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Aminoindoles, a Novel Scaffold with Potent Activity against *Plasmodium falciparum*[▽]†

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This study characterizes aminoindole molecules that are analogs of Genz-644442. Genz-644442 was identified as a hit in a screen of ~70,000 compounds in the Broad Institute's small-molecule library and the ICCB-L compound collection at Harvard Medical School. Genz-644442 is a potent inhibitor of *Plasmodium falciparum in vitro* (50% inhibitory concentrations [IC_{50}s], 200 to 285 nM) and inhibits *P. berghei in vivo* with an efficacy of >99% in an adapted version of Peters' 4-day suppressive test (W. Peters, Ann. Trop. Med. Parasitol. 69:155–171, 1975). Genz-644442 became the focus of medicinal chemistry optimization; 321 analogs were synthesized and were tested for *in vitro* potency against *P. falciparum* and for *in vitro* absorption, distribution, metabolism, and excretion (ADME) properties. This yielded compounds with IC_{50}s of approximately 30 nM. The lead compound, Genz-668764, has been characterized in more detail. It is a single enantiomer with IC_{50}s of 28 to 65 nM against *P. falciparum in vitro*. In the 4-day *P. berghei* model, when it was dosed at 100 mg/kg of body weight/day, no parasites were detected on day 4 postinfection. However, parasites recrudesced by day 9. Dosing at 200 mg/kg/day twice a day resulted in cures of 3/5 animals. The compound had comparable activity against *P. falciparum* blood stages in a human-engrafted NOD-*scid* mouse model. Genz-668764 had a terminal half-life of 2.8 h and plasma trough levels of 41 ng/ml when it was dosed twice a day orally at 55 mg/kg/day. Seven-day rat safety studies showed a no-observable-adverse-effect level (NOAEL) at 200 mg/kg/day; the compound was not mutagenic in Ames tests, did not inhibit the hERG channel, and did not have potent activity against a broad panel of receptors and enzymes. Employing allometric scaling and using *in vitro* ADME data, the predicted human minimum efficacious dose of Genz-668764 in a 3-day once-daily dosing regimen was 421 mg/day/70 kg, which would maintain plasma trough levels above the IC_{90} against *P. falciparum* for at least 96 h after the last dose. The predicted human therapeutic index was approximately 3, on the basis of the exposure in rats at the NOAEL. We were unable to select for parasites with >2-fold decreased sensitivity to the parent compound, Genz-644442, over 270 days of *in vitro* culture under drug pressure. These characteristics make Genz-668764 a good candidate for preclinical development.

Malaria continues to be a major global health burden, endemic in 87 countries with 2.5 billion people at risk (11). Widespread resistance to current antimalarials such as chloroquine (29, 40), atovaquone (33), pyrimethamine (39), and sulfadoxine (37) and, more recently, reduced efficacy of artemisinin derivatives (7, 38) emphasize the urgent need for new antimalarial drugs. At least in part because the majority of people affected live in the poorest parts of the world, the response of the pharmaceutical industry has been sparse or irregular. New collaborations combining the expertise of academia, industry, and public-private partnerships have been suggested to overcome these obstacles, and it is in that spirit

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that the Broad Institute, Genzyme Corporation, and Medicines for Malaria Venture have sought to develop novel therapies for malaria.

The aminoindole Genz-644442 was identified as a hit in a screen of ~70,000 compounds in the Broad Institute's small-molecule library and the ICCB-L compound collection at Harvard Medical School, showing 50% inhibitory concentrations (IC_{50}s) of 200 to 285 nM against *Plasmodium falciparum* blood stages *in vitro* (2, 36). *In vivo*, in an adapted version of the 4-day suppressive test (27, 28), Genz-644442 inhibited *P. berghei* with an efficacy of >99% (36). Subsequent *in vitro* studies showed that selectivity, drug-like properties, and metabolic stability were promising (see below). On the basis of these results and initial exploratory structure-activity relationship (SAR) analysis, the aminoindole series became the focus of hit-to-lead medicinal chemistry optimization which resulted in selection of Genz-668764 as a candidate for preclinical development. We demonstrate that this compound shows significantly greater potency against *P. falciparum* than the parent, is well tolerated in mice and rats, and can cure *P. berghei* infections.

MATERIALS AND METHODS

Chemical synthesis. Details of chemical synthesis are presented in the supplemental material and reference 36.

***In vitro P. falciparum* viability assay.** Potency against *P. falciparum in vitro* was assessed using a modified version of the method of Plouffe and coworkers (30). Parasites were cultured in the presence of drug in RPMI 1640 (Sigma) containing 4.16 mg/ml lipid-rich bovine serum albumin (Albumax II) in a total volume of 50 μl at a 2.5% hematocrit and an initial parasitemia of 0.3% in black Greiner GNF clear-bottom plates. Cultures were incubated for 72 h at 37°C under 95% N_2, 4% CO_2, and 3% O_2. At the end of the incubation, SYBR green was added to a dilution of 1:10,000 and plates were stored overnight (or until they were ready to be read) at −80°C. Just before they were read, plates were centrifuged at 700 rpm and fluorescence was read using 480-nm excitation and 530-nm emission frequencies. Compound concentrations that inhibit parasite replication reduce the fluorescence intensity of SYBR green bound to parasite DNA.

***In vitro P. knowlesi* viability assay.** Selected compounds were tested against *P. knowlesi* H1 strain parasites cultured in *Rhesus* blood cells as a surrogate for *P. vivax* infections as previously described (19). Briefly, *P. knowlesi* parasites were cultured in 2% *Rhesus* macaque erythrocytes (New England Primate Research Center) in RPMI 1640 culture medium containing 10% human type O-positive serum (Interstate Blood Bank). Schizont-stage parasites were purified by flotation in 60% Percoll (GE Life Sciences) and allowed to reinvade to generate a synchronous population of ring-stage parasites. Drug assays were performed by plating ring-stage parasites at 0.5% parasitemia in triplicate in RPMI 1640 containing 2.5 μg/ml hypoxanthine. Parasites were incubated for 24 h with serially diluted test compounds. After 24 h, thin smears were made to confirm that reinvasion had occurred, 0.5 μCi ^{3}H-labeled hypoxanthine (Perkin Elmer) was added to each well, and parasites were allowed to progress through S phase to early schizonts. Cells were then harvested via glass filter plates, and ^{3}H incorporation was measured by scintillation counting. Values were normalized to a percentage of the values for controls containing no drug, and IC_{50}s were generated (GraphPad Prism).

***In vitro P. berghei* viability assay.** Selected compounds were tested against *P. berghei* in an *in vitro P. berghei* viability assay using transgenic parasites containing a luciferase gene (8). This assay measures luciferase activity (luminescence), using a luciferase assay system kit (Promega), from transgenic blood-stage parasites that express LucIAV luciferase under the control of the schizont-specific (*ama-1*) promoter (transgenic parasite RMgm-32; http://www.pberghei.eu/index.php?rmgm = 32). In these studies, ring-stage parasites (13) were incubated for 24 h at 37°C in 24-well plates in complete culture medium (RPMI 1640 with 20% fetal calf serum [13]) containing serial dilutions of drugs. Synchronized ring-form parasites for these assays were prepared as previously described (14). Briefly, cultured and purified schizonts/merozoites (13) were injected intravenously (i.v.) into the tail veins of mice. In these animals, merozoites invade within 4 h after injection, giving rise to synchronized *in vivo* infections with a parasitemia of 0.5 to 3% containing mainly (>90%) ring-form parasites. Infected blood collected by cardiac puncture 4 h after the injection of the purified schizonts/merozoites was washed once with complete culture medium by centrifugation (450 × *g*, 8 min), and then erythrocytes were resuspended in complete culture medium at 1 to 3% parasitemia and 1% hematocrit and were mixed with serial solutions of the drugs in complete culture medium (final volume, 1 ml/well in 24-well plates). Plates were incubated for 24 h under standardized *in vitro* culture conditions (14), allowing the ring forms/young trophozoites to develop into mature schizonts. After incubation, 250 μl of the cell suspension from each well was transferred to Eppendorf tubes and cells were pelleted by centrifugation (13,000 × *g* for 1 min). Supernatant was removed and cells were lysed by addition of 100 μl cell culture lysis reagent (luciferase assay system kit; Promega). Ten microliters of lysate was transferred to wells of a 96-well plate, 100 μl luciferase assay substrate (luciferase assay system kit; Promega) was added, and the luminescence spectrum of each well was read for 10 s using a multiplate reader (Wallac 1420 multilabel counter; Perkin Elmer). Measurements of luciferase activity were expressed in relative light units (RLUs) and represent the average of triplicate samples at each drug dilution. Wells containing phosphate-buffered saline (PBS) (instead of infected cells) were used as negative controls.

Mammalian cytotoxicity. Compounds were tested at 10 dilutions against normal human renal proximal tubule cells (CC-2553; Clonetics) and dermal fibroblasts (normal human dermal fibroblasts [NHDFs]; CC-2509; Clonetics) by incubation for 4 days, until cell confluence. Viability was measured using the alamarBlue assay (Trek Diagnostic Systems). Cytotoxicity IC_{50}s were required to be ≥20-fold the potency against *P. falciparum* in the *in vitro* assay as a criterion for advancing a compound through distribution/metabolism/pharmacokinetic (DMPK) assays.

Erythrocyte lysis. Compounds were tested at 10 dilutions against fresh human erythrocytes at 1% hematocrit in Dulbecco's PBS in V-bottom plates for 24 h at 37°C. Plates were then centrifuged at 2,000 rpm for 5 min, and 50 μl of supernatant was transferred to a fresh flat-bottom plate. Hemoglobin was measured using a QuantChem hemoglobin assay kit (DIHB-250; BioAssay Systems).

***In vitro* absorption, distribution, metabolism, and excretion (ADME) properties.** Solubility, passive permeability, metabolic stability, log of the distribution coefficient (*D*), percent protein bound, and cytochrome P450 (CYP450) assays were performed as previously described (5).

hERG channel activity. CHOK1 cells that stably overexpress the hERG ion channel were tested in a whole-cell voltage clamp procedure at Genzyme by measuring the ability of a drug to inhibit the peak current flowing through the hERG channel upon depolarization of the membrane potential. Initially, recordings were made in the presence of control saline to establish a baseline (zero inhibition). This was followed by addition of four increasing concentrations of test drug. Each drug was tested in at least three different cells ($n \geq 3$), and the effect of each drug concentration was compared with the baseline to obtain a percent inhibition value. The dose-dependent percent inhibition was fitted by a Blotzmann function to generate a dose-response curve for the compound, and its IC_{50} was determined.

***In vitro* resistance selection.** Studies to select for resistance were performed utilizing successive cycles of drug exposure in which parasites were exposed to drug for 1 week at 10× the IC_{50}, followed by 2 weeks of culture in the absence of drug. *P. falciparum* was grown in four T75 flasks containing 1×10^9 Dd2 parasites (5% hematocrit; 5% parasitemia in 25 ml of medium). Test compound was added at 10× the IC_{50} and maintained during cell feeding every other day for a week; parasitemia was monitored daily by microscopy. During periods of drug exposure, parasites were generally not detectable after the 4th day. Following 1 week of exposure, drug pressure was removed and cultures were fed every other day until parasites reemerged (1 to 3 weeks). Drug pressure (10× IC_{50}) was then resumed, repeating the same protocol. Periodically, parasites were removed and assayed for altered susceptibility to the test compound. For Genz-644442, this exposure cycle was repeated ~10 times over 270 days; for Genz-668764 this was done for ~3 cycles (65 days).

***In vivo* studies: tolerability/plasma exposure.** To guide dose selection for efficacy studies, tolerability studies were conducted at Genzyme in CD-1 mice. Protocols were IACUC approved, and animals were housed in AAALAC-accredited facilities. Compounds were dosed orally (p.o.) twice, 8 h apart, to groups of 3 female 4-week-old CD-1 mice at 50, 100, or 200 mg/kg of body weight/day, in addition to a vehicle control group. The aminoindole analogs were formulated in 1.6% lactic acid mixed sodium salt, 1% Tween 80, 9% ethanol, and 20% hydroxypropyl β-cyclodextrin (Cerestar; Wacker Chemie) in water. Animals were observed for signs of overt toxicity/poor tolerability every 15 min for the first hour postdosing and then hourly for up to 4 h after the first dose. Blood was collected 1 h after each dose and 18 h after the final dose to measure plasma concentrations.

PK studies. Pharmacokinetic (PK) evaluation of compounds was first conducted in mice (the host species for the *P. berghei* efficacy model) and then for

selected compounds in rats, dogs, and monkeys. For studies in mice, the in-life portion was performed at Mispro Biotech Services, Inc., Montreal, Quebec, Canada. For dog studies, the in-life portion was performed at Absorption System, San Diego, CA, while rat and monkey studies were performed at Xenometrics, Stillwell, KS. All of these laboratories utilized IACUC-approved protocols and are AAALAC-approved facilities. Bioanalytical work was performed at Genzyme. In mice, once-per-day (QD) or twice-per-day (b.i.d.) dosing studies were performed using a group of 3 male CD-1 mice per dosing group. The test article was administered following a 2-h fast, and mice were given food access approximately 45 min postadministration. At specified intervals (0.083, 0.25, 0.5, 1, 2, 6, and 24 h) blood (25 to 30 μl per time point) was collected into K_2EDTA and processed to plasma. In the rat study, three (group 1) and two (group 2) Sprague-Dawley male rats (6 to 8 weeks old) were administered the test article at a dose of 3 mg/kg via oral gavage and a dose of of 1 mg/kg i.v., respectively. Vehicle was 0.5% lactic acid, 0.89% sodium lactate, 0.95% Tween 80, 4.5% ethyl alcohol, 9.5% hydroxypropyl β-cyclodextrin, and 83% water. The dose volumes were 3 ml/kg and 2 ml/kg for the p.o. and i.v. groups, respectively. Each animal was weighed and dosed following an overnight fast. Following dose administration, whole-blood samples (approximately 250 μl) were collected via a jugular vein catheter from each animal at the following time points: predose and 0.033 (i.v. only), 0.083, 0.167 (p.o. only), 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h postdose. Food was returned to the animals at approximately 1 h postdose. Whole-blood samples were collected into K_2EDTA and processed to plasma. For 0- to 24-h intervals following dose administration, each of the animals was housed in a metabolism cage to collect urine.

Dog PK studies were conducted in a crossover design (3-day washout between two p.o. doses and a 7-day washout for the i.v. dose). Three beagle male dogs (2 to 4 years of age, body weight of 8 to 12 kg at dosing, and at least 2 weeks free of any treatment prior to this study initiation) were administered the test article at 2 or 3 ml/kg for the p.o. doses and 1 ml/kg for the i.v. dose. Each animal was weighed and dosed following an overnight fast. Following dose administration, whole-blood samples (approximately 0.6 ml) were collected via from the jugular, cephalic, or saphenous vein from each animal at the following time points: predose and 0.083, 0.167, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 12, 16, 24, 36, 48, and 72 h postdose. Food was returned to the animals at approximately 4 h postdose. Whole-blood samples were collected into K_2EDTA and processed to plasma. During the 24 h prior to and 0- to 4-h, 4- to 8-h, 8- to 24-h, 24- to 48-h, and 48- to 72-h intervals following dose administration, each of the animals was housed in a metabolism cage to collect urine. To evaluate pharmacokinetics in nonhuman primates, three cynomolgus male monkeys (weight, 4 to 6 kg) were used in a crossover study design similar to that use for the dog study. Genz-668764 was administered at 1 mg/kg (i.v.) and 5 or 20 mg/kg (p.o.) in the same vehicle formulation used for the rat PK study.

PK studies on selected compounds were also conducted in *P. berghei*-infected mice. Malaria infection has been shown to alter quinine protein binding (20), reduce metabolic clearance of dihydroartemisinin (3), and affect the elimination half-life of primaquine, so it was important to determine whether metabolism/availability of the aminoindole compounds is also influenced by infection.

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was employed for the analysis of samples from all PK studies described above.

Seven-day safety study in rats. The in-life portion of the 7-day safety study in rats was performed at Lab Research, Inc., Laval, Quebec, Canada, using IACUC-approved protocols in AAALAC-accredited facilities. Four groups of 8 rats were dosed orally with vehicle alone (0.5% methylcellulose in water) or with Genz-668764 (the more active enantiomer from the Genz-666984 racemic mixture) at 100, 200, or 300 mg/kg/day QD for 7 days. Within each dosing group, animals were divided into safety assessment (5 animals) and toxicokinetic (TK) groups (3 animals). Clinical observations were made twice daily, and body weights were measured before treatment; at days 3, 6, 8, 9, and 13; and at final necropsy. Animals in the 100- and 200-mg/kg/day treatment groups were necropsied on day 8, as were 5 animals in the control and 300-mg/kg/day groups. Clinical chemistry and hematology parameters were assessed, while bone marrow, kidney, liver, and lymph nodes were observed for gross abnormalities and then were analyzed for histopathology. The remaining 3 animals in the 300-mg/kg/day and control groups were observed for an additional 7 days posttreatment and then were necropsied and analyzed as described above. To assess TK, blood samples (approximately 0.4 ml) were collected from the jugular vein into tubes containing K_3EDTA at the following time points: 0.5, 1, 2, 8, and 24 h after dosing on study days 1 and 7. Serial blood samples were collected from 3 animals/group/time point and were processed to plasma for subsequent LC-MS/MS analysis.

***In vivo* efficacy studies, *P. berghei*.** The *P. berghei* acute-infection model in rodents is adapted from Peters' 4-day suppressive test (27, 28). Most compounds were tested at the University of Puerto Rico (UPR) against parasites of the *P. berghei* N strain; however, in select cases, compounds were also tested at the Swiss Tropical and Public Health Institute (SwissTPH) against *P. berghei* ANKA strain parasites to confirm comparability of data across different laboratories and to examine efficacy against different parasite strains.

At UPR, all studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (24) and the Public Health Service (PHS) guidelines under protocols approved by the IACUC of the Medical Sciences Campus, University of Puerto Rico, and all work was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (24) and regulations of the PHS Policy on Humane Care and Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/references/phspolicylabanimals.pdf). Animals were maintained according to NIH guidelines and were allowed to acclimatize for 1 week prior to the commencement of studies. On day 0, groups of 4- to 6-week-old female Swiss albino mice ($n = 5$) were infected by tail vein injection with 0.2 ml heparinized blood diluted to contain 1×10^7 N-clone parasites. The aminoindole analogs were formulated in 1.6% lactic acid mixed sodium salt, 1% Tween 80, 9% ethanol, and 20% hydroxypropyl β-cyclodextrin (Wacker Chemie), and the formulations were administered by oral gavage. On day 0, a single dose was given at 6 h after initial infection, and over the subsequent 3 days the dose was split and administered b.i.d., with 6 h between doses. Animals in the control group received vehicle alone. Dose concentration and frequency of dosing were based upon preliminary tolerability/exposure studies. On day 4 postinfection (5th day of assay), blood was collected by tail nick, and thin-smear microscope slides were prepared and stained using Diff Quick stain. Parasitized erythrocytes were counted and compared with the total number of erythrocytes per microscopic field to determine the percent parasitemia. A minimum of 350 erythrocytes was counted. Animals showing no detectable parasites on day 4 were examined every 2 to 3 days to determine whether cure was sterile. Animals with no detectable parasites 28 days after cessation of dosing (study day 33) were considered cured; animals were euthanized at the end of the study.

In vivo studies at the Swiss Tropical and Public Health Institute were performed under a protocol reviewed and approved by the local veterinary authorities of Canton Basel-Stadt, Switzerland. NMRI mice were infected with 2×10^7 ANKA strain GFP MRA-865 parasites containing a constitutively expressed *GFP* gene (transgenic parasite RMgm-5; http://www.pberghei.eu/index.php?rmgm=5) (9). Dosing was twice/day by oral gavage, using the same formulation as that used at the University of Puerto Rico. Parasitological assessments were made by resuspending 1 μl tail blood in 1 ml PBS buffer and counting fluorescent cells using a FACScan apparatus (Becton Dickinson), examining a total of 100,000 red blood cells. Animals that had no detectable parasites on day 4 were followed through day 30 before being deemed cured of infection. In the SwissTPH study, 100-μl blood samples were collected at 5 time points to assess plasma exposure.

***In vivo* efficacy studies, *P. falciparum*.** Genz-668764 was tested for efficacy against *P. falciparum* $Pf3D7^{0087/N9}$ growing in immunodeficient NOD-*scid IL-2R*γ^{null} (NSG) mice (generously made available through a collaboration with Leonard D. Shultz from the Jackson Laboratory) engrafted with human erythrocytes (generously provided by the Spanish Red Cross Bank in Madrid, Spain) (1, 17). Briefly, groups of 3 animals were infected intravenously on day 0 with 2×10^7 *P. falciparum* parasites and were treated orally once per day for 4 days with 5, 25, and 100 mg/kg/day formulated as described above beginning on day 3 postinfection. In parallel, Genz-668764's efficacy was also tested against *P. berghei*, with nonengrafted NSG mice infected on day 0. Groups of 3 animals were infected intravenously on day 0 with 1×10^7 *P. berghei* ANKA strain parasites on day 0 and received the same doses beginning 1 h after infection (18). Parasitemia was assessed by fluorescence-activated cell sorter analysis as previously described (16). All the experiments performed were approved by the Diseases of the Developing World (DDW) Ethical Committee on Animal Research, performed at the DDW Laboratory Animal Science facilities accredited by AAALAC, and conducted according to European Union legislation and GlaxoSmithKline policy on the care and use of animals.

Pharmacokinetic studies, infected mice. Single-dose PK evaluation in infected NSG mice was also performed to determine the time course of Genz-668764 clearance in these animals. Groups of 3 erythrocyte-engrafted NSG mice were infected with *P. falciparum* via oral gavage as described above. Blood samples were collected at 5, 15, and 30 min and 1, 3, 6, 8, 24, and 48 h after administration, were mixed 1:1 with 0.1% saponin and water for lysis, and were stored at −70°C until LC-MS/MS analysis. The same experimental design was followed to assess the compound's clearance in nonengrafted NSG mice administered Genz-668764 1 h after infection with *P. berghei* as described above.

RESULTS

Identification and characterization of Genz-644442. The aminoindole Genz-644442 was identified as a hit in a screen of ~70,000 compounds tested against *P. falciparum* strains Dd2 and 3D7 (2, 36). *In vitro* potency of Genz-644442 ranged from 285 nM (Dd2) to 200 nM (3D7). This activity against *P. falciparum* was selective since cytotoxicity IC_{50}s against mammalian cells were 800-fold higher than the IC_{50}s against *P. falciparum* (Table 1). *In vitro* ADME assays (Table 2) showed that Genz-644442 was quite soluble, highly membrane permeant, and stable in liver microsomes and hepatocytes from rats and humans. In addition, the IC_{50} against the major human drug-metabolizing CYP450 enzymes was >5 μM for all isozymes tested, indicating a low likelihood of significant drug-drug interactions mediated by CYP450. On the basis of these characteristics (Tables 1 and 2), the aminoindole Genz-644442 was analyzed further for tolerability and plasma exposure in mice, for efficacy against *P. berghei* in mice, and for PK properties. In initial studies for tolerability and efficacy of Genz-644442, we used intraperitoneal (i.p.) administration to ensure systemic exposure. At 50 mg/kg/day there was complete suppression of parasitemia with no detectable parasites on day 4 (see Fig. S1A in the supplemental material), while dosing at 25 and 15 mg/kg/day suppressed parasitemia by 86% and 54%, respectively, resulting in a 50% effective dose (ED_{50}) of 15 mg/kg/day. However, all mice dosed with 50 mg/kg/day showed recrudescent parasites by day 9 (see Fig. S1B in the supplemental material), indicating that this dose did not result in cure of the infection.

Enantiomers of Genz-644442. Because of the promising characteristics of Genz-644442, the aminoindoles became the focus of medicinal chemistry optimization, generating 321 novel compounds.

The medicinal chemistry approach (C. Celatka et al., submitted for publication) consisted primarily of exploring various aspects of the structure to identify essential features while improving potency and metabolic stability of the new compounds. These molecules were initially synthesized and tested as racemates around the 3 position of the indole ring of Genz-644442 (Table 1); therefore, it was essential to determine potency and metabolic stability of individual enantiomers. Genz-644442 was synthesized as described above, and the 2 enantiomers (Genz-665848 and Genz-665849) were resolved by supercritical chiral chromatography (see the supplemental material). Genz-665848 and Genz-665849 were screened for *in vitro* potency against *P. falciparum* and *P. berghei*, and the IC_{50}s were in the same range (Table 1). However, when it was tested against *P. berghei in vivo* at 25 or 50 mg/kg/day (i.p.) for 4 days, Genz-665848 was far less effective than Genz-665849 in suppressing parasitemia (see Fig. S2A to C in the supplemental material). At 50 mg/kg/day, Genz-665849 suppressed parasitemia to undetectable levels in all 5 mice, and 2 mice were cured (see Fig. S2C in the supplemental material). No cure was obtained with Genz-665848. When Genz-665849 was dosed orally (see Fig. S3 in the supplemental material), efficacy in suppressing parasitemia and achieving cures was similar to that obtained using i.p. dosing (see Fig. S2 in the supplemental material). The increased efficacy of Genz-665849 appears to be due to greater plasma exposure compared to that of Genz-665848 (see Fig. S2D in the supplemental material) and lower plasma clearance (i.e., 8 ml/min/kg) than that of Genz-665848 (i.e., 37 ml/min/kg) in mice.

Screening analogs of Genz-644442: identification of lead compound Genz-668764. Observed differences in metabolic stability, *in vivo* systemic clearance, and efficacy of individual enantiomers shaped the flow of the medicinal chemistry lead optimization effort (C. Celatka et al., submitted for publication). Compounds were first screened against *P. falciparum in vitro* (Tables 1 and 2) and subsequently *in vivo* against *P. berghei* using 2 doses/compound (usually 100 and 200 mg/kg/day b.i.d.; Table 3). Compounds showing the best activity and *in vitro* metabolic properties (Table 2) were synthesized at a large scale (50 g), and enantiomers were separated by supercritical chiral chromatography for PK studies and for additional efficacy studies. These studies resulted in selection of Genz-668764 (enantiomer of Genz-666984) for further characterization on the basis of *in vivo* efficacy and preliminary safety studies (see below). This compound showed low IC_{50}s against *P. falciparum*, *P. berghei*, and *P. knowlesi in vitro* (Table 1). *P. knowlesi* infects both humans and nonhuman primates (15) and is used for *in vitro* drug susceptibility testing as a surrogate for the human parasite *P. vivax* (6, 19).

Pharmacological properties of Genz-668764. Pharmacokinetic parameters of the lead compound Genz-668764 in mice, rats, and cynomolgus monkeys are shown in Table 4. Clearance in rodents was high (57.6 and 45.8 ml/min/kg for mouse and rat, respectively) compared to that in monkeys (13.1 ml/min/kg), while the half-life ranged from 7.3 h (mouse) to 4.3 h (monkey). Bioavailability ranged from 20% (monkey) to 33% (rat). Pharmacokinetic studies of Genz-668764 were performed in both infected and uninfected mice (Fig. 1). The dose levels and administration were not identical between the 2 studies: infected animals received a single oral dose of 50 mg/kg, while uninfected animals received 2 split doses of 27.5 mg/kg 7 h apart via oral gavage. However, no significant differences in area under the curve (AUC) were observed. At 24 h postdosing, plasma concentrations ranged from 70 to 100 ng/ml by 24 h after dosing (Fig. 1), which is only ~2 times the ED_{90} for *P. falciparum in vitro*.

***In vitro* safety assessment.** Genz-668764 was tested in the ExpressS (Cerep, Redmond, WA) panel of 55 primary molecular targets at 10 μM. In only 3 cases (κ [KOP, kappa opioid receptor], 93%; μ [MOP, mu opioid receptor], 52%; and rat sodium channel, site 2, 71% inhibition) was inhibition of >50% observed, suggesting a low likelihood of off-target effects. Ames tests were negative, indicating no genotoxicity. Genz-668764 was also tested for hERG inhibition; the IC_{50} was 36.6 μM for Genz-668764 (Table 1), suggesting a low potential for arrhythmia for *in vivo* inhibition of hERG, based on human efficacious dose predictions.

Preliminary *in vivo* safety assessment. Three aminoindoles were evaluated in a preliminary 7-day rat safety study (see Table S2 in the supplemental material). Because Genz-666984-treated rats showed fewer clinical adverse findings than vehicle-treated controls, the enantiomer Genz-668764 was tested in a preliminary safety study in rats. Treatment for 7 days with 300 mg/kg/day Genz-668764 resulted in a 70% reduction in reticulocytes (Fig. 2A; $P \leq 0.01$, Dunnett's test). However, reticulocytes rebounded during the 8 days following cessation

TABLE 1. *In vitro* potency and selectivity

Genz no.	Structure[a]	Potency (IC_{50} [nM])				Selectivity[b]			
						Mammalian cytotoxicity (IC_{50} [μM])			hERG concn (μM)
		P. falciparum Dd2	*P. falciparum* 3D7	*P. knowlesi* H1	*P. berghei* ANKA	Dermal fibroblast	Kidney epithelial	Eryth. lysis	CHO
644442		285	200	336	1,560	>242	>242	>155	>25
665848	644442 enant	1,460	665	ND[c]	1,363	>242	>242	>155	ND
665849	644442 enant	675	313	ND	1,689	>242	>242	>155	ND
649969		37	30	ND	ND	24	24	>129	ND
665570		455	183	318	ND	>62.5	>62.5	>168	ND
665571		110	38	ND	ND	>62.5	>62.5	>147	ND
665572		347	108	190	2,987	>62.5	>62.5	>178	ND
667066		37	22	90	ND	>188	>188	>120	19
667664		81	21	ND	ND	>195	>195	>125	ND
666984		49	23	38	ND	>195	>195	>125	21
668762	666984 enant	58	18	ND	342	>195	>195	>125	ND
668764	666984 enant	65	28	26	331	>195	>195	>125	36.6

[a] The absolute stereochemistry of each enantiomer is currently being determined. 644442 enant, compound is an enantiomer of Genz-644442; structures are shown for the racemic mixtures.
[b] Dermal fibroblast, normal human dermal fibroblasts (CC-2509; Clonetics); kidney epithelial, normal human renal proximal tubule cells (CC-2553; Clonetics); Eryth. lysis, erythrocyte lysis.
[c] ND, not done.

TABLE 2. *In vitro* absorption, distribution, metabolism, and excretion properties[a]

Genz no.	Sol. μg/ml	Perm. (10^{-6} cm/s)	Log *D*	CL_{pred} (ml/min/kg)				Human CYP inhibition IC_{50} (μM)					% prot bnd	
				Human		Rat		1A2	2C19	2C9	2D6	3A4	Hum	Mse
				μsom	hep	μsom	hep							
644442	>35	59	3.0	8	7	32	<12	>5	>5	>5	>5	>5	89	93
665848	>35	48	2.8	12	8	34	22	>5	>5	>5	>5	>5	89	86
665849	>35	54	2.7	11	6	25	15	>5	>5	>5	>5	>5	85	90
649969	28	40	3.6	8	9	41	21	>5	>5	>5	>5	3.2	97	97
665570	20	63	2.3	7	6	40	ND	>10	>10	>10	>10	>10	78	74
665571	26	60	2.8	8	10	27	30	>5	>5	>5	>5	>5	84	86
665572	20	72	2.4	6	8	17	16	>5	>5	>5	>5	>5	67	69
667066	>40	111	3.1	9	13	34	21	>5	4.6	>5	>5	1.0	70	76
667664	15	37	2.2	8	6	33	ND	>10	>10	>10	>10	>10	70	76
666984	>40	47	3.1	11	9	32	18	>10	>10	>10	>10	4.6	89	92
668762	>40	43	3.0	9	8	39	ND	>10	>10	>10	>10	>10	89	94
668764	>40	43	3.0	10	<4	33	ND	>10	>10	>10	>10	2.2	91	94

[a] Sol., solubility; Perm., permeation in parallel artificial membrane permeation assay (PAMPA) membranes; CL_{pred}, predicted clearance; μsom, liver microsomes; hep, hepatocytes; % prot bnd, percent protein bound in plasma; Hum, human plasma; Mse, mouse plasma; ND, not done.

of dosing and by study day 15 exceeded levels found in control animals ($P \leq 0.01$). Treatment with 200 mg/kg/day resulted in a more modest reduction in reticulocytes on day 8 (33%; $P \leq 0.05$). Animals in the 300-mg/kg/day group showed a reduced rate of weight gain compared with controls ($P \leq 0.05$; Fig. 2B); however, the rate of weight gain mirrored that of controls following cessation of dosing. Liver weights were somewhat elevated in all treatment groups in a dose-dependent fashion on day 8 (Fig. 2C); however, by day 15, liver weights in the 300-mg/kg/day group returned to those found in control animals. On day 8, histopathology showed minimal hepatocellular hypertrophy in all animals treated with 200 or 300 mg/kg/day Genz-668764 and in one animal treated with 100 mg/kg/day, but again, by day 15 liver histology of treated animals became indistinguishable from that of controls. Animals treated with 200 or 300 mg/kg/day Genz-668764 showed significant ($P \leq 0.01$) thymus weight reductions of 35% and 44%, respectively, on day 8 (Fig. 2D). In some animals dosed at ≥200 mg/kg/day, mild lymphoid atrophy was observed on day 8, but by day 15 no differences in thymus weight or atrophy were observed in treated versus control animals. On the basis of all these findings, the no-observable-adverse-effect level (NOAEL) for Genz-668764 in rats is considered to be 200 mg/kg/day.

TABLE 3. *In vivo* efficacy of aminoindoles against *P. berghei*

Compound	Dose (mg/kg/day)[a]	% suppression on day 5[b]	Average day of recrudescence[c] (no. of animals/ total no.)	No. of animals cured[d]/ total no.
644442	50	100	D13 (5/5)	0/5
665848	50	80	NA[e]	0/5
665849	50	100	D12 (3/5)	2/5
	100	100	(0/5)	5/5
665570	100	100	D17 (2/5)	3/5
	200	100	(0/5)	5/5
665572	50	98	D10 (3/5)	0/5
	100	99	D13 (3/5)	0/5
667066	100	99	D6 (1/5)	0/5
667664	100	95	NA	0/5
	200	98	D17 (2/5)	0/5
666984	100	99	D11 (1/5)	0/5
	200	99	D13 (1/5)	3/5
668762	100	81	NA	0/5
668764	100[f]	>99	D14 (3/5)	2/5
	200[g]	100	D12 (2/5)	3/5

[a] The dose was administered orally in 2 split doses/day to groups of 5 mice.
[b] % suppression day 4, percent reduction in parasitemia compared with mean of untreated controls on day 4, approximately 17 h after the final dose. Values of 100% suppression indicate that no parasites were detected upon microscopic examination; animals may still contain parasites below the limit of detection.
[c] Average day of recrudescence is the average study day on which parasites were detected, preceded by the letter D. Numbers in parentheses are the number of animals in the dosing group that had recrudescent parasites/total number of animals.
[d] No. cured, number of animals with no detectable parasites through day 30 as a proportion of all the animals in the dosing group.
[e] NA, not applicable because all animals were parasitemic on day 4.
[f] Study with ANKA strain.
[g] Study with N clone of *P. berghei*.

Results from the toxicokinetic assessment of Genz-668764 in the 7-day preliminary safety assessment study are shown in Table 5. Drug appears to accumulate, based on the comparison of maximum concentration in plasma (C_{max}) and AUC within each dose treatment group for the 200-mg/kg and 300-mg/kg dose groups on day 1 versus day 7. The C_{max}s were approximately 1.8- and 2.3-fold higher on day 7 than on study day 1 for the 200-mg/kg and 300-mg/kg dose groups, respectively. The areas under the curve from time zero to 24 h (AUC_{0-24}s) were approximately 1.4- and 2.1-fold higher on day 7 than on study day 1 for the 200-mg/kg and 300-mg/kg dose groups, respectively.

***In vivo* efficacy of Genz-668764 against *P. berghei* and *P. falciparum*.** The efficacy of compounds was determined in the 4-day suppressive test. Mice infected with the *P. berghei* N clone were dosed orally twice/day with the enantiomers Genz-668762 and Genz-668764, and parasitemia was assessed on day 4 postinfection. The ED_{50} of Genz-668764 (50 mg/kg/day) was lower than that of Genz-668762 (72 mg/kg/day) (see Fig. S4A and B in the supplemental material). Similarly, Genz-668764 had a higher cure rate. All mice treated with 100 mg/kg/day of Genz-668762 were still parasitemic on day 4, whereas 2 animals treated with the same dose of Genz-668764 were free of parasites on day 4 and did not show recrudescent parasites until day 14 (see Fig. S4C in the supplemental material).

An additional study was performed with *P. berghei* ANKA

TABLE 4. Pharmacokinetic parameters of Genz-644442 and Genz-668764

Parameter[a]	Genz-644442			Genz-668764		
	Mouse	Rat	Dog	Mouse	Rat	Monkey
CL (ml/min/kg)	18.1	29.0 ± 18.7[b]	6.32 ± 0.32	57.6	45.8 ± 0.8	13.1 ± 3.2
CL_{renal} (ml/min/kg)	NA[c]	0.151 ± 0.050	0.00657 ± 0.00615	NA	0.121 ± 0.040	0.0162 ± 0.0126
V_{ss} (liters/kg)	5.32	4.73 ± 1.89	2.81 ± 0.15	13.5	6.47 ± 0.14	2.48 ± 0.52
$t_{1/2}$ (h)	4.21	6.30 ± 3.65	7.34 ± 1.00	7.30	2.58 ± 0.01	4.26 ± 0.14
F (%)	NA	23 ± 5	80 ± 25	NA	33 ± 8	21 ± 6

[a] CL, calculated rate of clearance of compound from plasma; CL_{renal}, clearance through kidney; V_{ss}, steady-state volume of distribution; $t_{1/2}$, terminal half-life of the compound; F, oral bioavailability.
[b] Values are standard errors of the means.
[c] NA, not available.

strain parasites. In this experiment, cures were observed in 3 out of 5 mice when they were dosed at 100 mg/kg/day b.i.d. (Fig. 3A), indicating that parasites of the ANKA strain are somewhat more sensitive to Genz-668764 than parasites of the N clone, where we did not observe cures at the same dose.

Efficacy of Genz-668764 was also tested in the human-engrafted *scid* mouse model that supports growth of *P. falciparum* erythrocytic stages. This study allows direct *in vivo* comparison of efficacy against the rodent parasite *P. berghei* and the human parasite *P. falciparum* in the same murine system. We observed an ED_{50} of 40 mg/kg/day against *P. falciparum*, which was slightly less efficacious than the ED_{50} of 26 mg/kg/day against *P. berghei* ANKA (Fig. 3C; Table 6). This lower efficacy for *P. falciparum* was unexpected, since *in vitro* the blood stages of *P. falciparum* show 4- to 10-fold higher susceptibility than *P. berghei* blood stages (Table 1).

Human PK parameters and efficacious dose prediction. A variety of human PK prediction approaches was employed (12). The predicted human systemic plasma clearance and volume of distribution ranged from 0.5 to 10 ml/min/kg and 1 to 5 liters/kg, respectively. The highest degree of uncertainty was in the human clearance prediction. Given the reasonable *in vitro-in vivo* correlation between *in vitro* metabolic stability in liver microsomes and hepatocytes and *in vivo* clearance in preclinical species and differences in metabolism between human and preclinical species, we utilized the clearance value predicted from the *in vitro* human microsomes and hepatocytes, which averaged 2 ml/min/kg. The other predicted human PK parameters used to project the minimum efficacious dose were 3 liters/kg for volume of distribution at steady state, 17 h for half-life, 1 h^{-1} for the oral absorption rate constant, and 50% for oral bioavailability. Employing a one-compartment model with first-order input and output and no lag time, the predicted human minimum efficacious dose of Genz-668764 in a 3-day QD dosing regimen was 421 mg/day/70 kg. This maintained the plasma trough level above the IC_{90} against *Plasmodium falciparum* for 96 h after the last dose.

***In vitro* selection of resistance to Genz-644442.** We attempted to select for parasites (*P. falciparum* Dd2 strain) that showed decreased sensitivity to Genz-644442 by cycling 4 independent cultures between periods of drug exposure (at 10× the IC_{50} for 1 week) and outgrowth (for 1 to 3 weeks). At regular intervals, the IC_{50}s of these parasites were determined. Over the course of 10 cycles (270 days), we were unable to select for parasites that showed an IC_{50} more than 2-fold higher than that against the wild-type Dd2 strain. Similar studies are being performed using Genz-668764, and while they have progressed through only 3 cycles (65 days), thus far there has been no change in ED_{50}.

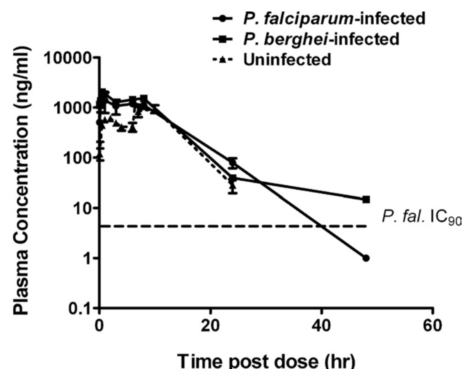

FIG. 1. Pharmacokinetics of Genz-668764 in infected and uninfected mice. Circles, plasma concentrations of Genz-668764 following single oral administration (50 mg/kg) to human-engrafted NSG mice (n = 3) infected with *P. falciparum*; squares, plasma concentrations of Genz-668764 following single oral administration (50 mg/kg) to human-engrafted NSG mice (n = 3) infected with *P. berghei*; triangles, plasma concentrations in uninfected CD-1 mice receiving 2 doses (27.5 mg/kg/dose) of Genz-668764 7 h apart; horizontal dashed line, concentration required to attain the *in vitro* IC_{90} value for *P. falciparum*.

DISCUSSION

We have followed two distinct strategies for identifying potential antimalarials. The first consists of screening compound libraries through whole-cell phenotypic assay to identify inhibitors of parasite growth; the second approach first screens libraries against validated biochemical targets and then co-optimizes using both biochemical and viability assays to develop SARs. The advantage of using viability screening as the primary assay is that it is independent of mechanism and thereby can identify compounds active against new biochemical pathways previously not associated with known antimalarial drugs. The disadvantage is that in the absence of a defined molecular target, target structure is unavailable to guide optimization efforts. The parent aminoindole molecule (Genz-644442) described here was identified in a live-dead screen of the small-molecule library at Harvard Medical School (ICCB-L) and the Broad Institute (36); results from a parallel program identifying inhibitors of *Plasmodium* dihydroorotate dehydrogenase (DHODH) are described elsewhere (5).

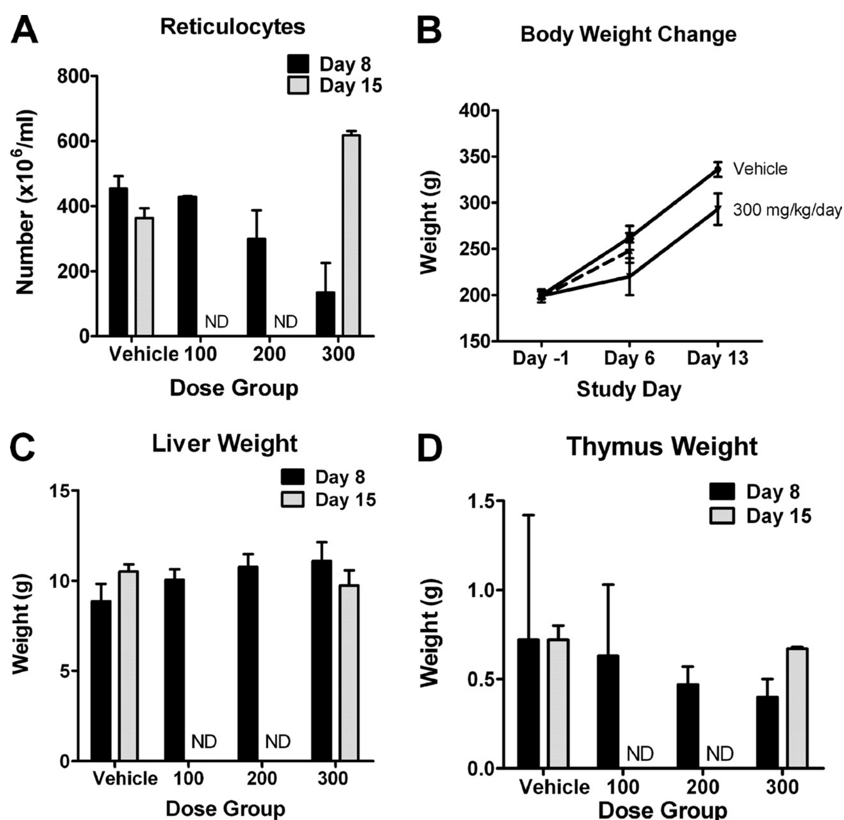

FIG. 2. Significant findings for Genz-668764 in preliminary 7-day rat safety study. (A) Total number of reticulocytes in each dosing group on day 8 (day following last dose) or day 15 (8 days after cessation of dosing). Dose group numbers indicate total daily dose (100, 200, or 300) in mg/kg/day. Only the vehicle control and the 300-mg/kg/day groups were tested on day 15; ND, not done, and rats were euthanized on study day 8. Error bars indicate standard error of the mean. (B) Average body weight of animals on the study day indicated. Circles, vehicle alone group; squares, animals receiving 100 mg/kg/day; dashed line, animals receiving 200 mg/kg/day; inverted triangles, animals receiving 300 mg/kg/day. (C) Average weight of liver on day 8 or day 15. (D) Average weight of the thymus on study day 8 and study day 15.

Genz-644442 was first run through a screening cascade designed to characterize potency, selectivity, and drug-like characteristics, which supported advancing the compound to efficacy studies against *P. berghei in vivo*, where 4 days' dosing either i.p. or orally resulted in significant suppression of parasitemia. The two enantiomers Genz-665848 and Genz-665849 did not differ significantly in potency against *P. falciparum in vitro* when they were tested individually (Table 1), but unexpectedly, Genz-665849 showed higher efficacy against *P. berghei in vivo*, most likely due to lower systemic plasma clearance and higher exposure *in vivo*.

Three hundred twenty-one aminoindole analogs were synthesized and screened to develop SARs both around *in vitro* potency against the parasite and for metabolic stability in hepatocytes and microsomes. Selected compounds were assessed for *in vivo* efficacy, for pharmacokinetic parameters, and in 7-day rat safety studies. This resulted in selection of a lead compound, Genz-668764. The potency of Genz-668764 against *P. falciparum* strain 3D7 is 28 nM, which is 7.1-fold more potent than Genz-644442 and within the 30 nM considered a threshold for potency of antimalarial compounds (22). Potency against the Dd2 strain was approximately 2-fold less, at 65 nM.

TABLE 5. Toxicokinetic parameters of Genz-668764 on study days 1 and 7

Study day	Mean target dose (mg/kg)	C_{max} (ng/ml)		C_{max}/dose (ng/ml)		T_{max}[a] (h)		AUC_{0-24} (ng · h/ml)		AUC/dose	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	100	2,450	285	24.5	2.85	8.0	0	35,668	5,131	357	51.3
	200	2,680	172	13.4	0.854	13.3	9.24	50,053	6,663	250	33.3
	300	2,850	718	9.51	2.42	18.7	9.24	51,425	8,247	171	27.5
7	100	2,180	21.2	21.8	0.212	8.0	0	32,239	482	322	4.8
	200	4,820	1,190	24.1	5.95	8.0	0	68,824	14,748	344	73.7
	300	6,660	381	22.2	1.31	8.0	0	106,973	4,607	357	15.4

[a] T_{max}, time of maximum concentration in plasma.

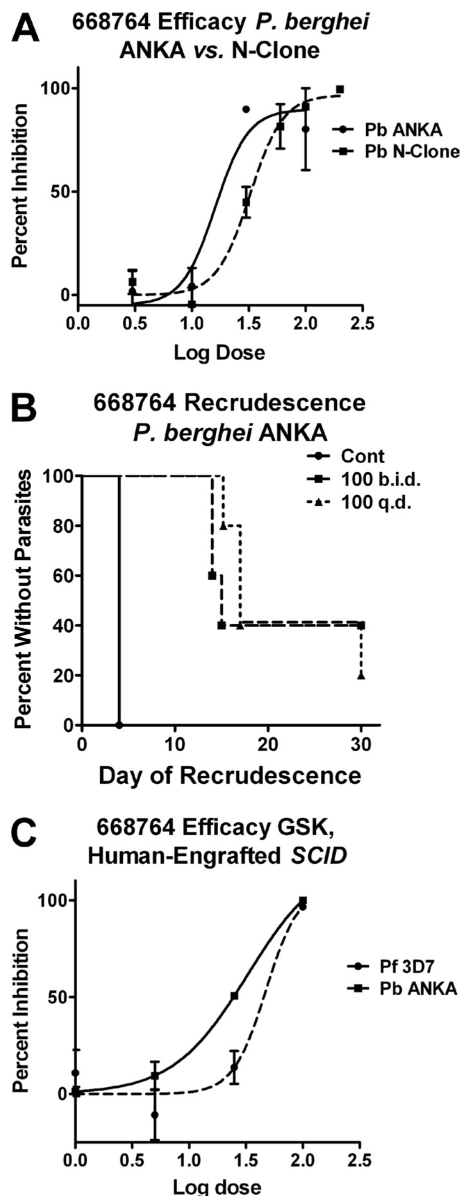

FIG. 3. *In vivo* efficacy against *P. berghei* ANKA strain and *P. falciparum.* Genz-668764 was tested against *P. berghei* ANKA strain (A and B) and in the NSG model against *P. falciparum* and *P. berghei* ANKA (C). (A) Dose-response curve showing percent inhibition (relative to vehicle-treated controls) on study day 4. Solid line, response of *P. berghei* ANKA strain (tested at SwissTPH); dotted line, response of *P. berghei* N clone (tested at UPR; data are shown for comparison). The ED_{50} of Genz-668764 against ANKA was 19 mg/kg/day, whereas the ED_{50} against the N clone was 32 mg/kg/day. Error bars are standard errors of the means. (B) Kaplan-Meier representation of day of recrudescence. Cont, vehicle-treated controls; dashed line; 100 b.i.d., animals dosed at 100 mg/kg/day; dotted line, 100 q.d., animals dosed at 100 mg/kg/day once/day. (C) Dose-response curve showing percent inhibition of parasitemia 24 h after 4 days of dosing in the human-engrafted NSG model (*P. falciparum*; circles, dashed line) and wild-type nonengrafted NSG mice (*P. berghei* ANKA strain; squares, solid line).

Potency was also assessed against *P. knowlesi in vitro*. The latter species infects both humans and nonhuman primates (15) and is used for *in vitro* susceptibility testing as a surrogate for *P. vivax* (6, 19). For *P. knowlesi*, the IC_{50} (26 nM) was very similar to that for *P. falciparum* strain 3D7, suggesting that this aminoindole might be active against blood stages of *P. vivax*. Selectivity studies *in vitro* showed that the potency of Genz-668764 against *P. falciparum* parasites was at least 1,900-fold greater than that against erythrocytes or mammalian primary cells. Further, the IC_{50} for hERG inhibition by Genz-668764 was 36.6 μM, indicating that this compound is unlikely to inhibit cardiac hERG channels *in vivo*.

As was the case for Genz-665848 and Genz-665849 (enantiomers of Genz-644442), the enantiomers Genz-668762 and Genz-668764 (enantiomers of Genz-666984) differed in their efficacy against *P. berghei in vivo*, with Genz-668764 being more active. The efficacy of Genz-668764 against *P. berghei* ANKA strain parasites was slightly greater than that against the N clone. The strain-specific difference in efficacy was similar to that observed when the efficacy of *Plasmodium* DHODH inhibitors against *P. berghei* was studied (5). While the reason for this is unclear, it may lie in the laboratory history of each strain: the N clone, derived from the K173 strain, has been maintained exclusively by syringe passage of blood-stage parasites (32), while ANKA strain infections are often maintained by sporozoite challenge (13). Interestingly, the ED_{50} of Genz-668764 against the *P. falciparum* 3D7 strain in the NSG model was very similar to that against *P. berghei* ANKA strain parasites in the same model. This was unexpected because blood stages of *P. berghei* showed 5- to 12-fold lower sensitivity *in vitro* than those of *P. falciparum* and illustrates the difficulty of extrapolating from the *in vitro* potency data.

Preliminary 7-day safety studies of different aminoindole analogs suggested that at high doses, reduced weight gain and reticulocytopenia appear to be class effects of the aminoindoles, while effects on white blood cells (WBCs) and lymphocytes are not and instead represent properties of individual analogs. Since the racemic mixture Genz-666984 had the fewest potentially adverse effects and had good efficacy (see Table S3 in the supplemental material), we purified the individual enantiomers and selected the more efficacious enantiomer, Genz-668764, for further testing. The 7-day rat safety study established the NOAEL at 200 mg/kg/day. As had been seen for the parent compound, the primary findings were reductions in rate of weight gain and in number of reticulocytes. However, 7 days after cessation of dosing, the rate of weight gain by animals treated with 300 mg/kg/day then became identical to

TABLE 6. Efficacy of Genz-668764 in different rodent models[a]

ED (mg/kg/day)							
UPR *P. berghei* N-clone		SwissTPH *P. berghei* ANKA strain GFP MRA-865		GSK *P. berghei* ANKA (QD)		GSK *P. falciparum* $Pf3D7^{0087/N9}$ (QD)	
50%	90%	50%	90%	50%	90%	50%	90%
32	68	19	30	26	48	40	74

[a] Efficacy in rodent models performed at UPR, SwissTPH, and GlaxoSmithKline (GSK). UPR and SwissTPH studies are nearly identical, but use N-clone and ANKA strain *P. berghei* parasites, respectively. In UPR and SwissTPH studies, animals were dosed b.i.d. GlaxoSmithKline uses NSG mice engrafted with human erythrocytes for *P. falciparum* assays and wild-type nonengrafted NSG mice for *P. berghei* evaluation. In these studies, animals were dosed QD. One study done at UPR looked at the effect of administering compound 3 times/day (t.i.d.), every 8 h.

that of untreated controls and the number of reticulocytes rebounded, indicating that these effects were transient. Because these findings were modest and were fully reversible, they do not represent an impediment to ongoing development of Genz-668764.

In vitro testing showed that Genz-668764 inhibited the cytochrome P450 enzyme CYP3A4/5 at 2.2 μM (Table 2). CYP3A4/5 is important in metabolism of antimalarials (34), including dapsone (21), proguanil (4), quinine (41), and halofantrine (10). To reduce the risk of selecting for resistance, no antimalarial is likely to be administered as monotherapy. Therefore, understanding possible drug-drug interactions will be important in selection of companion drugs, as is the case with any new antimalarial compound.

At present, the mechanism of aminoindole action against *Plasmodium* is unknown. On the basis of microscopic examination of synchronized *in vitro P. falciparum* cultures, the trophozoite stage appears to be the target (J. F. Cortese, unpublished data). The aminoindoles as a class are 2 to 3 times more potent in the wild-type 3D7 strain than in the multidrug-resistant Dd2 strain. Preliminary work implicates polymorphisms in the multidrug-resistant transporter *P. falciparum mdr1* (*pfmdr1*) in this differential activity, and not, for most of the aminoindoles, including Genz-668764, the chloroquine resistance transporter *pfcrt*. More detailed studies in progress will elucidate the precise mechanisms of this differential activity. The potency of Genz-668764 was shown to be essentially the same against *P. falciparum* D10 strain and the transgenic D10-scDHODH strain (A. B. Sidhu, unpublished data). The D10-scDHODH strain contains a cytoplasmic copy of *Saccharomyces cerevisiae* DHODH which can rescue parasites from compounds that inhibit the mitochondrial electron transport system (25, 26). The inability of the yeast enzyme scDHODH to rescue parasites from Genz-668764 suggests that the aminoindoles do not target the mitochondrial electron transport system. Finally, attempts to elucidate the mechanism of action have been rendered more difficult by our inability to select for resistance. Other studies have been able to utilize genomic differences between sensitive and resistant parasites to identify the putative target (31).

Genz-644442 (the parent compound) was exposed to 10 cycles of culturing parasites in the presence of $10\times$ IC_{50} for a week, followed by 2 weeks' outgrowth in the absence of drug pressure (270 days total). To date, Genz-668764 has been exposed to 3 cycles (65 days). In both cases, it has not been possible to select parasites with IC_{50}s >2 times that of the unselected parent. While it is hypothetically possible that this is because the drug is biostatic rather than biocidal, this does not appear to be the case, based upon microscopic examination, in which pyknotic forms are observed following drug exposure. Further, there is significant evidence both *in vitro* for pyrimethamine and mefloquine (23) and *in vivo* for artemisinin (35) that a subset of parasites may be dormant or quiescent, giving rise after removal of drug pressure to a recrudescent population of parasites with unaltered sensitivity to the drug. Whether this is the case for the aminoindoles awaits further confirmation, but the outgrowth of recrudescent populations *in vitro* following release from drug pressure would suggest that it is.

In summary, the aminoindoles represent a novel class of antimalarial compounds with good potency against *P. falciparum in vitro*. Genz-668764 is efficacious against both *P. falciparum* and *P. berghei* in the human-engrafted NSG model, and cure has been achieved against *P. berghei* in the adapted Peters rodent model (27). *In vitro* activity against *P. knowlesi* suggests that Genz-668764 might also be active as a schizontocide against *P. vivax*. Preliminary 7-day rat safety studies identified reversible reductions in weight gain and reticulocytopenia as the primary findings at exposures above those expected to be required for dosing in humans. The minimum efficacious human dose projection for Genz-668764 met the target product profile: oral daily dosing once/day of less than 1 g per day for 3 days maintaining trough levels above the IC_{90} for 4 days after the last dose. The compound exhibited a reasonable predicted human therapeutic index, based on the plasma exposure in rats at the NOAEL of 200 mg/kg/day. Finally, while the mechanism of action is unknown, the repeated failure to select for drug resistance *in vitro* over 270 days suggests that this could be an exciting companion drug for the next generation of antimalarials.

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