

Activity against Pneumocystis carinii infection in mice (20). Female BALB/c mice free of Pneumocystis, 6-8-weeks of age (Harlan Sprague Dawley) were immunosuppressed by the administration in drinking water of 1.2 mg/ml dexamethasone. After four days animals were trans-tracheally inoculated with $10^6$ P. carinii and were continued on immunosuppressive agents. At four weeks post inoculation, treatment was begun and continued for three weeks. There were ten 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, consumption for each group 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) (50/250 mg/kg/day) and NPC1161B.
(10) (0.25 mg/kg/day) treatments. At the end of three 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. 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 on G6PD deficient human red cells in NOD-SCID mice.** Eight to nine week old female NOD.CB17-Prkdc<sup>scid</sup>/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 (21). Approximately 5 µl of blood was analyzed for 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 FloJo software (TreeStar, Inc., Ashland, OR). Mice with peripheral huRBC levels greater than 60% were randomized for drug treatment with 4-5 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 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 (4<sup>th</sup>) 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 moribund; animals surviving to the end of 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 supplementary 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 at 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 potency of the racemate and (+)-PQ based on the parasitemia suppression data at the dose 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 day 5 and 7 whereas (-)-PQ showed only partial suppression. Racemic and (+)-PQ showed some signs of toxicity at 100 mg/kg dose. The mice treated with both racemic and (+)-PQ showed less mobility and grooming compared to the
animals in the control, chloroquine and (-)-PQ treated groups. The mice treated with (+)-PQ also underwent a reversible loss in body weight on day 4-6 post treatment (Fig. 2). The hematological 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 toxicity of racemic PQ and the enantiomers was evaluated separately in a beagle dogs and also in the humanized SCID mouse model.

**Comparative antimalarial causal prophylaxis in mice.** The difference in antimalarial potency between the two enantiomers was much more prominent on 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 (-)-PQ in this species. At a dose of 25 mg/kg/day, racemic PQ conferred 100% protection. The data were analyzed in GraphPad Prism by sigmoidal emax nonlinear regression, and the ED$_{80}$s are as follows: (+)-PQ ED$_{80} = 10.5$ mg/kg/d x 3 d; (-)-PQ ED$_{80} = 63.7$ mg/kg/d x 3 d. Racemic PQ dose response was not determined in this study, but in a previous study in our lab (AFRIMS), 20 mg/kg/d x 3 d was effective prophylaxis for 80% of the mice (Gettayacamin, Tungtaeng, unpublished data). Also, in 19 different experiments using 25 mg/kg/d x 3 d 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, Tungtaeng, unpublished data). 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 ED80 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 anti-pneumocystis activity in mice.** When the enantiomers were evaluated in 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 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 (Nanayakkara, unpublished) 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 towards baseline once dosing
stopped. Decreases in eosinophils and lymphocytes, and increases in leukocytes, neutrophils,
monocytes and reticulocytes were also observed but hemoglobin/hematocrit remained
unchanged. Animals treated with (+)-PQ consistently lost body weight (16-17% 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 the study days 1-8; the overall
weight loss in the (-)-PQ ranged from approximately 3-8%. All clinical chemistry and
hematological parameters in the (-)-PQ treated animals remained comparable to baseline values
through the end of study, although platelet counts appeared slightly decreased by day 8.

**Comparative hemolytic responses on G6PD deficient human RBCs in SCID mice.** To assess
if there were differences in hemolytic toxicity 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) (21) and with PQ
enantiomers at the same dose and at 6.25 mg/kg/day (Fig. 4). Treatment with the (+)-PQ
enantiomer resulted in similar hemolytic toxicity to racemic PQ, as indicated by the degree of
loss of huRBC after day 7 of treatment. In contrast, the levels of huRBC in mice treated with the
(-)-PQ enantiomer were not significantly different from the mice given PBS alone. The
hemolytic response of (+)-PQ was significantly higher compared to (-)-PQ.

**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 (22). 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 the (+)-PQ and more rapid
conversion of the (-)-PQ to the carboxyprimaquine metabolite (16-18). In an in vitro plasmodial
tissue schizontcidal 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. In two mouse models of malaria (blood and exo-
erythrocytic stages), we found that (+)-PQ was more potent than (-)-PQ. Similar findings were
observed in Pneumocystis pneumonia in mice. The improved activities we observed in mice for
(+)-(S)-PQ over the (-)-(R)- form contrasted with that for the enantiomers of the second
generation 8-AQ with 5-aryloxy substituent, NPC1161C (11). With these enantiomers (+)-(R)-
NPC1161B had better activity than (+)-(S)-NPC1161A for malaria and Pneumocystis pneumonia
in mouse models (1). 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 CD spectra with those of (+)-(S)-PQ and (-)-(R)-PQ (Fig. S2a in the supplementary material). We confirmed the absolute configuration of (+)-NPC1161A as S by X-ray crystallography of its (R)-(+)phenylethylurea derivative (Fig. S2b in the supplementary material). This observation indicates that the absolute configuration 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 in mice for enantiomers of PQ and NPC1161C were similar, where the (+)-(S)-enantiomer was more toxic. The systemic toxicity of (+)-PQ was 3-5 fold greater compared to (-)-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 activity 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 the racemic PQ in human (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-
aminooquinolines, (+)-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 (21). 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 report, 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 a companion paper (19), our groups have confirmed Schmidt’s findings
that both 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 non-human primate. To the extent that this reflects the potential
hemolytic toxicity, it would suggest that (-)-PQ may have a therapeutic index advantage over the
racemate in treatment of malaria in humans. Though the (-)-PQ gave evidence of greater liver
toxicity in the monkeys, this only occurred 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 (23). 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 (23, 24) and in vivo (25)
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 (26) and animals (27, 28). Biological studies have shown that carboxyprimaquine was inactive (29) and nonhematotoxic (30). In contrast, ring hydroxylated metabolites of PQ generated by CYP enzymes (23, 24) can undergo redox cycling generating reactive oxygen species (ROS) (31) which have been suggested as responsible for antimalarial activity (32) and hematotoxicity (30,33). Various PQ ring-hydroxylated metabolites and their oxidized products produced by CYP enzymes have been identified (23) and some of them have been detected as minor metabolites in human liver microsomes (34). Some of the hydroxylated PQ metabolites have been shown to be active in vitro (35) and in vivo (29) and more hematotoxic than the parent compound in in vitro assays (33, 35-37). Their activity and toxicity have been linked to their superoxide generation capacity (35). 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 (33, 36, 37). Synthetic 5-hydroxyprimaquine was found to be unstable (36) 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.
Even though ring-hydroxylated primaquines have been shown to cause hematoxicity 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 (18). In the companion paper to this report (19), we observed that in Rhesus monkeys, (+)-PQ had a somewhat longer half-life and much higher Cmax and AUC than (-)-PQ; though the (-)-PQ was preferentially converted to the carboxy metabolite than (+)-PQ, the differential was not as pronounced as that observed in 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 (40) 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 (26) even though the serum concentrations of the enantiomers were similar. As observed in other species, the (-)-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
erthrocytes is not known, the relative toxicity and efficacy of the two enantiomers in humans
remains a mystery. Currently PQ is administered at lower doses (15 mg/d) for long duration (14
days) as a precautionary measure to prevent hemototoxicity 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
(41), and would in addition give guidance for future 8-aminoquinoline drug development with
respect to stereochemistry issues in humans.

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and inhibition of monoamine oxidases A and B by exo-erythrocytic antimalarials. Optical
isomers of primaquine, N-acylated congeners, primaquine metabolites and 5-phenoxy-

glucose-6-phosphate dehydrogenase-deficient red cells to primaquine enantiomers and
two putative metabolites. I. Effect on reduced glutathione, methemoglobin content and


FIG 1 Structure of primaquine, NPC1161C and their enantiomers
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 three days (day 0, 1 & 2) (2 hours after the infection) with racemic PQ and PQ enantiomers. Chloroquine was tested as a control drug. The results shown here are for 100 mg/kg dose. The lower doses did not show any effect on total body weights. Each point shows mean ± S.D. values from 5 animals in each group.
FIG 3 Prophylactic antimalarial activity 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 post-infection. Graph represents % of mice surviving to day 31 (study end).
FIG 4 Hemolytic toxicity 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 values mean ± SD for four animals. The data were analyzed by the Students t test. P values PBS vs PQ (12.5)- <0.0001 (S); PBS vs (+)-PQ (12.5)- 0.003 (S); PBS vs (-)-PQ (12.5)- 0.05 (S); PBS vs (+)-PQ (6.25)- 0.0004(S); PBS vs (-)-PQ (6.25)- 0.38 (NS); PQ (12.5) vs (+)-PQ 12.5- 0.35 (NS); PQ (12.5) vs (-)PQ (12.5)- 0.0002 (S); (+)-PQ (12.5) vs (-)-PQ (12.5)- 0.007 (S).

S- Statistically significant difference; NS- Difference statistically not significant.
TABLE 1 Suppressive antimalarial activity of primaquine enantiomers in mice

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose mg/kg/day (x 3 days)</th>
<th>% Parasitemia suppression(^1)</th>
<th>Survival(^2) Day of Death (MST)(^3)</th>
<th>Cured/Treated (^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primaquine</td>
<td>11.1</td>
<td>97.47±4.47</td>
<td>3/5 21,21,28,28,28 (25.2±3.8)</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>33.3</td>
<td>100</td>
<td>5/5 (&gt;28)</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>5/5 (&gt;28)</td>
<td>4/5</td>
</tr>
<tr>
<td>(+)-Primaquine</td>
<td>11.1</td>
<td>79.85±20.65</td>
<td>0/5 15,18,18,21,21 (18.6±2.5)</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>33.3</td>
<td>100</td>
<td>4/5 24,28,28,28,28 (27.2±1.8)</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>5/5 (&gt;28)</td>
<td>2/5</td>
</tr>
<tr>
<td>(-)-Primaquine</td>
<td>11.1</td>
<td>13.34±3.91</td>
<td>0/5 12,14,14,18,18 (15.2±2.7)</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>33.3</td>
<td>90.35±1.38</td>
<td>4/5 8,28,28,28,28 (24.0±8.9)</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>5/5 (&gt;28)</td>
<td>2/5</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
<td>0/5 5/14/17/17/17 (14.0±5.2)</td>
<td>0/5</td>
</tr>
</tbody>
</table>

\(^{1}\) % suppression in parasitemia is calculated by considering the mean parasitemia in the vehicle control as 100%.

\(^{2}\) Number of animals that survived day 28/total animals in group (the day of the death post-infection)

\(^{3}\) MST- mean survival time (days)

\(^{4}\) Number of mice without parasitemia (cured) till day 28 post-infection

* values are mean±SD of five animals
TABLE 2 Effect of treatment of *Plasmodium berghei* infected mice with primaquine enantiomers on methemoglobin levels. The *P. berghei* infected mice were treated once daily for three days (day 0, 1 & 2) (2 hours after the infection) with racemic PQ and PQ enantiomers. Chloroquine was tested as a control drug. The results shown here are for 100 mg/kg dose. Blood was collected 20 hours after the last dose by tail snip and methemoglobin levels were estimated by co-oximeter. Values are mean ± S.D. of 5 animals in each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>% Methemoglobin</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle Control</td>
<td>0.82 ± 0.65</td>
<td>-</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.78 ± 0.22</td>
<td>0.9 (NS)</td>
</tr>
<tr>
<td>(+/-)Primaquine</td>
<td>1.22 ± 0.31</td>
<td>0.25 (NS)</td>
</tr>
<tr>
<td>(+) Primaquine</td>
<td>1.12 ± 0.25</td>
<td>0.36 (NS)</td>
</tr>
<tr>
<td>(-) Primaquine</td>
<td>0.66 ± 0.51</td>
<td>0.68 (NS)</td>
</tr>
</tbody>
</table>

NS- Not significant difference from the vehicle control
TABLE 3 Oral efficacy of primaquine enantiomers and racemate against pneumocystis infection in mice.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose mg/kg/day (x 21)</th>
<th>Activity Cure/Treated (Giemsa Stain)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primaquine</td>
<td>10</td>
<td>5/10</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0/10</td>
</tr>
<tr>
<td>(+)-Primaquine</td>
<td>10</td>
<td>9/10</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3/10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0/10</td>
</tr>
<tr>
<td>(-)-Primaquine</td>
<td>10</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0/10</td>
</tr>
<tr>
<td>TMP/SMX</td>
<td>50/250</td>
<td>9/10</td>
</tr>
<tr>
<td>NPC1161B</td>
<td>0.25</td>
<td>10/10</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0/10</td>
</tr>
</tbody>
</table>