Development of pyridyl thiosemicarbazones as highly potent agents for the treatment of malaria after oral administration


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Received 13 December 2018; returned 25 March 2019; revised 4 June 2019; accepted 5 June 2019

Objectives: Drug resistance exists to all current and investigational antimalarial drug classes. Consequently, we have set out to develop chemically and mechanistically discrete antimalarials. Here we report on the development of thiosemicarbazone (TSC) antimalarials, with TSC3 as the most advanced lead.

Methods: Thiosemicarbazones were generated through simple condensation reactions of thiosemicarbazides and ketones. TSC3 was selected and tested for in vitro antimalarial activities against MDR Plasmodium falciparum lines using the [3H]hypoxanthine growth assay, in vitro cytotoxicity against mammalian cell lines using the alamarBlue fluorescence cell viability assay, in vivo potency in the mouse–Plasmodium berghei model and blood exposure in mice measured by LC-MS for pharmacokinetic analysis.

Results: TSC3 showed potent in vitro activity against atovaquone-, dihydroartemisinin-, chloroquine- and mefloquine-resistant P. falciparum lines (EC50, 15 nM). The selectivity index (EC50 cells/EC50 Pf W2 line) of TSC3 was >500 in two of three mammalian cell lines. In P. berghei-infected mice, TSC3 showed potent activity in the Peters 4 day suppression test (ED50 1.2 mg/kg/day) and was as potent as artesunate and chloroquine in the curative modified Thompson test. A single oral dose of TSC3 at 16 mg/kg in healthy mice achieved a mean maximum blood concentration of 1883 ng/mL at 1 h after dosing and an elimination half-life of 48.7 h in groups of five mice.

Conclusions: TSC3 shows promise as a persistent, potent and orally effective antimalarial. This, coupled with the extremely low cost of synthesis, suggests that the further development of antimalarial thiosemicarbazones is clearly warranted.

Introduction

Malaria is the most important parasitic disease in the world. It is responsible for close to half a million deaths, with in excess of 200 million infections annually, and remains a target of critical interest in drug discovery.1 There is now good clinical evidence of drug resistance to all current antimalarial agents, including the transmission of resistant parasite strains.2–4 Consequently, the search for new antimalarials not subject to current resistance patterns with potentially new modes of action is of continued interest.

The current mainstays of antimalarial chemotherapy, the artemisinins (artesunate, artemether and dihydroartemisinin), are likely to exhibit their antimalarial activity through an interaction of the peroxidic function with ferrous iron and subsequent generation of reactive oxygen (as has been proposed for simple lipophilic peroxides), suggesting that the iron metabolism of the parasite can be targeted successfully.5–7

The activity of the quinoline antimalarials such as chloroquine has been attributed to an interaction with ferrirraptoporphyrin (or haem) resulting in a pH-dependent disruption in the parasitic haem detoxification mechanism.8–10 Considerable uncertainty, however, remains as to the actual nature of this process.11 The application of metal-chelating agents as antimalarials capitalizes on this concept.

Iron chelators figure predominantly as investigational antimalarials, with their probable mechanism of action being 2-fold: namely, depletion of labile iron required for metabolic processes and in situ formation of toxic iron species.12 Iron chelators such as desferrioxamine (1, Figure 1) inhibit parasite growth both in vitro

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(although at relatively poor potency compared with other drugs, EC50 of 20 μM averaged over 73 Plasmodium falciparum isolates) and in vivo with efficacy against human malaria in a clinical setting.13–16

The potency of other iron-chelating catechol-derived siderophores, such as FR160 and FBS0701 (2, Figure 1), is superior to desferrioxamine, but still relatively poor (EC50 of 1.5 μM averaged over 137 P. falciparum isolates).17,18 Hydrazones and thiosemicarbazones derived from phenolic aldehydes, such as 2-hydroxy-1-naphthaldehyde-4-phenyl-3-thiosemicarbazone (N4pT, 3, Figure 1), display similar anti-malarial potencies to FR160 and FBS0701 (2, Figure 1). For these latter agents, antiplasmodial activity is aided by lipophilic or bulky substituents, and potency is retained against chloroquine-resistant parasite strains.17–21

We recently reported that metal-coordinating bis-thiosemicarbazones with a pyridyl core have promising antimalarial activity22 (see Supplementary data available at JAC Online). A further 22 compound thiosemicarbazone test series was evaluated, from which 3 compounds exhibited potent in vitro antimalarial activities against the chloroquine-susceptible P. falciparum NF54 and the chloroquine-resistant P. falciparum Dd2 lines, with EC50S <30 nM. We herein report ongoing studies in the evolution of this class of antimarials, relating to thiosemicarbazones 8 (denoted BpNET) and 16 (denoted TSC3; see Supplementary data).

Materials and methods

Synthesis of chelators and copper(II) complexes thereof

All thiosemicarbazones and their copper(II) complexes were synthesized according to standard procedures23,24 (see also Supplementary data).

Rodent parasites and mice for tolerability, efficacy and pharmacokinetic studies

The chloroquine-susceptible Plasmodium berghei ANKA was obtained from the Liverpool School of Tropical Medicine and Hygiene (Liverpool, UK). Swiss outbred ARC (Animal Resource Centre, Murdoch, Western Australia) female mice (5–7 weeks of age) were used in the present study. The mice received a standard mouse pellet diet (Rat and Mouse Food from Laucke Mills, Daveyston, South Australia) and tap water ad libitum. The animals were caged in groups of up to six per cage and held at a temperature of 24°C ± 1°C at a relative humidity of 50% ± 10%. For the tolerability, efficacy and pharmacokinetic studies of the thiosemicarbazones, the mean (± SD) body weight of the mice was 28.4 ± 2.5 g (n = 429).

Drugs

Artesunate and dihydroartemisinin were obtained from DK Pharma (Hanoi, Vietnam). Chloroquine diphosphate, mefloquine hydrochloride and tafenoquine succinate were obtained from Sigma–Aldrich Chemical Co. (St Louis, MO, USA). For tolerability, efficacy and pharmacokinetic studies in mice, BpNET and TSC3 were prepared in 0.5% hydroxypropyl-methylcellulose, 10% ethanol, 10% Tween 80 and 79.5% distilled water (dosage volume 0.2 mL).

Parasite cultures of P. falciparum

The chloroquine- and pyrimethamine-resistant line W2 (Indochina), the MDR (atovaquone, chloroquine and mefloquine) TM90-C2B (Thailand) and the artemisinin-susceptible (MRA1239) and artemisinin-resistant (MRA1240) P. falciparum laboratory-adapted isolates from Cambodia (BEI Resources Repository, USA) were cultured as described by Chavchich et al.25

In vitro susceptibility of P. falciparum lines to thiosemicarbazone treatment

The activity of the drugs against the W2, TM90-C2B, MRA1239 and MRA1240 lines was assessed using the [3H]hypoxanthine growth inhibition assay.26 BpNET and TSC3 were dissolved in DMSO at a concentration of 1 mM for the in vitro studies. Residual methanol and DMSO concentrations are too low to cause assay interference (data not shown). Briefly, the assays (96-well plates) were initiated when the majority of parasites (>95%) were at the ring stage. Parasite cultures (100 μL/well) at 1% initial parasitaemia and 2% haematocrit in complete, but hypoxanthine-free, medium were exposed to ten 2-fold serial dilutions of the compounds for 48 h at 37°C with [3H]hypoxanthine (0.2 μCi/well) added 24 h after the beginning of the experiment. At least two independent experiments were carried out for each compound, with each assay performed in triplicate. The inhibitory concentrations (EC50 and EC90) were calculated by non-linear regression analysis using GraphPad Prism software V5.0 (GraphPad Software, Inc., CA, USA).

In vitro schizont maturation assay against P. berghei

The in vitro schizont maturation assay for testing BpNET and TSC3 was based on the procedure of Chang et al.27 BpNET and TSC3 and the reference drugs, artesunate and chloroquine, were prepared as 30 mM stocks in DMSO (except chloroquine, which was dissolved in water) and diluted in complete medium prior to use (RPMI 1640 with 10% FCS). Blood (500 μL) was collected from a mouse infected with P. berghei at 3%-5% parasitaemia and enriched for early-stage parasites by passing it through a magnetic-activated cell sorting (MACS) column. Washed parasitized cells were made to 2% haematocrit in complete medium and added to triplicate wells of drug-containing microplates with final drug concentrations of 16 μM to 240 μM. Microplates were incubated at 37°C in 5% CO2 for 22 to 24 h in a humidified environment.

Cells were subsequently gently resuspended to avoid rupture of schizonts, and 20 μL was transferred to a new microplate containing 180 μL of a 1/2000 dilution of Sybr Green (Invitrogen, Australia).26 After incubating in the dark for 30 min, 100,000 events/well were collected on a fluorescence-activated cell sorting (FACS) Canto II flow cytometer (Becton Dickinson, USA).
Development of antimalarial thiosemicarbazones

Australia. Giemsa-stained films of drug-free control wells were counted by microscopy to determine the percentage of schizonts, to allow for accurate setting of gates for statistical analysis of flow cytometry data. EC₅₀ values were calculated from non-linear regression analysis using GraphPad Prism software V5.0.

**In vitro cytotoxicity in mammalian cell lines**

The in vitro cytotoxicity of BpNEt and TSC3 against three mammalian cell lines (all sourced from the ATCC): HEK-293 (human embryonic kidney), HEP-G2 (human liver carcinoma) and BHK (baby hamster kidney) was determined using the alamarBlue (Invitrogen, Australia) fluorescence cell viability assay.²⁹ Cell cultures were maintained in complete RPMI 1640 medium (Sigma, USA) containing 10% FBS and 0.03% l-glutamine (complete medium) in 75 cm² flasks at 37°C with the medium changed twice weekly, as previously described.³⁰ BpNEt and TSC3 and the reference drugs, artesunate and chloroquine, were serially 2-fold diluted in triplicate using a master drug plate and tested at concentrations ranging from 120 μM to 60 nM.

**In vivo tolerability assessment in mice**

Tolerability assessment of BpNEt and TSC3 was performed in groups of three healthy mice to ensure that the compounds were tolerable. Four doses (oral gavage, volume 0.2 mL) administered at 24 h intervals were assessed (volume: 0.2 mL in physiological saline). Eight drug-treated groups with 2-fold increases in dose (oral gavage, volume 0.2 mL) were assessed for both thiosemicarbazones and the reference drugs, artesunate and chloroquine. Untreated vehicle control (0.5% hydroxypropyl-methylcellulose, 10% ethanol, 10% Tween 80 and 79.5% distilled water, dosage volume 0.2 mL) mice typically died between days 6 and 7 post-infection. If any animal exhibited clinical adverse event signs that appeared highly stressful, they were euthanized.

**In vivo efficacy in the Peters 4 day test**

The Peters 4 day test measures the suppressive activity of blood schizonticides over 4 days at doses that do not cause physical stress in healthy mice.³¹ BpNEt- and TSC3-treated and control female mice, in groups of six, were inoculated intraperitoneally with 2×10⁶ P. berghei-infected RBCs (volume: 0.2 mL in physiological saline). Eight drug-treated groups with 2-fold increases in dose (oral gavage, volume 0.2 mL) were assessed for both thiosemicarbazones and the reference drugs, artesunate and chloroquine. Untreated vehicle control (0.5% hydroxypropyl-methylcellulose, 10% ethanol, 10% Tween 80 and 79.5% distilled water, dosage volume 0.2 mL) mice typically died between days 6 and 7 post-infection. The drugs were administered ~2 h after parasite inoculation (DO) and then daily at 24 h intervals for three consecutive days. Blood samples for thin blood films were collected on D + 4 (with the number after the day, D, representing the total number of days since parasite inoculation).

**In vivo efficacy in the modified Thompson test**

The modified Thompson test determines the curative blood schizontocidal dose given daily over 3 days with an established infection of ~1%–3% parasitaemia.³² Based on the Peters 4 day test, the most potent thiosemicarbazone was selected for the modified Thompson test. Female mice in groups of six were infected with 2×10⁶ P. berghei-infected RBCs on DO (volume: 0.2 mL in physiological saline). By D + 3 post-infection, parasitaemia was typically ~1%–3%.

Five drug-treated groups with 2-fold increases in dose (1, 2, 4, 8 and 16 mg/kg/day, oral gavage, volume 0.2 mL, vehicle control as for Peters test) were evaluated. The reference drugs, artesunate and chloroquine, were used to gain insight into the performance of the modified Thompson test at an oral dose of 64 mg/kg/day. The drugs were administered on days D + 3, D + 4 and D + 5 post-infection at 24 h intervals. Blood samples for thin blood films were taken daily for 9–10 days and then twice weekly thereafter until the end of the test on day + 31.

**Parasitaemia monitoring in the mouse–P. berghei models**

The degree of infection (i.e. parasitaemia) was determined from blood thin films that were collected by clipping the mouse’s tail with a scalpel blade and ‘milking’ a drop of blood (~20 μL). The thin blood film slides were stained with 10% Giemsa for 15 min at room temperature and were read by a WHO-certified Level 1 malaria microscopist.

**Analysis of parasitaemia suppression and radical cure in the mouse–P. berghei models**

For the Peters 4 day test, inhibition of parasite growth in the BpNEt-, TSC3-, artesunate- and chloroquine-treated groups was calculated in relation to the non-treated control group. The suppressive difference between the mean parasitaemia value of the vehicle (control) group (taken as 100%) and those of the drug-treated groups was calculated and expressed as percentage reduction. Parasitaemia versus dose response and ED₅₀ (50% effective dose) and ED₉₀ (90% effective dose) values were calculated by non-linear regression analysis using GraphPad Prism V5.0. For the assessment of radical cure in the modified Thompson test, recurrence of P. berghei infection was tabulated for 31 days, at which time all mice surviving that were blood film negative were then deemed cured.

**Pharmacokinetics in mice**

The pharmacokinetics of the most potent thiosemicarbazone was determined in groups of five healthy female mice after a single oral dose of the compound. Blood samples (0.6 mL) were collected from each mouse by cardiac puncture at 0 (before dosing), 1, 3 and 6 h, and then at 1, 2, 3, 7, 10, 14, 21 and 28 days after dosing, and added to BD Microtainers containing EDTA as the anticoagulant (Becton Dickinson, Australia). Blood and plasma samples were stored at – 80°C until LC-MS analysis for measurement of the thiosemicarbazone concentrations. The LC-MS method is described in the Supplementary data.

The pharmacokinetic parameters derived from the blood and plasma thiosemicarbazone concentration data were Cmax, Tmax, AUC and elimination t½, determined by non-compartment analysis (PK Solutions 2.0, Summit Research Services, OH, USA).

**Ethical considerations**

The animal studies covering mice infected with P. berghei for the in vitro schizont maturation assay, tolerability studies, in vivo efficacy studies in the mouse–P. berghei model and pharmacokinetic studies in mice were approved by the Defence Animal Ethics Committee (DAEC 04–15, 12–15, 06–16 and 02–17).

**Results and discussion**

**Synthesis of the lead thiosemicarbazones**

BpNEt and TSC3 (Figure 2) were synthesized as part of a structurally diverse (Figure S1) thiosemicarbazone collection using a simple condensation protocol between a doubly aryl-substituted ketone and a thiosemicarbazide under acidic catalysis in refluxing ethanol according to literature protocols.²³,²⁴ The rationale behind the synthesis of the collection is described in the Supplementary data. Yields after crystallization for BpNEt and TSC3 were 47% and
61%, respectively. These two candidates were selected for further study on the basis of our antimalarial drug prioritization flowsheet (Figure 3).

**In vitro antimalarial activities of the lead thiosemicarbazones against P. falciparum lines**

As a follow-up to our earlier study,22 we set out to develop the thiosemicarbazone class as new antimalarials. The two lead TSCs (BpNEt and TSC3) were initially selected based on their in vitro potency against the two *P. falciparum* lines (NF54 and Dd2, Table S1). These studies revealed that the presence of two aryl residues appeared necessary for activity against the chloroquine-resistant *P. falciparum* Dd2 parasites. Of note, this structural feature was not included in an earlier series identified by Klayman et al.33 Further appraisal against MDR *P. falciparum* lines showed TSC3 to be markedly more potent in vitro than BpNEt by 3- to 4-fold based on EC50 values (Table 1). Furthermore, TSC3 was ~10-fold more potent than chloroquine and mefloquine against the highly resistant TM90-C2B and MRA1240 lines, but less potent than dihydroartemisinin.

**In vitro antimalarial activities of the lead thiosemicarbazones against P. berghei**

In addition to assessing the *in vitro* activities of the two thiosemicarbazones against *P. falciparum* lines, the compounds were assessed in the *in vitro* *P. berghei* schizont maturation assay for future planning for *in vivo* efficacy studies in the mouse--*P. berghei* model.

Unlike the results in the *in vitro* studies against the MDR *P. falciparum* lines, TSC3 (50 nM) exhibited EC50 values ~2-fold higher than that of chloroquine (32 nM) and artesunate (20 nM; Table 2). However, we concluded that the activity against *P. berghei* in vitro should be a good predictor of potency in the mouse--*P. berghei* model and potential efficacy against *P. falciparum* in an *in vivo* model. Furthermore, the activity against *P. falciparum* may, in fact, be superior to activity against *P. berghei* for this hit series.

**Detailed evaluation of mammalian cell-based toxicity in vitro**

Early single concentration mammalian toxicity studies of the thiosemicarbazone test series listed in the Supplementary data against MRC-5 human lung fibroblasts at 20 μM failed to indicate toxic effects. However, we chose to initiate an additional study of cytotoxic potential against three further mammalian cell lines to verify the early toxicity data using a multi-concentration (dose–response) approach described in this study. A neoplastic cell line was included in this assessment, the basis for this being that related drugs were developed to display selective cytotoxicity towards cancerous cells.33–36 While not strictly transferable to human toxicity, we also chose to use a non-transformed rodent cell line (BHK) prior to assessing tolerability in mice.

After calculating a selectivity index (SI; Table 3), we concluded that there would be a sufficient therapeutic window for evaluation of TSC3 as an antimalarial therapy.17 The *in vitro* data for TSC3 suggest that potency against the W2 line would be obtained at a concentration some 39 times lower than the onset of toxicity for the HEK-293 cells. This threshold is considerably wider for the BHK cells and the HEP-G2 cells, with SI values of 510 and 602, respectively. The average SI was ~1500 for the chloroquine-susceptible NF54 parasite and almost 400 for the chloroquine-resistant W2 parasite (see definition in the footnotes of Table 3). Accordingly, we decided that an *in vivo* study was warranted to assess the tolerability of the thiosemicarbazones.

**Tolerability studies in mice**

The tolerability of BpNEt and TSC3 was assessed in mice administered by oral gavage in four ascending daily doses of 8, 16, 32 and 64 mg/kg/day for BpNEt and 8, 16 and 32 mg/kg/day for TSC3. BpNEt was well tolerated in groups of three mice at all concentrations up to and including 64 mg/kg/day.

Some mice exposed to TSC3 at 32 mg/kg/day experienced adverse reactions after 3 days of dosing, showing symptoms of severe hunching, markedly decreased activity and excessive coat ruffling (these mice were euthanized). Nonetheless, TSC3 was well tolerated over the four dose regimen at 16 mg/kg/day. Consequently, all subsequent studies with TSC3 were carried out with a dosage ceiling of 16 mg/kg/day.

**Efficacy in the mouse--*P. berghei* model**

In the Peters 4 day test using a dose range of 0.125–16 mg/kg/day, TSC3 was 5.6-fold more effective in suppressing the lethal *P. berghei* ANKA strain than BpNEt (ED50 values: 1.2 mg/kg/day versus 7.8 mg/kg/day) (Table 4, Figure 4).

When compared with the two reference drugs, TSC3 had potency comparable to artesunate (ED50 1.2 mg/kg/day) and was ~2.4-fold more potent than chloroquine (ED50 3.3 mg/kg/day). However, the Peters 4 day test (carried out 24 h after the final dose of drug) does not cater to determining the suppression activity beyond the completion timepoint.

On the basis of these suppression data, we concluded that BpNEt was insufficiently potent after oral administration and, consequently, all further studies were conducted on the more potent agent, TSC3.

To further evaluate the therapeutic potential of TSC3, we assessed the ability of the thiosemicarbazone to effect radical cure in the modified Thompson test, which requires extended follow-up for 4 weeks post-treatment. Evaluation of a dose range of 1–16 mg/kg/day of TSC3 given daily for 3 days with continued parasitaemia monitoring after dosage provided the opportunity to assess the thiosemicarbazone’s capability to clear an established...
Preliminary in vitro screen of compound against CQ-sensitive NF54 strain. Is compound EC50 < 100 nM?

Yes

Preliminary in vitro screen of compound against CQ-resistant Dd2 strain. Is compound EC50 < 50 nM?

Yes

Preliminary in vitro assessment of compound against human lung fibroblast (MRC5). Do >75% of cells survive at 20,000 nM?

Yes

Broad in vitro screen of compound against MDR parasite strains. Is compound EC50 < 50 nM?

Yes

Toxicity screen against three mammalian cell lines: one rodent, one human non-cancerous, one human cancerous. Is average Selectivity Index > 100? SI = (EC50 cells/EC50 P. falciparum W2)

Yes

Assessment of compound against P. berghei (rodent malaria) in schizont maturation model against MDR parasite strains. Is compound EC50 < 100 nM?

Yes

Perform maximum tolerated dose study (multiple ascending doses) in mice (po)

No

Discard

Assess parasite suppression (P. berghei mouse, po) in Peters 4 day test. Establish ED50 and ED90 values. Are values less than double those of CQ and AS?

Yes

Assess radical cure potential (P. berghei mouse, po) in Thompson 3 day test with 30 day monitoring. How does candidate compare to CQ and AS?
Table 1. *In vitro* antimalarial activities (EC50, nM) of BpNEt, TSC3, chloroquine, dihydroartemisinin and mefloquine against MDR *P. falciparum* lines with varying drug resistance profiles

<table>
<thead>
<tr>
<th>Compound</th>
<th>W2 (EC50, nM)</th>
<th>TM90-C2B (EC50, nM)</th>
<th>MRA1239 (EC50, nM)</th>
<th>MRA1240 (EC50, nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BpNEt</td>
<td>23 ± 5</td>
<td>ND</td>
<td>27 ± 5</td>
<td>25 ± 7</td>
</tr>
<tr>
<td>TSC3</td>
<td>6.2 ± 0</td>
<td>13 ± 2</td>
<td>6.9 ± 0.5</td>
<td>9.1 ± 1.8</td>
</tr>
<tr>
<td>chloroquine</td>
<td>150 ± 30</td>
<td>144 ± 28</td>
<td>76 ± 1</td>
<td>97 ± 21</td>
</tr>
<tr>
<td>dihydroartemisinin</td>
<td>0.9 ± 0.2</td>
<td>1.7 ± 0.7</td>
<td>0.3 ± 0.2</td>
<td>1.0 ± 0.7</td>
</tr>
<tr>
<td>mefloquine</td>
<td>27 ± 4</td>
<td>130 ± 51</td>
<td>26 ± 4</td>
<td>84 ± 8</td>
</tr>
</tbody>
</table>

ND, not determined.
Mean ± SD EC50 values based on at least two independent experiments.

Table 2. *In vitro* antimalarial activities of BpNEt, TSC3, artesunate and chloroquine against *P. berghei* in the schizont maturation assay

<table>
<thead>
<tr>
<th>BpNEt EC50 (nM)</th>
<th>TSC3 EC50 (nM)</th>
<th>Artesunate EC50 (nM)</th>
<th>Chloroquine EC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>65.7 ± 26.8</td>
<td>50.0 ± 18.2</td>
<td>20.1 ± 4.1</td>
<td>32.3 ± 15.5</td>
</tr>
</tbody>
</table>

Mean ± SEM EC50 values based on three independent experiments.

Table 3. *In vitro* cytotoxicity: artesunate, chloroquine, BpNEt and TSC3 in BHK, HEK-293 and Hep-G2 cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>BHK mean</th>
<th>TSC3 mean</th>
<th>Artesunate</th>
<th>Chloroquine</th>
</tr>
</thead>
<tbody>
<tr>
<td>artesunate</td>
<td>86.09</td>
<td>53.92</td>
<td>7.92</td>
<td>3.16</td>
</tr>
<tr>
<td>chloroquine</td>
<td>32.59</td>
<td>2.81</td>
<td>7.25</td>
<td>1.24</td>
</tr>
<tr>
<td>BpNEt</td>
<td>1.35</td>
<td>46.14</td>
<td>0.28</td>
<td>0.24</td>
</tr>
<tr>
<td>TSC3 SI</td>
<td>0.54</td>
<td>8.73</td>
<td>0.24</td>
<td>0.19</td>
</tr>
<tr>
<td>Hep-G2 mean</td>
<td>6.48</td>
<td>15.04</td>
<td>2.25</td>
<td>3.73</td>
</tr>
<tr>
<td>TSC3 SI</td>
<td>2.74</td>
<td>2.74</td>
<td>1.76</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Mean (SEM) EC50 values were based on three independent experiments; SI, EC50 cells/EC50 *P. falciparum* W2 line.

Table 4. Parasite suppression of *P. berghei*-infected mice treated with BpNEt, TSC3, artesunate and chloroquine in the Peters 4 day test after oral dosing

<table>
<thead>
<tr>
<th>Compound</th>
<th>ED50 (mg/kg/day)</th>
<th>ED90 (mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BpNEt</td>
<td>7.8 ± 0.8</td>
<td>14.6 ± 0.1</td>
</tr>
<tr>
<td>TSC3</td>
<td>1.2 ± 0.4</td>
<td>4.4 ± 0.3</td>
</tr>
<tr>
<td>artesunate</td>
<td>1.2 ± 0.3</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td>chloroquine</td>
<td>3.3 ± 0.4</td>
<td>5.5 ± 0.2</td>
</tr>
</tbody>
</table>

Mean ± SD ED50 values based on two independent experiments.

P. berghei infection and to prevent recrudescence. These data were compared with a parallel assessment of the reference drugs artemisinin and chloroquine.

At doses of <4 mg/kg/day, TSC3 failed to elicit complete parasite clearance (data not shown). Clearance was observed on day 5 post-treatment in 50% of six mice at doses of 8 mg/kg/day, with reappearance of parasites on day 8 post-treatment (Table 5). At the highest dose tested, all six mice cleared the *P. berghei* infection at 16 mg/kg/day by day 4 post-treatment. However, one mouse had to be euthanized because of significant body weight loss (>16%), with recrudescence of a second mouse 10 days after commencing treatment. Four mice in this treatment cohort showed no recurrence of infection by day 31 and were deemed cured.

This study was repeated with similar results, with an overall cure rate of 58% (7/12 mice). In contrast, artesunate and chloroquine at a dose of 64 mg/kg/day cleared infection at days 1 and 3, with recrudescence occurring at days 3 and 8, respectively, with no mice surviving to day 31 (0% cure).

**Pharmacokinetic evaluation**

The assessment of *in vivo* potency for TSC3 suggested that the thiosemicarbazone was highly persistent in the mouse model. This pharmacodynamic finding suggests that TSC3 could have a relatively lengthy blood elimination half-life. The pharmacokinetic properties of TSC3 were evaluated in healthy mice administered a single oral dose of 16 mg/kg of TSC3. Calibration and quality control data for blood and plasma analyses can be viewed in the Supplementary data (Tables S2–S10). The mean (SD) blood and plasma concentration versus time profiles of TSC3 are shown in Figure 5 (the full data set is available in the Supplementary data, Tables S11 and S12).

The thiosemicarbazone was absorbed rapidly, followed by concentration-dependent clearance approximating behaviour characteristic of a single compartment model in both blood and plasma samples. The mean blood concentration data of TSC3 revealed a *C* max of 1883 ng/mL with a *T* max of 1 h and an elimination *t* 1/2 of 48.7 h (Table 6). Published values for the elimination *t* 1/2 of related thiosemicarbazones Triapine and Dp44mT are 1 h (human data) and 2.2 h (rat), respectively, illustrating that TSC3 is far more persistent *in vivo* than related drugs. 38,39 This level of persistence for TSC3 suggests that a refined dosage regimen...
for the in vivo studies may be desirable (the current dosage interval is ~50% of half-life) to avoid accumulation of the drug (a loading dose and smaller follow-up doses may be warranted). Toxicity of earlier thiosemicarbazones is highly dose dependent and varies substantially with drug structure, ranging from resolvable leucopenia to cardiotoxicity (reported only for Dp44mT after 2 weeks of intense, non-optimal dose administration).36,38–40 No evidence of cardiotoxicity was detected in the current study.

Table 5. In vivo efficacy of TSC3, artesunate and chloroquine in the modified Thompson test

<table>
<thead>
<tr>
<th>Activity</th>
<th>Compound dose</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting parasitaemiaa (%) for treatment at D0, mean (range)</td>
<td>TSC3 (16 mg/kg/day×3 days)</td>
<td>2.2% (1.9–2.9)</td>
<td>2.3% (1.5–3.0)</td>
</tr>
<tr>
<td></td>
<td>TSC3 (8 mg/kg/day×3 days)</td>
<td>D+4 (6 of 6 mice)</td>
<td>D+4 (6 of 6 mice)</td>
</tr>
<tr>
<td></td>
<td>artesunate (64 mg/kg/day×3 days)</td>
<td>D+3 (6 of 6 mice)</td>
<td>D+2 (6 of 6 mice)</td>
</tr>
<tr>
<td></td>
<td>chloroquine (64 mg/kg/day×3 days)</td>
<td>D+3 (6 of 6 mice)</td>
<td>D+3 (6 of 6 mice)</td>
</tr>
<tr>
<td>Day of parasite clearance after starting treatment</td>
<td>TSC3 (16 mg/kg/day×3 days)</td>
<td>D+10 (1 of 5 mice)</td>
<td>D+9 (1 of 4 mice)</td>
</tr>
<tr>
<td></td>
<td>TSC3 (8 mg/kg/day×3 days)</td>
<td>D+8 (3 of 3 mice)</td>
<td>D+5 (6 of 6 mice)</td>
</tr>
<tr>
<td></td>
<td>artesunate (64 mg/kg/day×3 days)</td>
<td>D+5 (6 of 6 mice)</td>
<td>D+5 (6 of 6 mice)</td>
</tr>
<tr>
<td></td>
<td>chloroquine (64 mg/kg/day×3 days)</td>
<td>D+8 (6 of 6 mice)</td>
<td>D+8 (6 of 6 mice)</td>
</tr>
<tr>
<td>Day of recrudescence after starting treatment</td>
<td>TSC3 (16 mg/kg/day×3 days)</td>
<td>D+10 (1 of 5 mice)</td>
<td>D+9 (1 of 4 mice)</td>
</tr>
<tr>
<td></td>
<td>TSC3 (8 mg/kg/day×3 days)</td>
<td>D+8 (3 of 3 mice)</td>
<td>D+5 (6 of 6 mice)</td>
</tr>
<tr>
<td></td>
<td>artesunate (64 mg/kg/day×3 days)</td>
<td>D+5 (6 of 6 mice)</td>
<td>D+5 (6 of 6 mice)</td>
</tr>
<tr>
<td></td>
<td>chloroquine (64 mg/kg/day×3 days)</td>
<td>D+8 (6 of 6 mice)</td>
<td>D+8 (6 of 6 mice)</td>
</tr>
</tbody>
</table>

Experiment 1: three of six mice on TSC3 (8 mg/kg) did not clear the infection; three recrudesced by D + 8.
Experiment 1: one mouse on TSC3 (16 mg/kg) was euthanized on D + 6: body weight loss >16%; one mouse recrudesced on D + 10.
Experiment 2: two mice on TSC3 (16 mg/kg) were euthanized on D + 9: body weight loss >16%; one mouse recrudesced on D + 9.
aMean values for the drug-treated and vehicle control groups of mice.

Table 6. Pharmacokinetic parameters of TSC3 in healthy mice administered a single oral dose of 16 mg/kg of TSC3

<table>
<thead>
<tr>
<th>PK parameters</th>
<th>TSC3 blood</th>
<th>TSC3 plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax (ng/mL)</td>
<td>1883</td>
<td>1260</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>AUC0–last (0–168) (ng·h/mL)</td>
<td>37594</td>
<td>18659</td>
</tr>
<tr>
<td>AUC0–last (0–336) (ng·h/mL)</td>
<td>40455</td>
<td>19586</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>48.7</td>
<td>48.3</td>
</tr>
<tr>
<td>Blood/plasma AUC0–last (0–168)</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>
Plasma TSC3 concentrations paralleled blood concentrations, with a blood to plasma concentration ratio based on AUC values of 2, suggesting an association of the thiosemicarbazone with blood cells.

**Ongoing research**

As the lead thiosemicarbazone, TSC3 has excellent potency and oral efficacy; current studies have been initiated to refine both pharmacodynamic and pharmacokinetic properties and to elucidate the mechanism of action of this antimalarial class.

**Conclusions**

Pyridyl thiosemicarbazones offer a promising alternative to current chemotherapeutic options for the treatment of malaria. Notably, thiosemicarbazones are simple to synthesize (resulting in an extremely low cost of goods) and chemically robust. Both these properties are desirable characteristics for development of antimalarial drugs. The lead thiosemicarbazone, TSC3, was highly potent against MDR *P. falciparum* lines and also demonstrated high in vivo potency in the mouse–*P. berghei* model. In fact, TSC3 was at least as potent as artesunate and chloroquine. This efficacy in vivo was at least as potent as artesunate and chloroquine. This efficacy in vivo high in mice.

**Acknowledgements**

We acknowledge the technical excellence of Kerryn Rowcliffe for *in vitro* drug testing and we thank Ivor Harris, Stephen McLeod-Robertson and Thomas Travers for assisting in the mouse studies. We are grateful to the Australian Red Cross Blood Service for the provision of human blood and sera for *in vitro* cultivation of *P. falciparum* lines. The opinions expressed are those of the authors and do not necessarily reflect those of the Australian Defence Organisation or any extant policy.

**Funding**

This study was partly funded by the South African Medical Research Council (MRC) in the form of a South African MRC Flagship Grant supporting the biological work reported herein (MRC-RFA-UFSP-01-2013; MALTB Redox: The development of oxidant and redox drug combinations for treatment of malaria, TB and related diseases) and the Charles Sturt University Pharmacy Foundation for the chemistry component. D. R. R. was supported by a National Health and Medical Research Council Senior Principal Research Fellowship (1062607). The *in vitro* and *in vivo* studies were funded by the Australian Defence Organization.

**Transparency declarations**

The authors report no conflicts of interest relating to this work.

**Author contributions**

C. J. P., R. K. H., D. R. R. and M. D. E. conceived and designed the study. G. W. B., M. C., D. M. and C. d.k. performed the studies. C. J. P., G. W. B., M. C., C. d.k. and M. D. E. analysed and interpreted the results. C. J. P. wrote the first draft of the manuscript and supplied all test compounds. All authors read and approved the final manuscript.

**Supplementary data**

*Supplementary data*, including Tables S1–S12 and Figure S1, are available at JAC Online.

**References**

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Development of antimalarial thiosemicarbazones


