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Antiparasitic Activities and Toxicities of Individual Enantiomers of the 8-Aminoquinoline 8-[(4-Amino-1-Methylbutyl)Amino]-6-Methoxy-4-Methyl-5-[3,4-Dichlorophenoxy]Quinoline Succinate▽

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8-Aminoquinolines are an important class of antiparasitic agents, with broad utility and excellent efficacy, but also limitations due to hematological toxicities, primarily methemoglobinemia and hemolysis. One representative from this class, (±)-8-[(4-amino-1-methylbutyl)amino]-6-methoxy-4-methyl-5-[3,4-dichlorophenoxy]quinoline succinate (NPC1161C), proved extremely efficacious in animal models of malaria and pneumocystis pneumonia. This racemic mixture was separated into its component enantiomers by chemical and chromatographic means. The enantiomers were evaluated for antiparasitic activity in murine models of *Plasmodium berghei*, *Pneumocystis carinii*, and *Leishmania donovani* infection, as well as the propensity to elicit hematotoxicity in dogs. The (−)-enantiomer NPC1161B was found to be more active (by severalfold, depending on the dosing regimen) than the (+)-enantiomer NPC1161A in all of these murine models. In addition, the (−) enantiomer showed markedly reduced general toxicity in mice and reduced hematotoxicity in the dog model of methemoglobinemia. It is concluded that the configuration at the asymmetric center in the 8-amino side chain differentially affects efficacy and toxicity profiles and thus may be an important determinant of the "therapeutic window" for compounds in this class.

8-Aminoquinolines are an important class of anti-infective drugs with promising utility in the treatment of malaria and other emerging infectious diseases (41). Primaquine (Fig. 1), the only 8-aminoquinoline derivative in clinical use, is the drug of choice for the radical cure of relapsing malaria (2). This drug is also reported to be effective against *Trypanosoma* (28) and *Leishmania* (39) species and, in combination with clindamycin, for prophylaxis and treatment of *Pneumocystis carinii* pneumonia (PCP) in animal models (7, 33). Several studies have shown that the primaquine-clindamycin combination was extremely effective for prophylaxis (23) and treatment (10, 11, 20, 23, 43, 44, 45) of mild to severe cases of pneumocystis pneumonia in AIDS patients, especially as a salvage therapy in cases where conventional therapy was ineffective or not tolerated. (Note that, in recent years, the nomenclature *Pneumocystis jirovecii* has been adopted for the human pathogen.) A serious limitation to widespread use of primaquine, however, is that it produces methemoglobinemia (16, 22, 40) and hemolysis (40), especially in individuals who suffer from hereditary glucose-6-phosphate dehydrogenase deficiency.

Over the years, several attempts have been made to improve the therapeutic index of primaquine. In a study sponsored by the U.S. Army, a large number of 8-aminoquinolines chemically related to primaquine have been synthesized and evaluated for antiparasitic activity (31). Some of these compounds were found to have superior activity compared to primaquine against *Leishmania* (25) and *Trypanosoma* (24) species and against several *Plasmodium* species (31) in animal models. Based on these results tafenoquine (WR 238605) was selected for clinical evaluation for the treatment of relapsing malaria (48) and sitamaquine (WR 6026) was selected for clinical evaluation for the treatment of leishmaniasis (17, 21, 37, 49). Structures of these two investigational drugs, along with those of primaquine and the analogs presented in this paper, are shown in Fig. 1.

Some new 8-aminoquinoline analogs, including tafenoquine and sitamaquine, were reported to be as active as a primaquine-clindamycin combination or trimethoprim-sulfamethoxazole (TMP-SMX) combination for the treatment of PCP in a rat model, even when they were used alone (5, 32, 34).

In an effort to optimize structural features of an 8-aminoquinoline analog for oral antimalarial activity against malaria parasites in animal models, we prepared a large number of 8-aminoquinoline analogs. As shown herein, we found that the racemic (±)-8-[(4-amino-1-methylbutyl)amino]-6-methoxy-4-methyl-5-[3,4-dichlorophenoxy]quinoline succinate (NPC1161C) had the best antimalarial activity in the assay where test compounds were administered orally in multiple doses, afforded a 100% cure rate at 1 mg/kg of body weight for 3 days, and did not show any toxicity at the highest dose tested (64 mg/kg for

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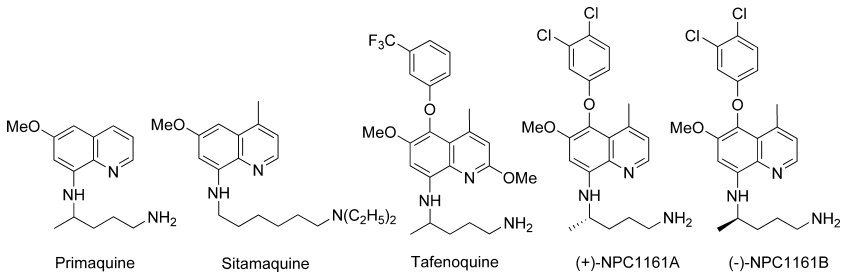

FIG. 1. Structures of primaquine and primaquine analogs.

3 days) (Table 1). The same compound had shown a similar activity/toxicity profile in a previous study where test compounds were administered subcutaneously (80% cure rate at 5 mg/kg and no toxicity at 640 mg/kg) (26). NPC1161C had earlier shown better tissue schizonticidal activity than primaquine had against *Plasmodium cynomolgi* in rhesus monkey models (26).

NPC1161C also showed potent activity against PCP in a mouse model. As indicated below, at a dose of 1.3 mg/kg/day it showed activity comparable to that of TMP-SMX at 50/250 mg/kg/day (see Table 4). Though this compound showed no appreciable toxicity in rodents, it induced a significant methemoglobinemia in a beagle dog model and was more toxic than primaquine or tafenoquine in this regard (see Fig. 4).

Although the influence of stereochemistry on pharmacological and toxicological activities is well established, this phenomenon has received relatively little attention in the case of the 8-aminoquinolines. Schmidt et al. (36) examined the relative curative and toxic activities of primaquine and its (+) and (−) isomers in mice and rhesus monkeys. They confirmed an earlier report that (+)-primaquine was more toxic than the (−) form in mice but found that the opposite was true in the rhesus monkey. All three forms of primaquine, the (+), (−), and (±) forms, showed essentially identical curative properties against sporozoite-induced *Plasmodium cynomolgi* infections (36). Different in vitro activity and toxicity profiles also have been reported for primaquine enantiomers (1, 14). Studies in our institute (4) have shown that (−)-primaquine is more susceptible to metabolic oxidation to carboxyprimaquine in rats as well as in liver microsomal preparations. These results were consistent with the rate of clearance previously reported by Nicholl et al. for individual enantiomers in an isolated perfused rat liver preparation (30).

To investigate the influence of stereochemistry on the activity and toxicity profiles of NPC1161C, we have separated this analog into individual stereoisomers and evaluated them for activity against *Plasmodium berghei*, PCP, and *Leishmania donovani* in murine models and their toxicities in rodent and beagle dog models.

TABLE 1. Suppressive antimalarial activities of NPC1161C, tafenoquine, and primaquine against *P. berghei* in mice

Compound	Dose (mg/kg/day)	Total dose (mg/kg)	ΔMST[a] (days)	No. of mice alive at day +60/total no. of mice
NPC1161C	64	192		7/7
	16	48		7/7
	4	12		7/7
	1	3		7/7
Tafenoquine	64	192	14.5	1/7
	16	48		7/7
	4	12	9.7	1/7
	1	3	6.6	0/7
Primaquine	64	192	9.5	3/7
	16	48	9.3	1/7
	4	12	3.8	0/7
	1	3	0	0/7
Control	0	0	0	0/7

[a] ΔMST, increase in mean survival time over that of the controls (8.4 days).

MATERIALS AND METHODS

8-Aminoquinolines. The synthesis of racemic NPC1161C was described earlier (29). The phthalimide analog of racemic (±) 8-[(4-amino-1-methylbutyl)amino]-6-methoxy-4-methyl-5-[3,4-dichlorophenoxy]quinoline (NPC1161C) was resolved into individual enantiomers by preparative high-pressure liquid chromatography using a Chiralcel OD column (Daicel Chemical Industries Ltd.) and hexane-isopropanol (92:8) as the solvent. Removal of the phthalimide protecting group yielded enantiomerically pure free amines which were converted to the succinate salts and crystallized from ethanol-ether to yield (+)-(*S*)-8-[(4-amino-1-methylbutyl)amino]-6-methoxy-4-methyl-5-[3,4-dichlorophenoxy]quinoline succinate (NPC1161A) and (−)-(*R*)-8-[(4-amino-1-methylbutyl)amino]-6-methoxy-4-methyl-5-[3,4-dichlorophenoxy]quinoline succinate (NPC1161B). Their identities were confirmed by nuclear magnetic resonance (NMR) (^{1}H NMR and ^{13}C NMR), mass spectrometry, and elemental analysis. Sitamaquine was prepared using the method previously described (15). Primaquine diphosphate was purchased from Aldrich Chemical Company, Milwaukee, WI. Tafenoquine was provided by the Walter Reed Army Institute of Research, Washington, DC.

Blood schizonticidal activity against *Plasmodium berghei* in mice. CD-1 male or female mice 5 weeks of age, in groups of five, were infected with 5×10^4 parasitized erythrocytes of *Plasmodium berghei* strain KBG-173. This strain was established in our laboratories (A. Ager) for its capability to produce reliable parasitemia and death in CD-1 mice in a modified Thompson test (42). The strain is maintained in vivo in Swiss mice and has been used for more than 20 years to test susceptibility to a wide range of antimalarial drugs. Drugs were mixed in 0.5% hydroxyethylcellulose and 0.1% Tween 80 and administered orally once a day on days 3, 4, and 5 postinfection. Blood films were taken on day +6 and weekly thereafter until day +60. Mortality data were tabulated for 60 days, at which time all mice surviving that were blood film negative were considered cured.

Duration of protection against *Plasmodium berghei* in mice. CD-1 male or female mice, 5 weeks of age, were infected with 5×10^4 parasitized erythrocytes of *P. berghei* strain KBG-173. Drugs were mixed in 0.5% hydroxyethylcellulose-0.1% Tween 80 and administered orally once a day on day 3, 2, 1, or 0 prior to the infection or 1 or 2 days postinfection. Blood films were taken on day +6 postinfection and weekly thereafter until day +30. Mortality data were tabulated for 30 days, at which time all mice surviving that were blood film negative were considered cured.

Activity against *Pneumocystis carinii* infection in mice (6, 7). 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 were administered in drinking water at a dose of 5.0, 1.0, 0.5, or 0.25 mg/kg/day. The drugs were prepared fresh daily, consumption for each group was monitored, and drugs were adjusted as needed to ensure proper dosing. A group of untreated animals served as a control, and a group of TMP-SMX-treated (50/250 mg/kg/day) animals served as a positive treatment control. 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 and modified methenamine silver nitrate. Slides were blinded as to treatment and examined microscopically by two experienced individuals, and severity of infections was graded 0 to 5 according to a roughly logarithmic scale based on the numbers of the organisms in 1,000× microscopic fields: >100 per field = 5, 11 to 100 per field = 4, 1 to 10 per field = 3, 2 to 9 per 10 fields = 2, ≤1 per 10 fields = 1, and 0 per 50 fields = 0. Scores for the group were averaged, and the standard error was calculated.

Prophylactic activity against *Pneumocystis carinii* infection in mice. 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. Prophylaxis regimens were begun and continued for 6 weeks. There were 10 mice in each group. Test compounds were administered in drinking water at doses of 0.25 and 0.1 mg/kg/day. The drugs were prepared fresh daily, consumption for each group was monitored, and drugs were adjusted as needed to ensure proper dosing. A group of untreated animals served as a control, and a group of TMP-SMX-treated (50/250 mg/kg/day) animals and a group of primaquine-treated (2.0 mg/kg/day) animals served as positive treatment controls. At the end of 6 weeks of prophylaxis, 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 and modified methenamine silver nitrate. Slides were blinded as to treatment and examined microscopically by two experienced individuals, and severity of infections was graded 0 to 5 according to the scale described above.

Antileishmanial activity against *L. donovani* HU3 in mice. Mice were infected with 1×10^7 *L. donovani* (MHOM/ET/67/HU3) amastigotes intravenously. Drug administration started 7 days postinfection. Mice were dosed daily, sodium stibogluconate was given subcutaneously for 5 days once/day, and the three other compounds were given orally for 5 days. Compounds were prepared in 0.25% methylcellulose-10% ethanol. Mice were sacrificed 14 days postinfection. Liver impression smears were made, and parasite burdens were counted and compared to the untreated control group. Fifty percent effective doses (ED_{50}s) were calculated by linear regression analysis (*xl*fit; Microsoft). Confidence limits for ED_{50} and ED_{90} values were derived using MS Excel and Prism.

Hematological toxicity after oral administration of 8-aminoquinolines in beagle dogs. Eight- to nine-month-old beagle dogs were treated with orally administered gelatin capsules containing the test compounds for four consecutive days. Dogs were observed daily for mortality and morbidity. Clinical signs were recorded approximately 1 and 4 h after each treatment with the test compounds and daily or more often as clinical signs warranted. Body weights were recorded on day 1 and weekly thereafter throughout the study. Blood samples for methemoglobin determination and hematology were collected from the jugular or cephalic vein prior to dosing on days 1, 2, and 4 and then on days 5, 7, 9, 11, 15, 18, 21, 25, and 29. Methemoglobin formation was determined by a cooximeter method that calculates the levels of hemoglobin, oxyhemoglobin, and methemoglobin based on absorbance at four specific wavelengths.

Dogs were treated with NPC1161C at 6.4 mg/kg/day (n = 4, two males and two females), tafenoquine at 6.7 mg/kg/day (n = 2 males), or primaquine at 5.3 mg/kg/day (n = 2 males), which is equivalent to 0.0116 mmol/kg/day (as the base) of each compound. The test compounds were administered as bulk drug loaded in gelatin capsules which were administered orally to dogs on days 1, 2, 3, and 4. The amount of drug loaded into the capsules was based upon the initial body weight determined for each dog on day 1.

In a separate follow-up study, the individual enantiomers of NPC1161C were administered to male and female beagle dogs. For each enantiomer, two dogs (one male and one female) were administered 0.64 mg/kg (0.5 mg/kg of base) orally daily for 4 days. Clinical observations and methemoglobin determinations were made at days −6, −5, and −4; then predose on dosing days 1, 2, and 4; and then on days 5, 6, 8, 10, 12, 15, 22, 29, and 36. After a 5-week washout, when methemoglobin levels had returned to 10% of baseline, each dog received 1.91 mg/kg of the same enantiomer, administered orally daily for 4 days, with the same sampling protocol.

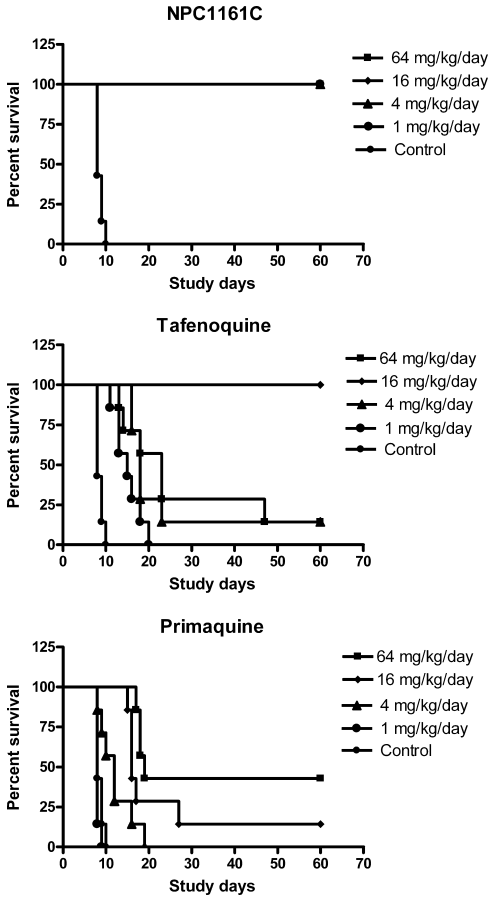

FIG. 2. Suppressive antimalarial activities of NPC1161C, tafenoquine, and primaquine against *P. berghei* in mice represented as survival curves.

RESULTS

Antimalarial activity in *P. berghei*-infected mice. NPC1161C was curative at all the doses tested (Table 1). Six out of seven mice treated with 64 mg/kg/day of tafenoquine died, apparently due to cumulative drug toxicity. This compound was effective in curing malaria-infected animals at a dose of 16 mg/kg/day but was ineffective at lower doses. Primaquine had no effect at 1- or 4-mg/kg/day doses and was partially effective at 16-mg/kg/day (cure rate of one/seven) and 64-mg/kg/day (cure rate of three/seven) doses. These results are also presented as Kaplan-Meier survival curves (Fig. 2).

Blood schizonticidal activities of the (+) enantiomer NPC1161A and (−) enantiomer NPC1161B were evaluated at 4, 1, and 0.25 mg/kg/day, whereas the racemate was evaluated at doses of 1 and 0.25 mg/kg/day. Mortality, increase in mean survival time over the control animals, and parasitemia on day 6 are summarized in Table 2. All the animals treated with

TABLE 2. Suppressive antimalarial activities of NPC1161A, NPC1161B, and NPC1161C against *P. berghei* in mice

Compound	Dose (mg/kg/day)	Total dose (mg/kg)	ΔMST[a] (days)	No. of mice alive at day +34/total no. of mice	Parasitemia (%) on day +6
NPC1161A	4	12	11	0/5	1.1
	1	3	6	0/5	27.4
	0.25	0.75	0	0/5	28.2
NPC1161B	4	12		5/5	0.0
	1	3	20.5	2/5	0.0
	0.25	0.75	11.6	0/5	0.2
NPC1161C	1	3	10.2	0/5	0.1
	0.25	0.75	11.6	0/5	4.8
Control	0	0	0	0/5	31.8

[a] ΔMST, increase in mean survival time over that of the controls (8.4 days).

enantiomer NPC1161A died of malaria before the end of the 34-day observation period. Parasitemias observed on day 6 for this enantiomer at doses of 0.25 and 1 mg/kg/day were only marginally different from that observed for untreated control. At 4 mg/kg/day there was a marked decrease in parasitemia on day 6 and an increase in mean survival time by 11 days over the control. The animals treated with 1 and 4 mg/kg/day of the enantiomer NPC1161B had no detectable parasites on day 6. All the mice treated with a 4-mg/kg/day dose and 40% of the mice treated with 1 mg/kg/day were cured. The mice which were not cured after the treatment with 1 mg/kg/day had their mean survival time increased by 20.5 days over the control. At a dose of 0.25 mg/kg/day of enantiomer NPC1161B, a marked decrease in parasitemia on day 6 compared to control was observed. All the animals in this group died during the 34-day observation period but had their mean survival time increased by 11.6 days over the control. A marked decrease in parasitemia was observed in the mice treated with 1 and 0.25 mg/kg/day of racemate NPC1161C. All of these animals died during the observation period. They showed a mean survival time increase of 10.2 and 11.6 days over the control, respectively (Table 2). These results are also presented as Kaplan-Meier survival curves (Fig. 3).

In the duration of protection study, treatment of animals with a dose of 2 mg/kg of the enantiomer NPC1161A on day −2, −1, 0, +1, or + 2 of the inoculation had little or no effect on the survival or extension of the mean survival time compared to that of the control group. In contrast, a single dose of 2 mg/kg of enantiomer NPC1161B protected 100% of the animals in the groups which were treated on day −1, 0, +1, or +2 of the inoculation. In the group which was treated with 2 mg/kg day on day −2 of the inoculation, 60% of the mice were protected and those that succumbed to the infection showed a mean survival time increased by 8 days (Table 3).

Antipneumocystis activity in mice. Both enantiomers and the racemate showed complete suppression of *P. carinii* at doses higher than 1.0 mg/kg/day. The (+) enantiomer was only partially effective at doses of 0.5 and 0.25 mg/kg/day, whereas NPC1161B totally suppressed *P. carinii* infection even at these doses. The positive control, TMP-SMX, was very effective at 50/250 mg/kg/day, and one animal showed very mild infection with Giemsa stain (Table 4). In the prophylactic assay both enantiomers and racemate prevented the infection at a dose of 0.25 mg/kg/day (Table 5). At a dose of 0.1 mg/kg/day NPC1161B totally protected animals against the infection whereas NPC1161A was only partially effective. At the same dose one of the animals treated with racemate showed very mild infection by Giemsa stain. One of the animals in the group treated with TMP-SMX at 50/250 mg/kg/day also showed mild infection. Primaquine was not effective at a dose of 2 mg/kg/day.

Antileishmanial activity against *L. donovani* HU3 in mice. All the mice treated with NPC1161A at a very high dose (100 mg/kg/day for 5 days) died due to apparent drug toxicity prior to evaluation (Table 6). However, with the (−) enantiomer, the drug was well tolerated. In the group treated with the racemic version, NPC1161C, two out of five died due to apparent drug toxicity and the animals that survived were free of parasites. At a dose of 10 mg/kg/day, all three compounds were effective and cleared the parasites after a 5-day course of treatment. Both NPC1161B and NPC1161C were effective at 5 mg/kg/day, whereas NPC1161A only partially cleared the parasites at this dose after 5 days of treatment. At lower doses, all three were only partially effective. In a comparative study, NPC1161B and sitamaquine (WR 6026) showed comparable activity profiles (Table 7). In this assay the ED_{50} values for

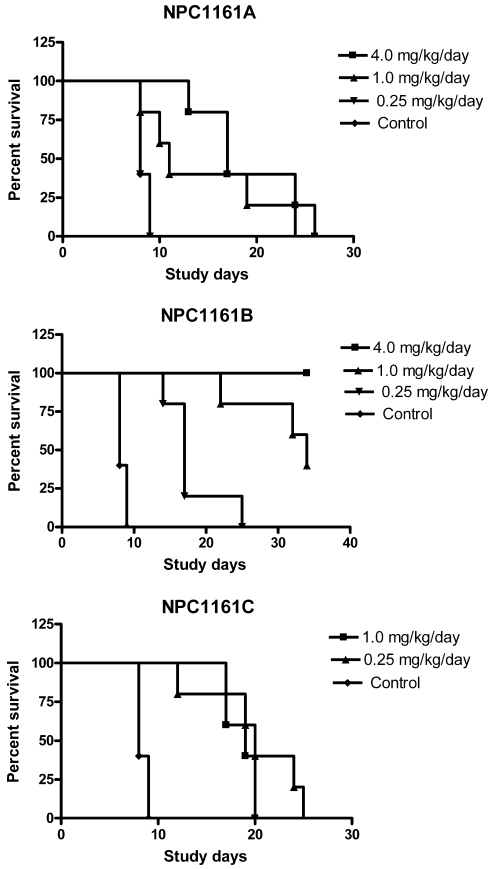

FIG. 3. Suppressive antimalarial activities of NPC1161A, NPC1161B, and NPC1161C against *P. berghei* in mice represented as survival curves.

TABLE 3. Duration of protection by NPC1161A and NPC1161B against *P. berghei* infection in mice

Day of treatment with compound[a]	ΔMST[b] (days)	No. of mice alive at day +30/total no. of mice
−3		
NPC1161A	0.0	0/5
NPC1161B	4.6	0/5
−2		
NPC1161A	0.0	0/5
NPC1161B	8.4	3/5
−1		
NPC1161A	0.6	0/5
NPC1161B		5/5
0		
NPC1161A	3.4	0/5
NPC1161B		5/5
+1		
NPC1161A	0.0	0/5
NPC1161B		5/5
+2		
NPC1161A	1.2	0/5
NPC1161B		5/5
Control	0.0	0/5

[a] A single dose of 2 mg/kg was administered on the day indicated. Controls received no treatment.
[b] ΔMST, increase in mean survival time over that of the controls (10.6 days).

NPC1161B and WR 6026 were 1.29 and 1.53 mg/kg, respectively. (Corresponding ED_{90} values were 3.66 and 3.95, respectively.) The ED_{50} value for sodium stibogluconate was 28.1 mg pentavalent antimony (Sb^V)/kg.

Hematological toxicity after oral administration of 8-aminoquinolines in beagle dogs. Dogs were treated orally with NPC1161C (as succinate salt) at 6.4 mg/kg/day, tafenoquine (succinate) at 6.7 mg/kg/day, or primaquine diphosphate at 5.3 mg/kg/day on days 1, 2, 3, and 4. All the animals survived for the duration of the study. No clinical signs of toxicity were observed in any of the animals during the study. Fluctuations in body weight were observed through the study in most treatment groups. The most consistent change was seen in dogs treated with primaquine, with about 10% loss of weight during the first 2 weeks of the study.

Methemoglobin levels were increased in the treated groups starting at study day 4 (Fig. 4). The amount of methemoglobin reached a peak in primaquine-treated dogs at approximately day 5 and slowly declined over 2 weeks. The peak in methemoglobin in dogs treated with NPC1161C and tafenoquine occurred between day 7 and day 11 and declined even more slowly to a baseline level. The maximum methemoglobinemia induced by treatment with NPC1161C was approximately 25% of the total hemoglobin, while with tafenoquine and primaquine, the increase was approximately 10%.

The racemic NPC1161C was separated into its two enantiomers, the (−) form, NPC1161B, and the (+) form, NPC1161A. These were compared for their propensities to generate methemoglobinemia. The dogs treated with 1.91 mg/

TABLE 4. Oral efficacies of NPC1161A, NPC1161B, and racemate against pneumocystis infection in mice

Compound	Nominal dose (mg/kg/day; for 21 days)	Result with stain:			
		Giemsa		Silver	
		I/T[a]	Score	I/T[a]	Score
NPC1161A	5	0/10	0.0	0/10	0.0
NPC1161B	5	0/10	0.0	0/10	0.0
NPC1161C	1.3	0/10	0.0	0/10	0.0
NPC1161A	1	0/10	0.0	0/10	0.0
NPC1161B	1	0/10	0.0	0/10	0.0
NPC1161A	0.5	2/10	0.20 ± 0.10	2/10	0.1 ± 0.1
NPC1161B	0.5	0/10	0.0	0/10	0.0
NPC1161A	0.25	4/10	0.55 + 0.16	9/10	1.15 ± 0.16
NPC1161B	0.25	0/10	0.0	0/10	0.0
TMP-SMX	50/250	1/10	0.03 ± 0.02	0/10	0.0
Control		10/10	4.43 ± 0.12	10/10	3.80 ± 0.08

[a] I/T, number of animals with organism after treatment/number of animals treated.

TABLE 5. Oral prophylactic efficacies of NPC1161A, NPC1161B, and racemate against pneumocystis infection in mice

Compound	Dose (mg/kg/day; for 21 days)	Result with stain:			
		Giemsa		Silver	
		I/T[a]	Score	I/T[a]	Score
NPC1161A	0.25	0/10	0.0	0/10	0.0
NPC1161B	0.25	0/10	0.0	0/10	0.0
NPC1161C	0.25	0/10	0.0	0/10	0.0
NPC1161A	0.1	4/10	0.3 ± 0.1	3/10	0.1 ± 0.0
NPC1161B	0.1	0/10	0.0	1/10	0.03 ± 0.02
NPC1161C	0.1	1/10	0.1 ± 0.1	0/10	0.0
Primaquine	2.0	10/10	4.3 ± 0.1	10/10	2.8 ± 0.1
TMP-SMX	50/250	1/10	0.03 ± 0.02	0/10	0.0
Control		10/10	4.3 ± 0.1	10/10	3.4 ± 0.1

[a] I/T, number of animals with organism after treatment/number of animals treated.

TABLE 6. Activities of NPC1161A, NPC1161B, and NPC1161C against *Leishmania donovani* infection in mice

Compound	Dose (mg/kg/day)[a]	% Inhibition of parasites in liver
NPC1161A	100	—[b]
NPC1161B	100	99.8
NPC1161C	100	99.0[c]
NPC1161A	10.0	99.58 ± 0.54
NPC1161B	10.0	99.29 ± 0.83
NPC1161C	10.0	99.76 ± 0.16
NPC1161A	5.0	64.98 ± 8.49
NPC1161B	5.0	98.0 ± 0.81
NPC1161C	5.0	99.24 ± 0.47
NPC1161A	0.4	41.17 ± 8.0
NPC1161B	0.4	61.49 ± 8.46
NPC1161C	0.4	34.09 ± 9.01
Sodium stibogluconate	45	99.29 ± 0.35
	15	59.4 ± 15.3
	5	13.83 ± 4.64

[a] Doses for the control treatment, sodium stibogluconate, are expressed in terms of pentavalent antimony (Sb^V).
[b] All mice died at the end of the treatment period prior to evaluation.
[c] Two/five mice died at the end of the treatment period prior to evaluation.

TABLE 7. Comparison of antileishmanial activities of NPC1161B and sitamaquine against *Leishmania donovani* infection in mice

Compound	Dose (mg/kg/day)[a]	% Inhibition (±95% CL[b])	ED_{50} (mg/kg) (95% CL)[c]	ED_{90} (mg/kg) (95% CL)[d]
NPC1161B	5.0	97.6 (1.35)	1.28 (0.92–1.77)	3.61 (1.28–10.19)
	1.0	36.0 (19.81)		
	0.2	6.5 (14.43)		
Sitamaquine	5.0	95.56 (1.35)	1.55 (1.12–2.15)	4.14 (1.86–9.19)
	1.0	25.76 (13.25)		
	0.2	15.62 (7.47)		
Sodium stibogluconate	45.0	67.05 (12.64)	27.7 (20.7–37.3)	>45
	15.0	29.32 (19.47)		
	5.0	0		

[a] Doses for the control treatment, sodium stibogluconate, are expressed in terms of pentavalent antimony (Sb^V).
[b] CL, confidence limit.
[c] Confidence limits derived in MS Excel.
[d] Confidence limits derived in Prism.

kg/day or 0.64 mg/kg/day of NPC1161B showed no appreciable increase in methemoglobin (Fig. 5). In contrast, dogs treated with NPC1161A had increased levels of methemoglobin starting at day 2 and peaked on day 6 or day 8. The maximum amount of methemoglobin induced by treatment with 1.91 mg/kg/day of NPC1161A was approximately 20% of the total hemoglobin, whereas for 0.64 mg/kg/day it was approximately 8%.

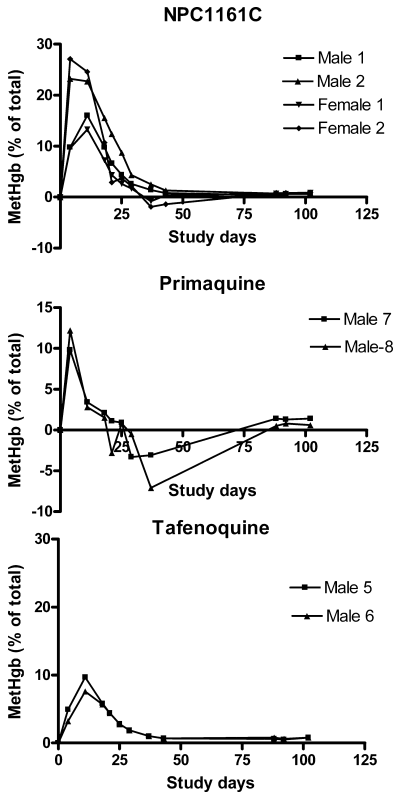

FIG. 4. Methemoglobin formation in dogs treated with NPC1161C succinate, tafenoquine succinate, or primaquine diphosphate, at 0.0116 mmol/kg orally on days 1 to 4. The doses in mg/kg for the salts are as follows: NPC1161C, 6.4; tafenoquine, 6.7; and primaquine, 5.3. Each curve represents data from a single dog.

DISCUSSION

Comparison of the antimalarial activities indicated that the racemic NPC1161C has an activity and toxicity profile in the mouse model superior to those of tafenoquine, which is currently undergoing phase 2 clinical trials (48) as a replacement for primaquine (Table 1). Tafenoquine showed toxicity at the highest dose tested (64 mg/kg/day) and had low activity at lower doses. In contrast, NPC1161C showed a 100% cure rate at all the doses and no observable toxicity, even at the highest doses tested. However, rodents are not good indicators of hematotoxicity, the dose-limiting toxicity of primaquine in humans. Evaluation of NPC1161C with tafenoquine and primaquine for methemoglobinemia in beagle dogs indicated that

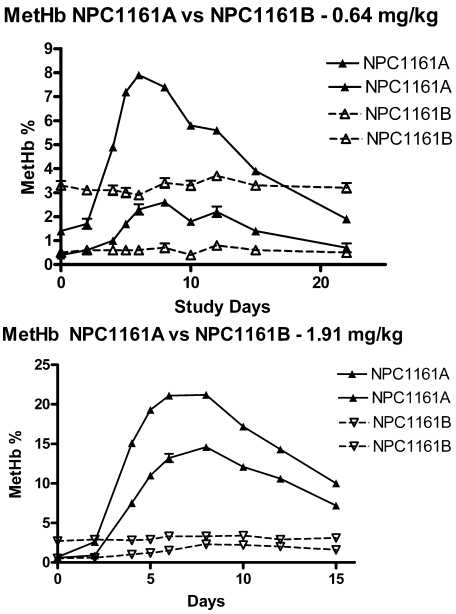

FIG. 5. Methemoglobin formation in dogs treated with NPC1161B or NPC1161A at 0.64 mg/kg of base (upper panel) or 1.91 mg/kg (lower panel) orally on days 1 to 4. Each curve represents data from a single dog, presented as mean + standard deviation of duplicate determinations.

NPC1161C showed a greater propensity for methemoglobinemia. The causal relationship of methemoglobinemia to intravascular hemolysis, which is the hemotoxicity of clinical significance, has not been established, but both are believed to be related to oxidative stress within erythrocytes.

Resolution of the racemate into individual enantiomers and evaluation of their oral antimalarial activities indicated a clear difference in activity profiles for the individual enantiomers. In the blood schizonticidal assay, the (−) enantiomer, NPC1161B, showed activity superior to that of the (+) enantiomer, NPC1161A, or the racemate, NPC1161C. Based on the parasitemias on day 6, NPC1161B appears to be at least 10-fold more active than NPC1161A. In addition to its potent blood schizonticidal activity, NPC1161B also appears to have a long duration of action. A single dose of 2 mg/kg could protect mice from lethal malaria infection if administered from 1 day prior to 2 days after infection. If the drug was administered 2 days prior to infection, 60% of the mice were protected, while the other enantiomer was ineffective.

A similar result was observed for antipneumocystis activity of these compounds. The (−) isomer, NPC1161B, was found to be two to four times as effective as the (+) enantiomer, NPC1161A, in this assay. The (−) enantiomer showed an activity at a dose of 0.25 mg/kg/day comparable to that shown by TMP-SMX at 50/250 mg/kg/day. Comparison of these results with those previously reported (5) for tafenoquine and sitamaquine shows that NPC1161B is about eight times more active than these compounds in this assay.

In the murine antileishmanial assay, NPC1161B also showed better activity than did NPC1161A. Though the differential between the two enantiomers for efficacy is somewhat less pronounced, it is of interest that at very high doses, the more effective enantiomer is also better tolerated by the mice. Comparison of activity showed that NPC1161B is as active as sitamaquine, which is currently in clinical development (17, 21, 37, 49).

Interestingly, though the racemic NPC1161C showed a high level of methemoglobinemia on administration to beagle dogs, resolution of the two enantiomers revealed a differential toxicological profile. NPC1161B, the more active enantiomer in all three infection models (malaria, leishmaniasis, and pneumocystis infection), showed much less methemoglobin toxicity in the dog model than did the less-active enantiomer NPC1161A. The latter would seem to account for the total methemoglobin toxicity caused by low doses of the racemate. The beagle dog has been used as a model for prediction of hematological toxicity because of its high sensitivity. It is generally believed that the sensitivity of humans to methemoglobin-generating drugs is lower. The basis for this is believed to be the marked biochemical differences between dog and human erythrocytes, with the former displaying very low levels of methemoglobin reductase (38) and a complete absence of cytosolic *N*-acetyltransferase activity (46).

The basis of the differential effects of the two enantiomers of NPC1161C has not been established. It appears likely that differences in metabolism or pharmacokinetics would be the most likely explanation; our preliminary evaluations in primates suggest that there are marked differences in the appearance in plasma of the carboxy metabolites of the two enantiomers of NPC1161C (18). Biological activities of individual enantiomers of other 8-aminoquinoline analogs have not yet been reported.

The observed late onset of methemoglobinemia and significantly low in vitro antimalarial (35) and hemolytic (19, 27) activities compared to those in vivo indicate that 8-aminoquinolines require metabolic activation for both antimalarial activity and hematological toxicity. The major human metabolite of primaquine, carboxyprimaquine, has lower antimalarial activity (8) and hemolytic toxicity (27) than does the parent drug. The metabolites that are responsible for activity and toxicity appear to be minor and highly labile. Several probable metabolites of primaquine have been proposed, but none of them have been fully characterized in humans or animal models (3, 12, 13, 19, 27).

The cause of methemoglobinemia and hemolytic toxicity (3, 12, 13, 19, 27), as well as the broad antiparasitic activity of the 8-aminoquinolines (9, 47), has been linked to formation of hydrogen peroxide and reactive oxygen intermediates by primaquine in erythrocytes. However, evidence suggests that these two processes are not directly correlated. Primaquine, which causes significant methemoglobinemia in humans, has very weak blood schizonticidal activity. This current study also demonstrates that the enantiomer which showed lower toxicity has higher blood schizonticidal activity. Primaquine is generally thought to elicit oxidative damage to red cells by phenolic (3, 12, 19, 27) or N-hydroxylated (12) metabolites. Though none of these metabolites have been conclusively demonstrated to play a role in humans or in animal models, synthetically prepared putative metabolites have been shown to be direct-acting hemolytic agents. Prominent among the phenolic metabolites is the 5-hydroxyprimaquine, which has been postulated to form reactive quinone-imines. However, in the case of the 5-aryloxy-substituted analogs, which are less toxic than primaquine but still able to elicit methemoglobinemia, this pathway is not likely relevant, since all available evidence suggests the metabolic stability of the 5-substituents. Perhaps hydroxylation of C-7 on the quinoline nucleus or N hydroxylation of the 8-aminoquinoline moiety after dealkylation of the side chain plays a role. These sites may be differentially influenced, based on the configuration of the asymmetric center in the side chain. Further studies are needed to explore these possibilities.

These results indicate that NPC1161B shows potential as a new drug from the 8-aminoquinoline class with a limited toxicity and enhanced efficacy, compared to those currently in use or under development, for the treatment of several parasitic diseases.

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