Activities of *N,N'*-Diarylurea MMV665852 Analogs against *Schistosoma mansoni*

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There is an unmet need to discover and develop novel antischistosomal drugs. As exemplified by MMV665852, *N,N'*-diarylureas have recently emerged as a promising antischistosomal chemotype. In this study, we evaluated the structure-activity relationships of 46 commercially available analogs of MMV665852 on newly transformed schistosomula (NTS) and adult *Schistosoma mansoni* worms *in vitro*. Active compounds were evaluated with a cytotoxicity assay, *in silico* calculations, metabolic stability studies, and an *in vivo* assay with mice harboring adult *S. mansoni* worms. Of the 46 compounds tested at 33.3 μ M, 13 and 14 compounds killed NTS and adult worms, respectively, within 72 h. Nine compounds had 90% inhibitory concentrations (IC₉₀s) of ≤ 10 μ M against adult worms, with selectivity indexes of ≥ 2.8 . Their physicochemical properties and permeation through an artificial membrane indicated good to moderate intestinal absorption. Their metabolic stabilities ranged from low to high. Despite satisfactory *in vitro* results and *in silico* predictions, only one compound resulted in a statistically significant worm burden reduction (66%) after administration of a single oral dose of 400 mg/kg of body weight to *S. mansoni*-infected mice. Worm burden reductions of 0 to 43% were observed for the remaining eight compounds tested. In conclusion, several analogs of the *N,N'*-diarylurea MMV665852 had high efficacy against *S. mansoni* *in vitro* and favorable physicochemical properties for permeation through the intestinal wall. To counteract the low efficacy observed in the mouse model, further investigations should focus on identifying compounds with improved solubility and pharmacokinetic properties.

Schistosomiasis is a parasitic disease caused by blood-dwelling flukes of the genus *Schistosoma*. An estimated 230 million people in 76 countries are infected, and 779 million people live at risk of infection (1). The clinically most relevant species are *Schistosoma mansoni*, *S. japonicum*, and *S. haematobium*. Preventive chemotherapy is the strategy of choice to control schistosomiasis, and in 2012, approximately 27.5 million people were treated (2). Treatment of schistosomiasis relies on a single drug: praziquantel. Despite praziquantel's single-dose efficacy, drug safety, and relatively low cost, new antischistosomal with differentiated modes of action need to be developed to address emergent drug resistance (3). Indeed, drug pressure on the worms increases continuously, and cases of praziquantel resistance in *S. mansoni* have already been reported (4). Additionally, a clear disadvantage of praziquantel is its lack of activity against the early developing schistosoma stage, also highlighting the need to develop novel antischistosomal drugs with multistage activity (5).

We recently screened the Medicines for Malaria Venture (MMV) Malaria Box (6) of 400 commercially available malaria-active compounds for antischistosomal activity (7). This library was initially assayed *in vitro* against schistosomula, and then active compounds were tested against adult worms; from this screen, selected compounds progressed to *in vivo* evaluation. MMV665852 was the most promising lead identified in that work. This *N,N'*-diarylurea inhibited worm viability *in vitro* by 50% at 0.8 μ M (50% inhibitory concentration [IC₅₀]), and it reduced worm burden in *S. mansoni*-infected mice to 53% (7).

N,N'-Diarylureas display a broad spectrum of biological activities and have been investigated for their potential use for tuberculosis (8), malaria (9), HIV (10), immunology (11), and, most extensively, oncology (12).

The aim of the present work was to conduct an initial structure-activity relationship (SAR) study of the *N,N'*-diarylurea MMV665852 against *S. mansoni*. A search of commercially available compound libraries for structures similar to that of MMV665852, using a Tanimoto-Rogers similarity coefficient of 0.85 as the cutoff, identified 46 compounds. These were tested against the larval and adult stages of *S. mansoni*. Hits progressed into a cell-based toxicity assay. In addition, physicochemical properties important for oral bioavailability according to Lipinski's "rule of five" (13) were calculated, intestinal wall permeation was assessed using a parallel artificial membrane permeation assay (PAMPA) (14), and metabolic stability was determined before the compounds were tested *in vivo*.

MATERIALS AND METHODS

Drugs and culture media. Identification of molecules chemically similar to MMV665852 was performed in the eMolecules database, using a Tanimoto-Rogers similarity coefficient of 0.85 as the cutoff, followed by a visual inspection and selection. The 46 identified compounds (Fig. 1) and

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FIG 1 Compound structures.

diuron were purchased from Specs, Sigma-Aldrich, or MolPort. For *in vitro* tests, compounds were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). For *in vivo* tests, compound 37 (15) was synthesized according to the methods of Chen et al. (16) and Hwang et al. (17). The culture media were prepared from medium 199 or RPMI 1640 (Life Technologies) with L-glutamine (Sigma-Aldrich), heat-inactivated fetal calf serum (FCS), penicillin, and streptomycin, which were purchased from LuBioScience.

Mice and parasites. *In vivo* experiments were approved by the veterinary authorities of Canton Basel-Stadt, Switzerland (license no. 2070). Female outbred NMRI mice ($n = 52$) were purchased from Charles River (Sulzfeld, Germany), kept at 22°C and 50% humidity with an artificial 12-h–12-h day-night cycle, and provided with water and rodent diet *ad libitum*. The 4-week-old mice were subcutaneously infected with 100 *S. mansoni* (Liberian strain) cercariae, which were collected from *S. mansoni*-infected *Biomphalaria glabrata* snails, as previously described (18).

Newly transformed schistosomula (NTS) drug assay. Cercariae were collected from *S. mansoni*-infected *B. glabrata*, mechanically transformed to schistosomula (19), and stored for 12 to 24 h at 37°C and 5% CO₂ in medium 199 supplemented with 5% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin before use.

For the NTS drug assay, 100 NTS per well were incubated in a 96-well plate (BD Falcon) with 33.3 µM compound diluted in supplemented medium 199. Wells containing NTS exposed to drug-free DMSO at a volume equivalent to the highest drug concentration in the assay (0.3%) served as controls. The assay was performed twice in triplicate. The efficacy of the drugs was judged by scoring their overall viability, using phenotypic reference points such as motility, morphology, and granularity. The following scoring scale was used: 3 = normal motility and morphol-

ogy, with no granularity; 2 = movements slowed down, first morphological changes, and signs of granularity were visible; 1 = highly reduced motility and/or altered morphology, with granularity; and 0 = complete immotility, altered morphology, and granularity. Parasites were judged via microscopic readout (magnification, ×80 to ×120; Carl Zeiss, Germany) 24, 48, and 72 h after incubation. Compounds that killed the NTS after 72 h were subsequently tested three times in triplicate, in 2-fold serial dilutions from 33.3 to 0.26 µM, for IC₅₀ and IC₉₀ determinations.

Adult *S. mansoni* drug assay. Adult *S. mansoni* were collected from mice by dissection at 49 to 70 days postinfection and were maintained in RPMI 1640 culture medium supplemented with 5% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C and 5% CO₂. Compounds were diluted to 33.3 µM in supplemented RPMI medium in 24-well plates (BD Falcon), to which three worms of each sex were added per well. Worms incubated with drug-free DMSO (0.3%) served as a control. The viability of *S. mansoni* adult was assessed via microscopic readout (in the same manner as described for NTS) at 1, 24, 48, and 72 h postincubation. For compounds that killed the worms after 72 h, IC₅₀ and IC₉₀ values were assessed using 3-fold serial dilutions from 33.3 to 0.41 µM in duplicate, and this was repeated once (20). Fast-acting compounds (100% immobilization after 1 h) were monitored 1, 2, 4, 7, 10, 24, 48, and 72 h after incubation. Slow-acting compounds (100% immobilization in 1 to 72 h) were evaluated 4, 24, 48, and 72 h after incubation.

Rat skeletal myoblast L6 cytotoxicity. Rat skeletal myoblast L6 cells (ATCC) were seeded at 2×10^3 cells/well in 96-well plates (BD Falcon) in RPMI 1640 medium supplemented with 10% FCS and 1.7 µM L-glutamine. Following a 24-h adhesion period at 37°C and 5% CO₂, cells were incubated with 3-fold serial drug dilutions, starting at 90 µM, for IC₅₀ determinations. As a positive control, the IC₅₀ of podo-

phyllotoxin (Sigma-Aldrich) was measured in every experiment, using 3-fold serial dilutions starting at 100 ng/ml. Along with every drug dilution, which was tested in duplicate, one cell-free drug dilution served as baseline. After 70 h of drug incubation, resazurin (Sigma-Aldrich) was added to the assay and incubated for another 2 h. The plates were then read using a SpectraMax M2 plate reader (Molecular Devices) and Softmax software (version 5.4.1), with an excitation wavelength of 536 nm and an emission wavelength of 588 nm. The assay was repeated at least twice (21).

Calculation of physicochemical properties. The numbers of hydrogen bond acceptors (HBA) and donors (HBD) were calculated using ChemBioDraw Ultra software by CambridgeSoft. The *in silico* prediction tool ALOGPS 2.1 was used to determine calculated log P (clogP) values of the compounds (22; <http://www.vcclab.org>). Drug-likeness was assessed according to Lipinski's "rule of five" for desirable physicochemical properties, i.e., molecular weight (MW) of ≤ 500 g/mol, logP value of ≤ 5 , HBD number of ≤ 10 , and HBA number of ≤ 5 (13).

Kinetic aqueous solubility. Compound kinetic aqueous solubility measurements were performed at two pH values (6.0 and 7.4) in triplicate. Compound stock solutions (10 mM) were diluted to 0.1 mM in an aqueous universal buffer solution (Pion). Benzthiazide (Sigma-Aldrich) served as a positive control. After 15 h, 300 μ l of solution was filtered (96-well, 0.2- μ m-filter plates; Corning) using vacuum filtration (Whatman Ltd.) to remove precipitates. Equal amounts of filtrate and *n*-propanol (Sigma-Aldrich) were mixed and transferred to 96-well plates for UV detection of the compound spectra (250 to 500 nm) (SpectraMax 190; Molecular Devices). The amount of compound dissolved was calculated by comparing the sample spectra with UV spectra obtained for references and blanks with μ SOL Explorer solubility analyzer software (Pion, version 3.4.0.5).

PAMPA and quantification using LC-MS. Effective permeation (log P_c) was determined in a 96-well format using a parallel artificial membrane permeation assay (PAMPA) (23). The PAMPA sandwich (P/N 110 163), system solution, GIT-0 lipid solution, acceptor sink buffer, and Gut-Box apparatus were purchased from Pion. For each compound, measurements were performed at two pH values (pH 6.0 and pH 7.4) in quadruplicate. Compounds were diluted with system solution to 10 μ M. Samples (150 μ l) were withdrawn for reference, and a further 200 μ l was transferred to donor plate wells of the PAMPA sandwich. The filter membranes at the bottom of acceptor plates were infused with 5 μ l of GIT-0 lipid solution, and 200 μ l of acceptor sink buffer was added to the acceptor wells. The sandwich was assembled and placed in the GutBox apparatus. After 16 h, the sandwich was disassembled, and samples (150 μ l) from donor and acceptor wells, as well as the reference wells, were quantified by liquid chromatography-mass spectrometry (LC-MS).

LC-MS measurements were performed using an 1100/1200 series high-pressure liquid chromatography (HPLC) system coupled to a model 6410 triple-quadrupole mass detector (Agilent Technologies) equipped for electrospray ionization. The system was controlled with Agilent Mass Hunter workstation data acquisition software (version B.01.04). The column used was an Atlantis XBridges amide column (2.1 $\times$ 50 mm) with a 3.5- μ m particle size (Waters). Two mobile phases were prepared to create an organic solvent gradient, i.e., mobile phase A (H₂O containing 0.1% [vol/vol] formic acid) and mobile phase B (acetonitrile containing 0.1% [vol/vol] formic acid), running at 0.6 ml/min. MS parameters, such as fragmenter voltage, collision energy, and polarity, were optimized individually for each analyte, and the molecular ion was followed for each compound, using the multiple-reaction monitoring mode.

Log P_c values were calculated from the determined concentrations by using PAMPA Explorer software (version 3.5; Pion) (14).

Mouse liver microsome stability assay. To assess metabolic stability, each compound (1 μ M) was incubated with liver microsomes (Xenotech) at 37°C and a 0.4-mg/ml protein concentration. The metabolic reaction was initiated by the addition of an NADPH-regenerating system and quenched at various time points over a 60-min incubation period by the

addition of acetonitrile containing diazepam as an internal standard. Control samples (containing no NADPH) were included (and quenched at 2, 30, and 60 min) to monitor for potential degradation in the absence of the cofactor. The samples were analyzed by ultraperformance LC-MS (UPLC-MS) (Waters/Micromass Xevo G2 QTOF instrument) under positive electrospray ionization, and MS spectral data were acquired in the mass range of 80 to 1,200 Da. The microsome-predicted hepatic extraction ratios (E_H), obtained based on the relative rates of test compound degradation *in vitro*, were used to classify compounds as low (<0.3), intermediate (0.3 to 0.7), high (0.7 to 0.95), and very-high (>0.95) extraction compounds.

***In vivo* studies.** Compounds ($n = 9$) were dissolved in DMSO (5% of the final volume) and diluted with 1% (mass/vol) hydroxypropyl methylcellulose (HPMC) (Sigma-Aldrich) in distilled water to a final concentration of 60 mg/ml. On postinfection day 49, *S. mansoni*-infected mice ($n = 4$ /group) were treated with single oral doses of 400 mg/kg of body weight. The control group of mice ($n = 8$) remained untreated. At 17 to 21 days posttreatment, the mice were dissected, and the worms residing in the mesenteric veins and the liver were counted and sexed (18).

Statistics. *In vitro* activities of the compounds against NTS and adult *S. mansoni* were calculated from the mean viability values in relation to the control value, as follows: drug effect = $1 - (\text{score}_{\text{drug}}/\text{score}_{\text{control}})$. From the obtained drug effects, IC₅₀ and IC₉₀ values were calculated using CompuSyn software (version 3.0.1; ComboSyn). The linear correlation coefficient (r value) reflects the conformity of the experimental data (24). IC₅₀s against the mammalian cell line and R^2 values (goodness of fit) were generated by the software Softmax. The selectivity index (SI) was calculated by dividing the L6 cytotoxicity IC₅₀ by the adult *S. mansoni* IC₅₀. To assess the *in vivo* efficacy, the mean values for living worms of each treatment group were compared to that of the control group, and worm burden reductions (WBR) were calculated as follows: WBR (%) = $100\% - 100\% \times (\text{WB}_{\text{treated mice}}/\text{WB}_{\text{control mice}})$. The statistical significance of the *in vivo* treatments was determined using the Kruskal-Wallis test (at a significance level of 0.05 [P value]) (StatsDirect, version 2.7.2; StatsDirect Ltd.).

RESULTS

***In vitro* activity against NTS.** Of the 46 compounds tested at 33.3 μ M for activity against NTS, 13 killed NTS within 72 h (Table 1). Of these compounds, two (compounds 10 and 37) killed NTS even at the lowest concentration tested (0.26 μ M). Six compounds (compounds 1, 29, 38, 40, 45, and 46) were characterized by IC₅₀s of ≤ 0.5 μ M and IC₉₀s of ≤ 1.4 μ M, and five (compounds 2, 25, 32, 41, and 44) had IC₅₀s ranging from 1.0 to 9.2 μ M.

***In vitro* activity against adult *S. mansoni*.** Fourteen of 46 compounds tested at 33.3 μ M for activity against adult *S. mansoni* killed the worms when incubated for 72 h (Table 1). Of these, eight compounds (compounds 1, 2, 10, 29, 37, 38, 40, and 45) resulted in death of adult *S. mansoni* within 1 h and had IC₅₀s of ≤ 0.8 μ M and IC₉₀ values of ≤ 2.4 μ M after a 72-h incubation period. Figure 2 presents the IC₅₀s of these derivatives between 1 and 24 h postincubation. The compounds acted quickly against adult *S. mansoni*: IC₅₀s measured after 4 h were similar to those measured after 72 h. The IC₅₀ of *N*-phenyl benzamide 29 reached the nanomolar range within 1 h. Compounds 3, 4, 22, 25, 26, and 46 were schistosomicidal at 1 to 72 h postincubation, with calculated IC₅₀s of 1.8 to 7.4 μ M.

Antischistosomal selectivity. Compounds ($n = 14$) that demonstrated IC₅₀s of ≤ 10 μ M against adult worms after 72 h were tested for cytotoxicity by use of L6 rat skeletal muscle cells to select compounds for *in vivo* testing. The antischistosomal selectivities of these compounds appeared to be rather low, with selectivity indexes ranging from 0.8 to 11.6 (Table 1).

TABLE 1 *In vitro* activities of compounds active against NTS and adult *S. mansoni*^a

Compound	NTS			Adult <i>S. mansoni</i> worms			L6 cells		SI
	IC ₅₀	IC ₉₀	<i>r</i>	IC ₅₀	IC ₉₀	<i>r</i>	IC ₅₀	<i>R</i> ²	
MMV665852 ^b	4.7		1.0	0.8	2.2	1.0	1.5	0.99	1.9
1	0.07	0.3	0.76	0.4	1.4	0.92	3.2	1.00	7.3
2	1.3	2.8	0.90	0.7	2.2	0.94	3.7	0.99	5.4
3	ND			3.6	9.6	0.90	3.3	0.97	0.9
4	ND			7.0	14.7	0.92	5.2	0.98	0.8
10	<0.26			0.2	0.8	0.92	1.4	1.00	7.2
22	ND			6.0	13.9	0.93	5.2	0.99	0.9
25	4.3	8.5	0.97	6.7	13.2	0.91	12.4	0.93	1.9
26	ND			7.4	17.1	0.92	10.4	0.97	1.4
29	0.03	0.2	0.76	0.1	0.5	0.80	1.4	1.00	11.6
32	2.6	501	0.83	ND			ND		
37	<0.26			0.3	1.1	0.89	0.9	1.00	2.8
38	0.2	0.7	0.87	0.6	2.4	0.92	4.0	0.99	4.9
40	0.07	0.3	0.76	0.6	1.8	0.92	2.3	1.00	4.0
41	9.2	30.2	0.87	ND			ND		
44	1.0	3.1	0.97	ND			ND		
45	0.5	1.4	0.93	0.7	2.3	0.93	2.1	1.00	2.9
46	0.2	0.8	0.93	1.8	4.9	0.95	2.1	0.98	1.2

^a Data are presented for compounds showing activity against NTS and/or adult worms (lethal at 33.3 μ M after 72 h of incubation). Worms were incubated in 3-fold serial dilutions of compounds, starting at 33.3 μ M, for 72 h, and IC₅₀ and IC₉₀ values were calculated. *r*, linear correlation coefficient (values of ≥ 0.85 are acceptable); *R*², goodness of fit; SI, selectivity index (IC₅₀ against L6 cells divided by IC₅₀ against adult *S. mansoni*); ND, not done (due to lack of activity at 33.3 μ M).

^b See reference 7.

Physicochemical properties, solubility, and passive intestinal permeability. For compounds (*n* = 9) with IC₉₀ values of ≤ 10 μ M within 24 h against both NTS and adult *S. mansoni*, and with selectivity indexes of ≥ 1 , physicochemical properties, solubilities, and PAMPA permeability values were determined (Table 2). MWs and numbers of HBA and HBD of all nine lead candidates were in the acceptable ranges for drug-likeness. Most compounds showed desirable clogP values of ≤ 5 . The aqueous solubilities were generally low (<0.3 μ M). The passive permeability of most compounds was predicted to be “good” ($-\log P_e < 5.7$). However, *N,N'*-diaryleurea 1 showed a decreased permeability potential at pH 7.4, with a $-\log P_e$ value of 6.37 ± 0.35 , and arylphenylcarbamate 45 showed moderate to poor permeability potentials at pH 6.0 and pH 7.4, with $-\log P_e$ values of 6.23 ± 0.40 and 6.41 ± 0.35 , respectively.

Metabolic phase I stability. Metabolic stability with mouse liver microsomes was assessed for the nine lead compounds (Table 3). Compound 45 showed the least *in vitro* degradation, with an *E_H* value of 0.22. Compounds 2, 1, and 40 revealed intermediate degradation, while compounds 10, 37, 29, 38, and 46 revealed high levels of degradation. The metabolic stability for compound 46 could not be determined (due to low solubility).

***In vivo* studies.** All nine compounds progressed to *in vivo* studies (Table 4), where they were tested in mice harboring adult *S. mansoni*. *N*-Phenyl benzamide 38 was the most active compound, with a worm burden reduction (WBR) of 66% (*P* < 0.05). For comparison, praziquantel at 400 mg/kg is characterized by a WBR of 94% (25). Other compounds resulted in statistically insignificant (*P* > 0.05) WBRs, as follows: compound 2, 43%; compound 10, 36%; compound 40, 36%; compound 46, 15%; and

compound 29, 12%. *N,N'*-Diaryleurea 1 and aryl *N*-phenylcarbamate 45 did not reduce worm burdens. Toxicity was observed for *N*-phenyl benzamide 37; two mice died, at 1 h and 3 h post-treatment, and one mouse had to be euthanized after 2 h. The majority of compounds did not dissolve completely in the 1% HPMC formulation.

DISCUSSION

Schistosomiasis is a neglected tropical disease, and research for new antischistosomal drugs is sparse. There is no antischistosomal drug in the developmental pipeline, which is potentially perilous should praziquantel resistance arise. By screening MMV's Open Access Malaria Box, we identified *N,N'*-diaryleureas as a new, orally active chemotype against *Schistosoma mansoni* (7).

In this follow-up study, we selected 46 commercially available compounds with chemical similarity to the lead *N,N'*-diaryleurea, MMV665852, by using the Tanimoto-Rogers similarity algorithm with a similarity coefficient cutoff of 0.85; these compounds encompassed 13 *N,N'*-diaryleureas, 12 *N*-aryl,*N'*-alkyleureas, 17 *N*-phenyl benzamides, and 4 aryl *N*-phenylcarbamates. All compounds were first tested *in vitro*, on larval (NTS) and adult schistosomes. Active compounds (IC₅₀ ≤ 10 μ M) were tested for cytotoxicity, and the most selective and active were assessed for drug-likeness (numbers of HBA and HBD, clogP value, solubility, and gastrointestinal permeation [estimated by PAMPA]), followed by microsomal stability determination and oral application to mice harboring chronic *S. mansoni* infections.

Nine compounds had IC₉₀ values of ≤ 10 μ M against both *S. mansoni* stages after 24 h of incubation, of which 8 were characterized as fast acting. Of these, 7 compounds had higher antischistosomal activity than the lead compound MMV665852 against adult *S. mansoni* and NTS. We observed that fast-acting compounds were also the most potent; compounds that killed adult worms at 33.3 μ M within 1 h had IC₅₀s of ≤ 0.8 μ M and IC₉₀ values of ≤ 2.4 μ M (determined after 72 h of incubation), whereas slow-acting compounds (worms were killed within 1 to 72 h at 33.3 μ M) had IC₅₀s of 1.8 to 7.4 μ M. Fast drug action is obviously important for compounds with fast plasma clearance in order to

FIG 2 IC₅₀s of 8 fast-acting compounds against adult *S. mansoni* observed between 1 and 24 h postincubation.

TABLE 2 Physicochemical properties, solubilities, and intestinal wall permeability potentials of lead candidates^a

Compound	MW (g/mol)	clogP	No. of HBA	No. of HBD	No. of rule-of-5 violations	Exptl solubility (μM)	PAMPA result (−log <i>P_e</i>) (cm/s)
1	315.58	4.71 ± 0.46	3	2	0	<0.3 (pH 6.0)	5.65 ± 0.64 (pH 6.0)
						<0.3 (pH 7.4)	6.37 ± 0.35 (pH 7.4)
2	299.13	4.28 ± 0.37	3	2	0	<0.3 (pH 6.0)	4.98 ± 0.18 (pH 6.0)
						<0.3 (pH 7.4)	4.83 ± 0.20 (pH 7.4)
10	351.56	4.89 ± 0.38	3	2	0	<0.3 (pH 6.0)	5.70 ± 0.25 (pH 6.0)
						<0.3 (pH 7.4)	5.65 ± 0.32 (pH 7.4)
29	315.67	4.40 ± 0.32	3	2	0	<0.3 (pH 6.0)	5.17 ± 0.28 (pH 6.0)
						<0.3 (pH 7.4)	5.13 ± 0.22 (pH 7.4)
37	383.67	5.34 ± 0.53	3	2	0–1	<0.3 (pH 6.0)	4.93 ± 0.02 (pH 6.0)
						<0.3 (pH 7.4)	4.67 ± 0.07 (pH 7.4)
38	315.67	4.38 ± 0.27	3	2	0	<0.3 (pH 6.0)	4.82 ± 0.06 (pH 6.0)
						<0.3 (pH 7.4)	4.62 ± 0.04 (pH 7.4)
40	315.67	4.43 ± 0.35	3	2	0	<0.3 (pH 6.0)	5.18 ± 0.30 (pH 6.0)
						<0.3 (pH 7.4)	5.27 ± 0.43 (pH 7.4)
45	436.22	6.23 ± 0.54	3	1	1	<0.3 (pH 6.0)	6.23 ± 0.40 (pH 6.0)
						<0.3 (pH 7.4)	6.41 ± 0.35 (pH 7.4)
46	332.28	4.09 ± 0.34	4	1	0	<0.3 (pH 6.0)	4.46 ± 0.17 (pH 6.0)
						<0.3 (pH 7.4)	4.53 ± 0.26 (pH 7.4)

^a MW, molecular weight; HBA, hydrogen bond acceptors; HBD, hydrogen bond donors; clogP, calculated partition coefficient for octanol-water; PAMPA, parallel artificial membrane permeation assay. Permeation values are interpreted as follows: poor, values of >6.3; moderate, values of 6.3 to 5.7; and good, values of <5.7.

impair schistosomes sufficiently before the drug is removed from the body.

High *in vitro* activity against both stages was observed for compounds of three chemical classes: *N,N'*-diarylureas 1, 2, and 10; *N*-phenyl benzamides 29, 37, 38, and 40; and aryl *N*-phenylcarbamates 45 and 46. Ureas with a nonaromatic ring on one side were less active, and those with a hydrogen and/or alkane residue were not active at all. The preferred positions of the halogen groups in the active *N,N'*-diarylureas seemed to be *meta* and *para* rather than *ortho*. *N,N'*-Diarylurea 1 (triclocarban) is an antibacterial agent used in consumer soaps and deodorants. It is mainly active against Gram-positive bacteria but also has slight activity against Gram-negative bacteria and fungi (26). Diuron (com-

pound 23), an herbicidal *N*-aryl,*N'*-alkylurea used in agriculture, was inactive on both parasite stages.

N-Phenyl benzamides with 2-hydroxy and 4-chloro substituents at the benzoyl substructure (salicylanilides) and a trifluoromethyl at the *N*-phenyl substructure (e.g., compounds 29 and 37) demonstrated excellent antischistosomal activity. Methylation of the *N*-phenyl benzamide phenol functional group lowered the antischistosomal activity (e.g., compound 38 > compound 39, and compound 41 > compound 42), indicating the importance of the salicylanilide. It is interesting that the active salicylanilides 29, 37, 38, and 40 closely resemble niclosamide, an old nitro-substituted salicylanilide teniacide thought to function by uncoupling oxidative phosphorylation. Due to the low antischistosomal selectivity of these salicylanilides, consistent with previous investigations (15) of this compound class, salicylanilides will be challeng-

TABLE 3 Metabolic stability *in vitro* and calculated *in vivo* hepatic extraction for lead candidates^d

Compound	<i>t</i> _{1/2} (min) ^a	CL _{int} (μl/min/mg protein) ^b	<i>E_H</i> ^c
1	33	52	0.53
2	45	39	0.45
10	8	222	0.83
29	8	223	0.83
37	16	111	0.70
38	6	294	0.86
40	23	77	0.62
45	129	13	0.22
46	UD		

^a *t*_{1/2} (degradation half-life) = ln 2/*k*.

^b CL_{int} = CL_{int, in vitro} × liver mass (g)/body weight (kg) × microsomal protein mass (mg)/liver mass (g).

^c *E_H* = predicted *in vivo* hepatic extraction ratio, determined as follows: *E_H* = CL_{blood}/Q = CL_{int}/(Q + CL_{int}); CL_{blood} = Q × CL_{int}/(Q + CL_{int}). *E_H* values were interpreted as follows: low, values of <0.3; intermediate, values of 0.3 to 0.7; high, values of 0.7 to 0.95; very high, values of >0.95.

^d The following scaling factors were applied to mouse liver microsome calculations: liver mass = 54.9 g liver/kg body weight; microsomal protein mass = 47 mg/g liver mass; and hepatic blood flow (Q) = 120 ml/min/kg body weight (32). UD, unable to be determined.

TABLE 4 Worm burden and worm burden reductions of lead candidates in mice harboring a chronic *S. mansoni* infection^c

Compound	No. of mice investigated	Avg (SD) no. of worms	WBR (%)
MMV665852 ^a			53
Praziquantel ^b			94
Control ¹	8	26.9 (20.3)	
Control ²	8	34.2 (10.3)	
38 ¹	4	9.3 (3.3)	66*
2 ^{1,2}	3	18.0 (4.2)	43
10 ¹	4	17.3 (12.4)	36
40 ^{1,2}	4	19.5 (8.7)	36
46 ²	4	29.0 (10.5)	15
29 ¹	4	23.8 (7.0)	12
45 ²	4	35.5 (3.5)	0
1 ¹	4	29.5 (15.3)	0
37 ²	4	21.8 (11.3)	36

^a See reference 7.

^b See reference 25.

^c SD, standard deviation; WBR, worm burden reduction. *, *P* < 0.05. Values in superscript refer to the corresponding control group.

ing starting points for further optimization. Since only four carbamate isosteres were assessed in this compound set, no conclusions about their SAR can be drawn. However, they may represent an alternative to ureas.

As we noted above for the salicylanilides, we observed relatively low antischistosomal selectivities for the tested compounds by using cytotoxicity data generated from a single mammalian cell line (L6). Indeed, there was a strong correlation ($r = 0.819$; $P < 0.01$) between *S. mansoni* adult IC_{50} s and L6 cell IC_{50} s. Interestingly, we previously observed (7) that MMV665852 was an order of magnitude less cytotoxic against the MRC-5 cell line. Thus, obtaining a true picture of cytotoxicity for these compounds will likely require data from multiple mammalian cell lines. Although physicochemical property calculations as well as PAMPA experiments suggested a high potential for intestinal absorption for most of the lead compounds, their measured aqueous solubilities were low. In the microsomal (phase I) stability assay, half of the tested compounds were characterized by good to intermediate stability. Some parameters that were not investigated were efflux effects or protein binding, which can also lower drug plasma levels.

Disappointingly, despite the promising *in vitro* antischistosomal activity of 14 compounds, only *N*-phenyl benzamide (salicylanilide) 38 had a significant WBR (66%) after a single oral dose of 400 mg/kg, which is slightly higher than the WBR of 53% achieved with the same dose of MMV665852 (7). For salicylanilide 38 and its analogs, inactive metabolites due to phase II glucuronidation (11) of the phenol functional group may account for the *in vitro/in vivo* discrepancy of this compound class. Moreover, triclocarban was described to be quickly biotransformed to *N*-glucuronides, leading to fast renal excretion (27, 28). On the other hand, the most likely reason for the *in vitro/in vivo* discrepancy of *N,N'*-diarylureas 1, 2, and 10 is their low aqueous solubility, a liability previously noted (9) in a SAR investigation of antimalarial *N,N'*-diarylureas. As no significant differences were observed between the IC_{50} s of the *N,N'*-diarylurea MMV665852 in media supplemented with differing amounts of serum (7), protein binding (29) is unlikely to account for the low *in vivo* antischistosomal activity of these *N,N'*-diarylureas. However, structural optimization of *N,N'*-diarylureas can improve their bioavailability, increase drug solubility and plasma peak areas, and decrease drug clearance (30, 31).

In conclusion, we identified several new *N,N'*-diarylureas, aryl *N*-phenylcarbamate, and *N*-phenyl benzamide derivatives of MMV66582 as having high activities against NTS and adult *S. mansoni* worms in *in vitro* experiments. One derivative, *N*-phenyl benzamide 38, had a significant WBR (66%) after a single oral dose, which is a promising result for further investigations. Achieving overall high *in vivo* efficacy and antischistosomal selectivity for these compound classes will likely require identification of more hydrophilic derivatives.

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