Lead Optimization of a Pyrazole Sulfonamide Series of *Trypanosoma brucei* N-Myristoyltransferase Inhibitors: Identification and Evaluation of CNS Penetrant Compounds as Potential Treatments for Stage 2 Human African Trypanosomiasis


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Supporting Information

**ABSTRACT:** *Trypanosoma brucei* N-myristoyltransferase (*Tb*NMT) is an attractive therapeutic target for the treatment of human African trypanosomiasis (HAT). From previous studies, we identified pyrazole sulfonamide, DDD85646 (1), a potent inhibitor of *Tb*NMT. Although this compound represents an excellent lead, poor central nervous system (CNS) exposure restricts its use to the hemolymphatic form (stage 1) of the disease. With a clear clinical need for new drug treatments for HAT that address both the hemolymphatic and CNS stages of the disease, a chemistry campaign was initiated to address the shortfalls of this series. This paper describes modifications to the pyrazole sulfonamides which markedly improved blood−brain barrier permeability, achieved by reducing polar surface area and capping the sulfonamide. Moreover, replacing the core aromatic with a flexible linker significantly improved selectivity. This led to the discovery of DDD100097 (40) which demonstrated partial efficacy in a stage 2 (CNS) mouse model of HAT.

**INTRODUCTION**

Human African trypanosomiasis (HAT) is caused by two subspecies of the protozoan parasite *Trypanosoma brucei*, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, transmitted by the bite of an infected tsetse fly.1,2 The disease is fatal unless treated. It has two stages: an initial (hemolymphatic) peripheral infection during which the parasites are found in the bloodstream and gives rise to nonspecific symptoms, and a second stage during which the parasites enter the central nervous system (CNS), giving rise to the classic symptoms of HAT, eventually leading to coma and death. Currently, there are five treatments available, although none of them are satisfactory due to toxicity, treatment failures, and the requirement for parenteral administration that is inappropriate in a rural African setting.

N-Myristoyltransferase (NMT) catalyzes the cotranslational transfer of myristate from myristoyl-CoA to the N-terminal glycine of a large number of proteins, a modification which is implicated in localization and/or activation of the substrate.3,4 The enzyme operates via a Bi−Bi mechanism in which it first binds myristoyl-CoA, causing a conformational rearrangement, which subsequently reveals the peptide binding site.5 In *T. brucei*, RNAi knockdown of NMT has been shown to be lethal in cell culture6 and to abrogate infectivity in animal models of HAT.8 Bioinformatics analysis suggests that about 60 proteins are myristoylated in the parasite,9 although there is incomplete knowledge of the downstream targets.10 NMT has also been investigated as a potential target for the treatment of other parasitic diseases including malaria,11 leishmaniasis,12 and Chagas disease.13
Recently, we have reported the discovery of DDD85646 (1, Figure 1), a very potent inhibitor of T. brucei NMT (TbNMT)

\begin{center}
| DDD85646 (1) | \\
| TbNMT, EC\(_{50}\) 0.002\(\mu\)M | \\
| HsNMT, IC\(_{50}\) 0.003\(\mu\)M | \\
| T. brucei, EC\(_{50}\) 0.002\(\mu\)M | \\
| MRC5, EC\(_{50}\) 0.3\(\mu\)M | \\
| Brain : blood < 0.05 (mouse) | \\
| Mw, 495.4 | \\
| PSA, 92 | \\
| clogP, 3.1 | \\
| Plasma protein binding, 94\% (mouse) | \\
\end{center}

Figure 1. Biological and physicochemical profile of the prototypical NMT inhibitor 1.

(IC\(_{50}\) = 0.002 \(\mu\)M) and of the growth of T. brucei (EC\(_{50}\) = 0.002 \(\mu\)M), which delivers potent activity in mouse models of stage 1 HAT (fully curative at 12.5 mg/kg b.i.d. po for 4 days, T. b. brucei S427; fully curative at 50 mg/kg b.i.d. po for 4 days, T. b. rhodesiense STIB900).\(^{14}\) Combined with further biological mode of action studies, this has provided excellent validation of NMT as a therapeutic target for the peripheral stage of HAT.\(^{15}\)

Unfortunately, 1 is not active in the stage 2 HAT mouse model (T. b. brucei GVR35; 100 mg/kg b.i.d. po for 5 days).\(^{14,15}\) However, this was unsurprising, as 1 poorly penetrates the blood–brain barrier and is a substrate for P-glycoprotein, thus preventing delivery of efficacious free brain concentration at a tolerated dose (blood:brain ratio is <0.05 in mouse and <0.09 in rat, rising to 0.27 in rat in the presence of the Pgp inhibitor GF120918;\(^{16}\) experimental in Supporting Information). Furthermore, 1 has relatively poor/no selectivity at the enzyme level compared to the two human orthologues HsNMT1 and HsNMT2 (IC\(_{50}\) = 0.003 \(\mu\)M), although this did not translate to low selectivity at the cellular level (cellular selectivity ratio, MRC5 EC\(_{50}\)/T. brucei EC\(_{50}\) = 150). While there was no observable toxicity in rodents at therapeutic doses, there was a relatively low therapeutic index (stage 1 HAT minimal curative dose = 12.5 mg/kg b.i.d. po [T. b. brucei S427] or 50 mg/kg b.i.d. po [T. b. rhodesiense STIB900]; MTD = 100 mg/kg b.i.d. po). It is unknown if the low safety margin in vivo is caused by inhibition of mouse NMT or another off-target effect. However, to try and eliminate the former, it was decided to improve the enzymatic selectivity. Typically, selectivity between two enzymes of interest is expressed as a ratio of their biochemical IC\(_{50}\) values (which in this case would be HsNMT/TbNMT). However, during the course of this study, we discovered that many compounds were very potent inhibitors of TbNMT, which due to the enzyme concentration used in the assay (10 nM) have IC\(_{50}\) values approaching the observable tight-binding limit (as evidenced by Hill slopes \(>1\)) and there is thus very little differentiation between the most potent compounds. Because we have already shown for this series that TbNMT IC\(_{50}\) is proportional to T. brucei EC\(_{50}\) our preferred means of defining selectivity (S) was therefore to use activity against the parasite as a surrogate for TbNMT IC\(_{50}\) when comparing activity against inhibition of HsNMT, to give a good rank order:

\[
S = \text{IC}_{50}\text{HsNMT}/\text{EC}_{50}\text{T. brucei}
\]

In this paper, we report the systematic optimization of both the blood–brain barrier penetration and selectivity of 1, which has led to the discovery of CNS penetrant TbNMT inhibitors suitable for progression into in vivo proof-of-concept studies in mice for the second stage of HAT. A summary of our structure–activity findings is shown in Figure 2.

**RESULTS AND DISCUSSION**

In this paper, we report the systematic optimization of both the blood–brain barrier penetration and selectivity of 1, which has led to the discovery of CNS penetrant TbNMT inhibitors suitable for progression into in vivo proof-of-concept studies in mice for the second stage of HAT. A summary of our structure–activity findings is shown in Figure 2.

Unfortunately, we were unable to obtain crystal structures of TbNMT. However, we were able to obtain structural information for the Leishmania major NMT (LmNMT) and Aspergillus fumigatus NMT (AfNMT). The TbNMT, LmNMT, AfNMT, and HsNMT have very high sequence identity in the binding site for these class of inhibitors (Supporting Information, Table S7). Therefore, we decided to use the LmNMT and AfNMT as surrogates. These should be of use in determining the binding mode of the inhibitors and suggesting vectors for chemistry optimization. However, given the similarities of the active sites, using the crystallography to derive selectivity may be very challenging, especially where factors such as the conformational flexibility of the protein and interactions with water may play a key role.

**Optimization of the Pyrazole Head Group.** We had previously shown that we were unable to vary the pyrazole headgroup for other heterocycles without significant loss in potency and that removal of the N-methyl or replacements with other alkyl groups gave a significant loss in activity.\(^{14}\) From the Leishmania major cocrystal structure with 1, the N1-methyl group binds in a hydrophobic pocket and is important in fixing N2 as hydrogen bond acceptor in a key hydrogen bonding interaction with the hydroxyl side chain of a highly conserved serine residue. However, substituents bigger than methyl on N1 would be expected to suffer a clash in the active site (see Figure 3).

The importance of both the pyrazole 3- and 5-methyl groups can be seen by comparing 2, 3, and 4 with 1 (see Table 1), which were prepared according to Scheme 1. It was possible to remove each of the methyls independently without affecting the enzyme potency, but removal of both methyls caused a significant loss in enzyme potency. Interestingly, removal of the 3-methyl group appeared to cause a reduction in parasite potency in contrast to removal of the 5-methyl group (i.e., 2 vs 3). The 3- and 5-methyl groups both make hydrophobic contacts with the protein. It was possible to increase the size of the substituent at either the 3- or 5-positions (5 and 6),

**Figure 2. Summary of structure–activity relationships of (1).**

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although there was no apparent increase in enzyme potency. However, the iso-butyl substituent on the 3-position (6) gave a significant increase in activity against the parasite. As well as being one of the most active compounds from this series, compound 6 also benefited from having nearly a 10-fold higher oral exposure in mouse than 1 (10 mg/kg po AUC0−8, 3400 μg min/mL cf. 392 μg min/mL; bioavailability 93% cf. 20%) and correspondingly demonstrated superior efficacy to 1 in the stage 1 model in mice infected with T. b. rhodesiense STIB900 (minimal curative dose was 6 mg/kg cf. 50 mg/kg when dosed for 4 days b.i.d. po). Although it had higher efficacy than 1, compound 6 was nonselective, with no overall improvement in stage 1 HAT therapeutic window (2−3-fold; MTD in mice 20 mg/kg b.i.d. po). Indeed, the very high potency of 6 for HsNMT could be the driving force for a high antiproliferative effect against the MRC-5 cell line (EC50 = 0.090 μM), thus eliminating it from further progression for HAT. Moreover, compound 6 also had very low brain penetration in mouse (brain:blood ratio <0.1). So, although potency was increased, there was no overall gain in terms of selectivity or brain penetration.

Investigation of SAR at the Central Aromatic Ring.

One or both of the chloro substituents on the central aromatic ring of 1 were replaced with fluorine, methyl, or hydrogen, intended to modulate localized van der Waals interactions, but this did not have a significant effect on activity or selectivity for HsNMT.

Table 1. Modifications to the Head Group Pyrazole

<table>
<thead>
<tr>
<th>No.</th>
<th>R</th>
<th>T. bINMT IC50 (μM)</th>
<th>HsNMT IC50 (μM)</th>
<th>T. brucei EC50 (μM)</th>
<th>S</th>
<th>B:B</th>
<th>MRC-5 EC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>0.002a</td>
<td>0.003</td>
<td>0.002</td>
<td>1.5</td>
<td>&lt;0.1</td>
<td>0.30</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.005</td>
<td>0.009</td>
<td>0.024</td>
<td>&lt;1</td>
<td>nd</td>
<td>1.23</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.004</td>
<td>0.012</td>
<td>0.002</td>
<td>6.0</td>
<td>nd</td>
<td>0.25</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.039</td>
<td>0.059</td>
<td>0.37</td>
<td>&lt;1</td>
<td>nd</td>
<td>10.3</td>
</tr>
<tr>
<td>5</td>
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<td>0.025</td>
<td>0.005</td>
<td>5.0</td>
<td>nd</td>
<td>0.64</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.002a</td>
<td>0.004</td>
<td>0.0004</td>
<td>10</td>
<td>&lt;0.1</td>
<td>0.09</td>
</tr>
</tbody>
</table>

aIC50 values are shown as mean values of two or more determinations. Standard deviation is typically within 2-fold from the IC50. bCompounds with Hill slopes >1.S = IC50 HsNMT/EC50T. brucei. B:B is the brain:blood ratio in mouse. nd = not determined.
TbNMT (see Scheme S1 and Table S1, Supporting Information).

**Optimization of the Piperazine Ring.** It was decided to investigate the influence of substituents $\alpha$- to the terminal nitrogen of the piperazine on the selectivity of 1. Reference to the structure of 1 in LmNMT showed a pocket which was not accessed but could accommodate extra functionality (see Figure S1, Supporting Information). This investigation found that small substituents were tolerated but did not give a significant rise in selectivity, $S$. Larger substituents caused a loss in activity (see Scheme S2 and Table S2, Supporting Information).

The effect of removing one or both of the pyridyl and internal piperazine nitrogens (Scheme 2) was investigated with the aim of reducing the PSA to increase CNS penetration because high PSA is well-known to negatively correlate with passive membrane permeability (see Table 2). Removal of the pyridyl nitrogen atom (i.e., 8) had virtually no effect on activity at either enzyme or cellular level. Removal of the piperazine nitrogen atom in addition to the pyridine (i.e., 9) did not significantly affect activity against TbNMT, although there was a reduction in activity (~10-fold) against the parasite. Although 8 and 9 have reduced PSA compared to 1 (PSA = 79 and 76 Å$^2$ vs 92 Å$^2$, respectively), the desired improvement in the brain:blood ratio was not forthcoming with 8. Capping (alkylating) of the piperazine nitrogen (10) caused a noticeable reduction in potency against the parasite.

**The Effect of Modifications to the Linker.** Previous work$^{14}$ had indicated that compounds with an alkyl chain replacing the lower biaryl aromatic linker of 1 had a better degree of selectivity (up to 60-fold) at the enzyme level (see compounds in Figure 4). Therefore, it was decided to investigate modification of the linker. Scheme 3 summarizes the synthetic routes employed to prepare linker-modified compounds 12−23. Compounds 12 and 13 were prepared using the Sonogashira reaction$^{19}$ of sulfonamide 7 with 5-

**Table 2. Modifications to the Piperazine**

<table>
<thead>
<tr>
<th>No.</th>
<th>R</th>
<th>TbNMT IC$^{50}$ (pM)$^a$</th>
<th>HsNMT IC$^{50}$ (pM)$^a$</th>
<th>T. brucei EC$^{50}$ (pM)</th>
<th>$S$</th>
<th>B:B</th>
<th>MRC-5 EC$^{50}$ (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$X = N$</td>
<td>0.002$^2$</td>
<td>0.003</td>
<td>0.002</td>
<td>1.5</td>
<td>&lt; 0.1</td>
<td>0.30</td>
</tr>
<tr>
<td>8</td>
<td>$X = C$</td>
<td>0.007$^3$</td>
<td>0.017</td>
<td>0.006</td>
<td>2.8</td>
<td>&lt; 0.1</td>
<td>0.73</td>
</tr>
<tr>
<td>9</td>
<td>$X = C$</td>
<td>0.007</td>
<td>0.023</td>
<td>0.023</td>
<td>1.0</td>
<td>nd</td>
<td>1.5</td>
</tr>
<tr>
<td>10</td>
<td>$X = N$</td>
<td>0.015</td>
<td>0.025</td>
<td>0.020</td>
<td>1.3</td>
<td>nd</td>
<td>0.14</td>
</tr>
</tbody>
</table>

$^a$IC$^{50}$ values are shown as mean values of two or more determinations. Standard deviation is typically within 2-fold from the IC$^{50}$. $^b$Compounds with Hill slopes > 1.5. $S = IC^{50}_{TbNMT}/IC^{50}_{HsNMT}$ T. brucei. B:B is the brain:blood ratio in mouse. nd = not determined.

![Scheme 2. Synthesis of Compounds in Table 2](image-url)
ethynyl-1-methyl-1H-imidazole and 1-methyl-4-(prop-2-yn-1-yl)piperazine, respectively. In general, sulfonamides \(14-23\), which bear a pendant amine attached via a saturated alkyl chain, were prepared using a 9-BBN mediated boron-alkyl Suzuki-type coupling reaction\(^{20}\) between aryl bromide \(7\) or \(11\) and the appropriate alkene, i.e. compounds \(14-17\). Compounds \(20\) and \(21\) were prepared by direct addition of the appropriate alkene while secondary amines \(18\), \(19\), \(22\), and \(23\) were prepared in two steps by coupling with the corresponding BOC-protected alkenes, followed by deprotection with TFA/DCM.

Rigid propargylic systems bearing protonatable tail groups did not have appreciable activity (i.e., \(12\) and \(13\)) (see Table 3). Attachment of the terminal piperazine to the aryl core via a flexible propyl linker (i.e., \(14\)) not only retained reasonable activity but resulted in a marked increase in isoform selectivity (\(>100\)-fold) relative to \(1\). We cannot rationalize the origin of this selectivity by crystallography, as we do not have structures of the \(Tb\)NMT and \(Hs\)NMT complexes. Homology models predict very similar structures in the active sites; therefore, the selectivity may be due to differences in the protein structures remote from the active site, differential protein dynamics\(^{21}\) or the differential effects of interactions with water in the active site.

The structure of \(14\) bound to \(Af\)NMT (Figure 5) confirmed that substitution of the rigid biaryl system with a flexible linker does not alter the binding mode with the piperazine NH interacting with the C-terminal carboxylate via a conserved water molecule, as described for \(1\).

Compound \(14\) was selected as a new lead because, in addition to having significantly improved selectivity (\(S = 20\)), it was fully curative in a stage 1 mouse model (\(T. b. brucei\) S427) at a dose of \(4 \times 50\) mg/kg b.i.d po for 4 days. Work subsequently focused on increasing CNS penetration (brain-blood ratio of \(14 < 0.1\)) and selectivity of this new framework. Removal of the internal piperazine nitrogen of \(14\) resulted in a 10-fold improvement in potency (\(16\) (\(EC_{50} = 0.002\) \(\mu\)M) and also a small reduction in PSA. Although deletion of this nitrogen appeared to cause a reduction of selectivity at the biochemical level compared to \(14\) (from \(>100\)-fold to approximately \(16\)-fold), comparison of selectivity (\(S\)) shows that this modification is selectivity neutral.

Extending the linker by an additional methylene unit (i.e., \(20\) cf. \(16\) and \(22\) cf. \(18\)) increased activity substantially, resulting in subnanomolar potency against the parasite, although notable gains in selectivity were not achieved with the increased molecular flexibility. Comparison of the structures of \(19\) and \(23\) in \(Ln\)NMT shows that the extra methylene in the linker does not significantly impact the binding mode of the inhibitor (data not shown). Removal of the piperidine \(N\)-methyl groups of \(16\) and \(20\) appeared to reduce potency marginally. Removal of the chlorine atoms from \(14\), \(16\), \(18\), \(20\), and \(22\) consistently reduced potency by at least 10-fold (i.e., \(14\) vs \(15\); \(16\) vs \(17\) and \(20\) vs \(21\) etc.). Indeed, this appeared to be a general phenomenon within this subseries. The impact of removing the piperidine methyl group on selectivity was greater for the three-carbon linker than the four-carbon linker. Unfortunately, none of the compounds in Table 3 have a brain:blood ratio >0.1 (\(14\), \(16\), and \(17\) were tested).

Improving CNS Penetration by “Capping” the Secondary Sulfonamide. It is notable that there are very

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**Scheme 3. Synthesis of Compounds in Table 3\(^{\ast}\)**

\(\text{(i)}\) Alkene, 9-BBN, THF, 90 °C, then aryl bromide \(7\) or \(11\), Pd(PPh\(_3\))\(_4\), K\(_3\)PO\(_4\), THF/H\(_2\)O; \((\text{ii})\) alkene, 9-BBN, THF, 90 °C, then aryl bromide \(7\) or \(11\), Pd(PPh\(_3\))\(_4\), K\(_3\)PO\(_4\), THF/H\(_2\)O followed by TFA, DCM, \((\text{iii})\) \(7\), alkyne, CuI, Pd(PPh\(_3\))\(_4\), HNiPr\(_2\).
few examples of CNS penetrant secondary sulfonamides in the literature.22−24 The secondary sulfonamide has an appreciable acidity, particularly in compounds where the sulfonyl aromatic bears two halogen atoms (i.e., $pK_a$ of 7 = 6.9 ± 0.5, $pK_a$ of 1 = 7.0 ± 0.5, calculated using ACDlabs software, version 11.02), suggesting the sulfonamide is significantly ionized at physiological pH. Moreover, the secondary sulfonamide exerts a considerable contribution to the overall PSA of compounds in this series (approximately 54 Å$^2$). Therefore, the sulfonamide was substituted with a variety of capping groups designed to simultaneously reduce PSA and preclude deprotonation of the sulfonamide.

Capping the sulfonamide nitrogen of 7 with a methyl group (i.e., 24) was found to significantly enhance brain penetration in mice (brain:blood ratio of 7 = <0.1 cf. brain:blood ratio of 24 = 3.7) without unduly affecting potency. Indeed, this modification caused a significant enhancement in CNS penetration across a variety of inhibitors bearing different terminal amines, implicating the sulfonamide moiety as the principal cause of the poor CNS penetration of the parent compounds (see Table 4).

In general, the compounds bearing a tethered amine in Table 4 were prepared via a Suzuki-type reaction $^{20}$ with sulfonamides 24−28, which had been modified by N-alkylation of sulfonamide 7 (Scheme 4). For instance, compound 40 was prepared by the 9-BBN mediated addition of 4-allyl-1-methylpiperidine with 28. Notably, we believe this is the first

<table>
<thead>
<tr>
<th>No.</th>
<th>R</th>
<th>X</th>
<th>T$b$NMT $IC_{50}$ (pM)$^a$</th>
<th>H$n$NMT $IC_{50}$ (pM)$^a$</th>
<th>T.brucei $EC_{50}$ (pM)</th>
<th>S</th>
<th>MRC-5 $EC_{50}$ (pM)</th>
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<td>0.029</td>
<td>0.009</td>
<td>3.2</td>
<td>5.3</td>
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$^a$IC$\text{_{50}}$ values are shown as mean values of two or more determinations. Standard deviation is typically within 2-fold from the IC$\text{_{50}}$. $^b$Compounds with Hill slopes >1.5. S = IC$\text{_{50}}$ H$n$NMT/EC$\text{_{50}}$ T. brucei.

Figure 5. Binding mode of 14 (C atoms gold) bound to AfNMT showing the interaction between the piperazine NH and a water molecule coordinated with the C-terminal carboxylate. The binding mode of 1 bound to LmNMT (C atoms slate) is shown for comparison of the piperazine moieties.
reported application of the difluoromethylation of a secondary sulfonamide, achieved by reaction of 7 with sodium chlorodifluoroacetate. Methylation of the sulfonamide of lead compound 14 (i.e., 33) demonstrated that a compound within this series could deliver the desired profile for potency (TbNMT IC₅₀ = 0.003 μM, EC₅₀ = 0.010 μM), selectivity (S = 19), and good brain penetration (brain:blood ratio = 0.6). However, 33 exhibited very poor metabolic stability when incubated with mouse microsomes relative to its parent secondary sulfonamide 14 (CLI = 7.4 cf. 1.7 mL/min/g, respectively). While both microsomal stability and CNS exposure could be improved by resorting to the ethyl sulfonamide analogue 34 (brain:blood ratio = 1.3 cf. 0.6; CLI = 3.1 cf. 7.4 mL/min/g for 34 and 33, respectively), microsomal instability was still higher than compound 1, and this compound had poor oral exposure in mice. Similarly, the more potent piperidine congener of 33, i.e., 37, also exhibited a higher microsomal turnover relative to its parent secondary sulfonamide (i.e., compare 37 vs 16, CLI = 7.3 cf. 4.3 mL/min/g, respectively). An hepatic portal vein study in male Sprague–Dawley rat dosed orally with 33 (3 mg/kg) indicated almost total hepatic extraction of the parent (see Figure S2 in Supporting Information), and sulfonamide dealkylation was suspected as the principal mode of elimination. Incubation of compounds 33, 34, 37, and 39 with mouse microsomes, which showed rapid disappearance of parent and the N-dealkylated sulfonamide as the predominant metabolite for all compounds, supports this theory (see the metabolite identification study, Table S4 in Supporting Information).

**Optimization of the Sulfonamide Capping Group.**

A variety of compact fluoroalkyl groups were surveyed as sulfonamide caps with the aim of improving metabolic stability and therefore oral exposure (compounds 40 to 42 in Table 4). The 2-fluoroethyl analogue was not included in this study due to its reported potential for conversion to fluoroacetic acid, a metabolic poison. On the basis of higher potency and lower PSA, piperidine 37 instead of 33 was adopted as the test-bed for this investigation. Replacement of the methyl group of 37 with a 2,2-difluoroethyl group (i.e., 41) and 2,2,2-trifluoroethyl group (i.e., 42) successfully resulted in a reduction in microsomal turnover as desired (CLI = 7.3, 2.2, and 1.1 mL/min/g, respectively), albeit at the expense of potency, which was reduced approximately 5-fold in both cases. Encouragingly, however, the novel difluoromethylated sulfonamide 40 demonstrated a potency profile similar to the parent compound 37 and had significantly improved stability (i.e., CLI = 2.5 mL/min/g compared to 7.3 mL/min/g for 37). In addition to benefiting from greater mouse microsomal stability compared to 33, 34, 37, and 39, compound 40 did not undergo sulfonamide N-dealkylation (see metabolite identification study, Table S4 in Supporting Information). Consistent with greater sulfonamide stability relative to 33, compound 40 demonstrated good oral exposure in mouse and crucially

### Table 4. Effect on the Brain:Blood Ratio of “Capping” the Sulfonamide with an Alkyl Group

<table>
<thead>
<tr>
<th>No.</th>
<th>R</th>
<th>X</th>
<th>TbNMT IC₅₀ (pM)</th>
<th>HbNMT IC₅₀ (pM)</th>
<th>TbBrain EC₅₀ (pM)</th>
<th>CLI (mouse)</th>
<th>B:B</th>
<th>MRC-5 EC₅₀ (pM)</th>
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<tr>
<td>7</td>
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<tr>
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<td>5.1</td>
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<td>27.2</td>
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<td>H</td>
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<td>0.003</td>
<td>0.002</td>
<td>&lt;1.0</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
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<td>0.004</td>
<td>0.002</td>
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<tr>
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<td>0.2</td>
<td>1.2</td>
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</tr>
<tr>
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<td>0.043</td>
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<td>0.087</td>
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<td>0.023</td>
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<td>&lt;0.1</td>
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<td>0.010</td>
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<td>H</td>
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<td>0.002</td>
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<td>1.1</td>
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<tr>
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<td>0.039</td>
<td>0.19</td>
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<td>0.003</td>
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<td>0.012</td>
<td>0.001</td>
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<td>1.6</td>
<td>1.0</td>
<td>38</td>
</tr>
<tr>
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<td>0.018</td>
<td>0.013</td>
<td>2.2</td>
<td>nd</td>
<td>3.8</td>
<td>38</td>
</tr>
<tr>
<td>42</td>
<td>CH₅F₂</td>
<td>0.004</td>
<td>0.25</td>
<td>0.011</td>
<td>1.1</td>
<td>0.7</td>
<td>19</td>
<td>19</td>
</tr>
</tbody>
</table>

*IC₅₀ values are shown as mean values of two or more determinations. aY = CI for all compounds except compounds 30, 31, 32, and 38, where Y = H. bThe blood:brain ratio was unchanged when measured in wild-type and mdr1a-deficient mice, indicating that neither are Pgp substrates. cCompounds with Hill slopes >1.5. dCLI measured in mL min⁻¹ g⁻¹. B:B is the brain:blood ratio in mouse. nd = not determined.*
retained appreciable CNS penetration (brain: blood ratio = 1.6). Unfortunately, some of the compounds described above showed significant inhibition of hERG, which would need further investigation if these compounds were to be progressed (i.e., hERG IC50 = 0.6 μM cf. hERG IC50 = 28 μM).

Further Optimization of the Linker and Amine on a Tertiary Sulfonamide. Due to its superior potency and improved metabolic stability the difluoromethyl group was retained as the preferred sulfonamide cap in a final study sought to further optimize potency and selectivity of 40 by making modifications in both the tether and the pendant amine portions. The crystal structure of 43 bound to AfNMT confirms that the binding mode of the sulfonamide is largely unaffected by capping with the CHF2 group (see Figure 6).

Compounds were prepared using analogous procedures to those already described according to the conditions shown in Scheme 5. This study resulted in the identification of a large number of highly trypanocidal compounds, some with good CNS penetration (see Table 5 and Table S3, Supporting Information). Unfortunately, however, it proved challenging to obtain substantial selectivity at the enzyme level and there was not an observable trend. Although primary potencies against TbNMT remain substantially unvaried in the low nanomolar range, there is wider variation in activity against the parasite; this is probably a consequence of the inability of the enzyme

Figure 6. Stereo diagram showing comparison of binding modes of 43 (C atoms gold) and 1 (C atoms cyan) showing retention of sulfonamide configuration after capping with a CHF2 group.

Scheme 4. Synthesis of Compounds in Table 4

```
(i) NaH, DMF, Mel or EtBr; (ii) CF3CH2OMs, K2CO3, CH3CN; (iii) CHF2CH2I, K2CO3, CH3CN; (iv) Na+ CF2ClCO2−, K2CO3, CH3CN; (v) alkene, THF, 9-BBN, then aryl bromide 24−28, Pd(PPh3)4, K3PO4, THF/H2O; (vi) 3-formylphenyl boronic acid, Pd(PPh3)4, K3PO4, THF/H2O; (vii) piperazine, NaBH(OAc)3, CHCl3; (viii) Pd(PPh3)4, 2-(1-piperazinyl)pyridine-4-boronic acid pinacol ester, K3PO4, THF/H2O; (ix) pent-4-en-1-ol for compound 35 or hex-5-en-1-ol for compound 36, THF, 9-BBN, then aryl bromide 24, Pd(PPh3)4, K3PO4, THF/H2O; (x) MsCl, NEt3, pyridine, then imidazole.
```
assay to discriminate the most potent compounds due to the tight binding nature of enzyme inhibition (many compounds had high Hill slopes, i.e. $H_s ≫ 1$). 

Key points noted from these studies:

- In the one example for which there is data, converting the terminal tertiary amine to a secondary amine (40 cf. 43) caused a large drop in the brain:blood ratio (falls from 1.6 to 0.03).
- Replacement of the piperidine of 40 with a piperazine (i.e., 44) caused an approximately 10-fold reduction in cellular potency, consistent with previous observations.
- Compound 53, a bicyclic analogue of 44, exhibited the best CNS penetration observed within this series (brain:blood ratio = 2.6). However, this compound suffered from high microsomal clearance ($Cl_h = 15 \text{ mL/min/g}$) and no improvement to oral exposure in mouse compared to the lead compound 40.
- An increase in the length of the propyl tether of 40 by an additional methylene (i.e., 46), or an oxygen atom (i.e., 47) resulted in similar or slightly better trypanocidal activity while retaining brain:blood ratio.
- Removal of the piperidine ring and replacement by an acyclic linker of comparable length to 46 and 47 was also tolerated (i.e., 48, $T. \text{ brucei EC}_{50} = 0.002 \mu \text{M}$). However, these acyclic linkers were very sensitive to the substituents on the terminal amine, with potency decreasing rapidly as the size of substituents on the basic nitrogen is increased (see Table S3 in Supporting Information). While this makes sense intuitively, because the N-substituent could potentially impede contact between the basic and acidic centers, it was hoped to strike a potential balance between potency and selectivity by branching into unaccessed space.

It was decided to see if selectivity ($S$) could be improved by the introduction of chiral amines, with substituents which could potentially access different subpockets in the region bound by the terminal amine (see compounds 49–53 in Table 5). These modifications resulted in some compounds with subnanomolar potency against $T. \text{ brucei}$ (see Table S3, Supporting Information) and compounds with high selectivity at the biochemical level (i.e., $Hs_{NMT}/TbNMT$ is $>100$-fold for 50). However, there was no substantial improvement in selectivity ($S$) relative to 40, with these compounds having a limited range of varying degrees of selectivity for compounds 50 ($S = 47$), nor a substantially greater window at the whole protein level (i.e., $T. \text{ brucei EC}_{50}$ vs MRC-5) achieved compared with 40, which already has an approximately 1000-fold differential. Additionally, these compounds generally exhibited higher microsomal intrinsic clearances than 40 and were therefore ruled out of selection for progression into animal models. Comparison of compounds 49–52 (i.e., 49 and 50, cf. 51 and 52) suggests that the $S$-enantiomers show a marginally greater effect on selectivity ($S$) compared to the R-enantiomers.

In addition to those compounds described in Tables 4 and 5, a variety of other amine-bearing cores appended via flexible linkers of varied length to the benzenesulfonamide were evaluated, although none were able to provide the desired profile in terms of potency and selectivity (see Table S3, Supporting Information).

**Efficacy in a Stage 2 Model of HAT for “Capped” Sulfonamide Lead Compounds.** On the basis of their trypanocidal activity, oral pharmacokinetics, brain:blood ratio, and confirmed fully curative activity in the stage 1 mouse model ($T. \text{ b. brucei S427}$, 50 mg/kg b.i.d. po for 4 days), compounds 40, 46, and 42 were selected for evaluation in the stage 2 mouse model of HAT ($T. \text{ b. brucei GVR35}$). With the assumption that the brain interstitial fluid is the biophase for effective cure of stage 2 HAT and that $EC_{99}$ drug levels are required, it was calculated (using $T. \text{ b. brucei S427 EC}_{50}$ mouse oral pharmacokinetics, measured brain total concentration, and brain free fraction) that an oral dose of 20 mg/kg b.i.d., 15 mg/kg b.i.d., or 250 mg/kg b.i.d. for 40, 46, and 42, respectively, would need to be delivered to deliver stage 2 cures based on maintaining the brain free concentration (as a surrogate of brain interstitial fluid levels ($C_{fs}$)) above the $T. \text{ brucei EC}_{99}$ (see Table S5, Supporting Information for dose calculations). All the compounds were dosed in the stage 2 mouse model of HAT ($T. \text{ b. brucei GVR35}$) at the MTD (100, 50, or 150 mg/kg b.i.d. po for 40, 46, and 42, respectively) for 5 days. However, some
toxicity was observed at this dose regimen in infected mice for both 40 (1/5 mice) and 46 (2/5 mice). Although 42 was well tolerated, none of the mice were cured, which was somewhat expected based on the above calculation. For 40 and 46, further dosing regimens were tested in order to try and achieve in vivo proof-of-concept in a stage 2 HAT model (40: 50 mg/kg b.i.d. po for 10 days, 100 mg/kg b.i.d. po for 8 days, and three rounds of 3 day dosing at 100 mg/kg with a two day “drug holiday” between rounds. 46: 25 mg/kg b.i.d. for 10 days, 50 mg/kg b.i.d. po for 8 days). Encouragingly, the two 100 mg/kg extended dosing schedules for 40 were partially efficacious (1/5 mice). However, no cures were achieved at 50 mg/kg b.i.d. po for 10 days with either dose regimen of 46. Indeed, significant toxicity was observed for the extended 50 mg/kg dose regimen of 46. Kaplan–Meier graphs for efficacy of compound 40 in continuous and pulsed dosing regimens are shown in Figure 7.

Although this outcome is encouraging for 40, it was disappointing that full cures were not achieved considering a 20 mg/kg b.i.d. oral dose was predicted to be efficacious. A possible explanation for this is the difference observed in minimal curative efficacy to different parasite strains, as has already been seen for key NMT compounds. For example, an efficacy study of compound 46 showed approximately a 4-fold reduction in efficacy in an acute model of T. b. brucei GVR35 (minimum curative dose 25 mg/kg b.i.d. po for 4 days) compared to T. b. brucei S427 (minimum curative dose 6 mg/kg b.i.d. po for 4 days). The predicted efficacy of 40 was based on T. b. brucei S427 data, and if efficacy to T. b. brucei GVR35 is
4–5-fold less sensitive (not tested), as was observed for 46, a 100 mg/kg b.i.d. po dose regimen of 40 would be on the cusp of efficacy. It was therefore unfortunate that a lack of tolerability prevented higher dosing to deliver full cures. While compounds 49 and 50 have better selectivity (S) than 40 and may therefore be better tolerated at a higher dose required for efficacy, their high metabolic instability ruled them out from further evaluation.

## CONCLUSIONS

In this article, we describe optimization of the lead pyrazole sulfonamide 1 which resulted in compound 40, which has markedly increased blood–brain barrier penetration and improved selectivity. We have a more complete understanding of the SAR of this series and how to achieve blood–brain barrier penetration by capping the sulfonamide group which reduces the PSA and acidity. While a greater degree of selectivity has also been obtained, achieved through introduction of a flexible linker between the pyrazole and amine, the reasons for this are not fully understood. It is important to note that the downside of these modifications to 1 is a significant increase in lipophilicity (cLogP of 1 and 40 is 3.1 and 4.7 respectively), which, when combined with a reduction in PSA, potentially increases the risk of off-target activity.27 This may be the cause of the poor tolerability of compound 40.

Evaluation of key tool compounds from this series in a CNS model of HAT resulted in partial efficacy. Lack of full cure is likely to be a consequence of insufficient therapeutic window to be able to deliver fully efficacious free drug concentration to the brain at a tolerated dose, further confounded by the lower efficacy of these compounds toward the T. b. brucei GVR35 strain compared to the T. b. brucei S427 strain. Compounds with better selectivity would hopefully allow dosing at either higher levels or for longer duration and therefore result in a fully curative stage 2 TbNMT lead. It is, however, encouraging that some cures and prolongation of survival in the stage 2 model is observed via blockade of TbNMT because this increases confidence in the validity of this target for the development of therapeutics for stage 2 HAT. The compounds described above, including compound 40, and the increased understanding of how to optimize compounds for both BBB penetration and selectivity represent a very useful platform for further studies.

## EXPERIMENTAL SECTION

General Experimental Information. Chemicals and solvents were purchased from the Aldrich Chemical Co., Fluka, ABCR, VWR, Acros, Fisher Chemicals, and Alfa Aesar and were used as received unless otherwise stated. Air- and moisture-sensitive reactions were carried out under an inert atmosphere of argon in oven-dried glassware. Analytical thin-layer chromatography (TLC) was performed on precoated TLC plates (layer 0.20 mm silica gel 60 with fluorescent indicator UV254, from Merck). Developed plates were air-dried and analyzed under a UV lamp (UV2 54/365 nm). Flash column chromatography was performed using prepacked silica gel cartridges (230–400 mesh, 40–63 μm, from SiliCycle) using a Teledyne ISCO CombiFlash Companion or CombiFlash Retrieve. 1H NMR, 13C NMR, 19F NMR, and 2D-NMR spectra were recorded on a Bruker Avance DPX 500 spectrometer (1H at 500.1 MHz, 13C at 125.8 MHz, 19F at 470.5 MHz). Chemical shifts (δ) are expressed in ppm recorded using...
allowed to warm to rt over 4 h. The reaction was concentrated to 111.7, 109.1, 108.9, 42.7, 42.0, 36.2, 10.4. HRMS (ES+, 70 V) 404.1 (MH+).

Prototypical Procedure for Preparation of a Sulfonamide from an Amine and a Sulfonil Chloride: 4-Bromo-N-(1,3,5-trimethyl-1H-pyrazol-4-yl)-benzenesulfonamide (TI). 4-Bromobenzensulfonyl chloride (5.0 g, 19.6 mmol) was added portionwise to a stirred solution of 4-amino-1,3,5-trimethyl-1H-pyrazol-4-yl (2.45 g, 19.6 mmol) in pyridine (50 mL) at rt. The reaction was stirred for 24 h then concentrated to dryness in vacuo. The resulting residue was diluted with DCM (100 mL), washed with aqueous sodium hydroxide solution (0.5M, 100 mL), organic phase separated, dried (MgSO4), and concentrated in vacuo. Trituration of the residue with diethyl ether gave a precipitate, which was collected by vacuum filtration. Microwave-assisted chemistry was determined using this analytical LC–MS method. Microwave-assisted chemistry was performed using a Biotage Initiator Microwave Synthesizer. The reaction mixture was then concentrated in vacuo, diluted with DCM (100 mL) and water (100 mL), the organic phase separated, dried (MgSO4), and concentrated in vacuo. Chromatography (SiO2, 40 g, 92.2% purity, 2.1 mm, 3.5 μm particle size; J. Med. Chem. 28 (13) 3032–3035) gave the title compound in 14.8 mmol, 79%). 1H NMR (500 MHz, DMSO-d6): 6.97 (9H, s), 2.08 (3H, s), 1.61 (3H, s). HRMS (m/z): [M + H]+ calcld for C22H31N4SO2Cl2F2, 523.1507; found, 523.1490. 13C NMR (125 MHz, DMSO-d6): δ 140.3, 135.2, 132.0, 129.5, 111.1, 105.9, 53.3, 42.5, 36.5, 34.6, 33.8, 32.4, 32.0, 26.8, 10.2, 8.6. 19F NMR (470 MHz, DMSO-d6): δ −92.2, J = 6.03 Hz 207.8 Hz, 1F). −94.4 (dd, J = 60.3 Hz 207.5 Hz, 1F), spectrum was not 'H-decoupled.

Enzyme Inhibition Assay. N-Myristoyltransferase is an enzyme that catalyzes the addition of myristic acid from myristoyl-CoA to the N-terminal glycine residue of numerous substrate proteins and peptides with the subsequent release of coenzyme A. H+-labeled myristoyl-CoA (GE Healthcare) can be used in the reaction to transfer H+-myristic acid to a biotinylated substrate peptide (GCGGSKVQPPQQAK(biotin)-amide, Pepceuticals Inc.). The reaction can be measured by the subsequent binding of the labeled peptide to streptavidin-coated scintillation proximity assay (SPA) beads (GE Healthcare) and monitoring of β-particle excitation of the embedded scintillant. Measurement of the ability of compounds to inhibit the N-myristoyltransferase enzyme(s) of human (HNM1-NT and HNM1-NT2) and kinetoplast (T. brucei, T. cruzi, and L. major) species was performed using a modification of the scintillation proximity assay platform described previously by Panethymitaki et al.28 as follows: Compounds were solubilized in DMSO at a concentration of 10 mM and serially diluted in half-log steps to achieve a range of final assay concentrations of 100 μM to 1 nM. Compound at each concentration was added to white 384-well plates in a volume of 0.5 μL. N-Myristoyltransferase enzyme (HNM1-NT, HNM1-NT2, TcNMT, TbNMT, or LmNMT), dissolved to a working concentration of 10 nM in assay buffer (30 mM Tris-HCl, pH 7.4, 0.5 mM EGTA, 0.5 mM EDTA, 1.25 mM DTT, 0.1% Triton X-100), was then added to columns 1–11 and 13–23 of the plates in a volume of 2.6-Dichloro-N-(difluoromethyl)-N(1,3,5-trimethyl-1H-pyrazol-4-yl)-benzenesulfonamide (28). A well-stirred slurry of 4-bromo-2,6-dichloro-N-(1,3,5-trimethyl-1H-pyrazol-4-yl)-benzenesulfonamide (3.0 g, 7.62 mmol), potassium carbonate (3.0 g, 21.0 mmol), and sodium chlorodifluoracetate (3.3 g, 21.0 mmol) in acetonitrile (100.0 mL) was heated to 60 °C for 48 h. The resulting slurry was then concentrated in vacuo, diluted with DCM (100 mL) and water (100 mL), the organic phase separated, dried (MgSO4), and concentrated in vacuo. Tritruration of the residue with diethyl ether gave a precipitate, which was collected by vacuum filtration and dried to give the title compound as a white fine powder (2.0 g, 4.43 mmol, 62%). 1H NMR (500 MHz, CDCl3): δ 7.61 (2H, s), 7.34 (1H, dd, J = 59.4 Hz 61.2 Hz, 2.63 (t, J = 5.9 Hz, 1H), 8.20 (s, 2H), 7.61 (s, 1H), 7.40 (d, J = 5.9 Hz, 1H), 4.08 (s br, 4H), 3.63 (s, 3H), 3.28 (s br, 4H), 2.00 (s, 3H), 1.77 (s, 3H). 13C NMR (125 MHz, DMSO-d6): 147.5, 147.3, 143.8, 137.4, 136.3, 135.2, 129.8, 111.8, 111.7, 109.1, 108.9, 42.7, 42.0, 36.2, 10.4. HRMS (m/z): [M + H]+ calcld for C22H31N4SO2Cl2F2, 523.1507; found, 523.1490. 13C NMR (125 MHz, DMSO-d6): δ 140.3, 135.2, 132.0, 129.5, 111.1, 105.9, 53.3, 42.5, 36.5, 34.6, 33.8, 32.4, 32.0, 26.8, 10.2, 8.6. 19F NMR (470 MHz, DMSO-d6): δ −92.2, J = 6.03 Hz 207.8 Hz, 1F). −94.4 (dd, J = 60.3 Hz 207.5 Hz, 1F), spectrum was not 'H-decoupled.

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20 μL. To columns 12 and 24, 20 μL of assay buffer was added to provide a no-enzyme control. Following a 5 min incubation at room temperature, the substrates (GCGGSKVKPQPPQKAK-biotin)-amide and myristoyl-CoA, dissolved in assay buffer, were added to all wells in a volume of 20 μL to start the reaction. The final concentrations of peptide and 2H-myristoyl-CoA were 0.5 μM and 125 nM, respectively, and the specific activity of the radiolabel was 8 Ci/mmol. Plates were then incubated at room temperature for up to 50 min (dependent upon the period of linearity for the different enzyme species) before SPA beads, suspended to 1 mg/mL in a stop solution (200 mM phosphoric acid/NaOH, pH 4, 750 mM MgCl2), were added in a volume of 40 μL. Plates were then read on a TopCount microplate luminometer and data analyzed by calculating the percentage inhibition compared to the maximum and minimum assay controls. Concentration effect curves were fitted using nonlinear regression using XLFit 4.2, and IC50 values were determined.

Cell Viability Assay. Measurement of the ability of the compounds to inhibit human (MRC5, human lung fibroblast cells) and trypanosome (T. b. brucei, BSF427, VSG118) cell growth was performed using a modification of the cell viability assay previously described by Raza et al.16 Compounds were dissolved in DMSO at a top concentration of 10 mM and serially diluted in half log steps to achieve a range of final assay concentrations of 50 μM to 0.5 nM. Compound at each concentration (200-fold final) was added to cell culture plates in a volume of 1 μL. Then 2000 cells per well in relevant growth medium (HMI-9T for clearances (Cl) of each test compound was then calculated using the compound to internal standard at each time point. The rate of intrinsic clearance (Cl) in brain (ng/g) was divided by the concentration in blood (ng/mL) to give a brain: blood ratio. The mean value obtained was quoted.

Efficacy Experiments. Stage 1 efficacy experiments using T. b. brucei S427 were performed according to methods described by Frearson et al.177 T. b. brucei GVR35 Stage 2 Efficacy Protocol. NMRI mice used in the experiment were purchased from Harlan, UK. Mice were maintained under standard temperature/lighting conditions and given food and water ad libitum. The trypanosomes used in this study were of the cloned stablile T. b. brucei GVR35, originally derived from trypanosomes isolated from a wildebeest in the Serengeti in 1966.18 By 13 days postinfection, parasites entered the CNS.19 If treatment is delayed until 21 days after infection, successful cure is therefore dependent on the ability of the compound to penetrate through the blood–brain barrier.20 This model is widely used to evaluate the efficacy of new compounds for treatment against stage 2 HAT.21 Cryopreserved T. b. brucei GVR35 trypomastigotes were diluted to ∼5 × 106 cells/mL in 20 mM Hanks Balanced Salt Solution with glucose, and 0.2 mL of that dilution was used for infection (∼1 × 104 cells/mL per animal). All animals in the groups were intraperitoneally infected (ip) on day 0. After infection, parasitaemia was monitored by examination of tail blood smears. On day 21, post infection animals were randomly allocated to five mice per group. Infected animals were dosed with one of the following: dexamethasone diacetate (ip), 40 mg/kg once only as a HAT stage 1 control (early stage trypanocidal to clear the parasites in the systemic circulation and in tissues other than CNS), melasporol (ip), 10 mg/kg once daily for 5 days as a positive control for HAT stage 2; or test compound (gavage) twice daily for 10 days (i.e., 40, 42, or 46) or twice daily for 3 x 3 days with 2 days rest between dose intervals (i.e., 40). One group of five mice was an untreated control group. The dose solutions were prepared daily, using 5% DMSO:40% PEG400:55% Milli-Q water for compound (i.e., 40) and 10% DMSO:90% peanut oil for control drugs. To measure the response to treatment, parasitaemia was monitored twice per week until day 50. After day 50, the mice were checked only once per week until day 180 post treatment. Mice surviving to the end of experiment were purchased from Harlan, UK. Mice were maintained under standard temperature/lighting conditions and given food and water ad libitum. In stage 1 and stage 2 HAT efficacy models. Blood samples were taken from each mouse at 2 (IV only), 5 (IV only), 15, and 30 min, 1, 2, 4, 6, 8, and 24 (po only) hours postdose and mixed with two volumes of distilled water. After suitable sample preparation, the concentration of test compound in blood was determined by UPLC-MS/MS using a Quattro Premier XE (Waters, USA). Pharmacokinetic parameters were derived from the mean blood concentration time curve using PKSolutions software v 2.0 (Summit Research Services, USA).

Mouse Pharmacokinetics. Test compound was dosed as a bolus solution intravenously at 3 mg free base/kg (dose volume, 5 mL/kg; dose vehicle, saline or 5% DMSO; 40% PEG400; 55% saline) to female NMRI mice (n = 3) or dosed orally by gavage as a solution at 10 and/or 50 mg free base/kg (dose volume, 5 mL/kg; dose vehicle, 5% DMSO; 95% distilled water or 5% DMSO; 40% PEG400; 55% distilled water) to female NMRI mice (n = 3/dose level). Female NMRI mice were chosen as these represent the sex and strain used for the stage 1 and stage 2 HAT efficacy models. Blood samples were taken from each mouse at 2 (IV only), 5 (IV only), 15, and 30 min, 1, 2, 4, 6, 8, and 24 (po only) hours postdose and mixed with two volumes of distilled water. After suitable sample preparation, the concentration of test compound in blood was determined by UPLC-MS/MS using a Quattro Premier XE (Waters, USA). Pharmacokinetic parameters were derived from the mean blood concentration time curve using PKSolutions software v 2.0 (Summit Research Services, USA).

Mouse Brain Penetration. Each compound was dosed as a bolus solution intravenously at 2 mg free base/kg (dose volume, 5 mL/kg; dose vehicle, 5% DMSO; 5% saline) to female NMRI mice (n = 6). At 5 and 30 min following intravenous bolus injection of test compound, mice (n = 3/time point) were placed under terminal anesthesia with isoflurane. A blood sample was taken by cardiac puncture into two volumes of distilled water and the brain removed. After suitable sample preparation, the concentration of test compound in blood and brain was determined by UPLC-MS/MS using a Quattro Premier XE (Waters, USA). For each mouse at each time point, the concentration in brain (ng/g) was divided by the concentration in blood (ng/mL) to give a brain: blood ratio. The mean value obtained was quoted.

Intrinsic Clearance (Cl) Experiments. Test compound (0.5 μM) was incubated with female CD1 mouse liver microsomes (Xenotech LLC; 0.5 mg/mL 50 mM potassium phosphate buffer, pH 7.4) and the reaction started with addition of excess NADPH (8 μg/mL 50 mM potassium phosphate buffer, pH 7.4). Immediately, at time zero, then at 3, 6, 9, 15, and 30 min, an aliquot (50 μL) of the incubation mixture was removed and mixed with acetonitrile (100 μL) to stop the reaction. Internal standard was added to all samples, the samples centrifuged to sediment precipitated protein, and the plates then sealed prior to UPLC-MS/MS analysis using a Quattro Premier XE (Waters corporation, USA).

XLfit (IDBS, UK) was used to calculate the exponential decay and consequently the rate constant (k) from the ratio of peak area of test compound to internal standard at each time point. The rate of intrinsic clearance (Cl) of each test compound was then calculated using the equation Cl (mL/min/g liver) = k × V × microsomal protein yield, where V (mL/g protein) is the incubation volume/mg protein added and microsomal protein yield is taken as 52.5 mg protein/g liver. Verapamil (0.5 μM) was used as a positive control to confirm acceptable assay performance.

Electronic Supplementary Information

Table S6 (Supporting Information).

Fig 1A–C. In the systemic circulation and in tissues other than CNS, Melasporol (ip), 10 mg/kg once daily for 5 days as a positive control for HAT stage 2; or test compound (gavage) twice daily for 10 days (i.e., 40, 42, or 46) or twice daily for 3 x 3 days with 2 days rest between dose intervals (i.e., 40). One group of five mice was an untreated control group. The dose solutions were prepared daily, using 5% DMSO:40% PEG400:55% Milli-Q water for compound (i.e., 40) and 10% DMSO:90% peanut oil for control drugs. To measure the response to treatment, parasitaemia was monitored twice per week until day 50. After day 50, the mice were checked only once per week until day 180 post treatment. Mice surviving to the end of experiment and blood smear aparasitaemic were considered cured.

All regulated procedures in living animals were carried out under the authority of licenses issued by the Home Office under the Animals (Scientific Procedures) Act 1986, as amended in 2012 (and in compliance with EU Directive EU/2010/63). License applications for the use of animals included in this study are held by the respective institute. No specific license or approval was required for the use of parasitic or plasmodial material.
were approved by the University’s Ethical Review Committee (ERC) before submission to the Home Office.

**ASSOCIATED CONTENT**

**Supporting Information**

Synthetic details for all compounds, supplementary data tables, additional information on ADMET and pharmacology, and crystallographic data tables. This material is available free of charge via the Internet at http://pubs.acs.org.

**Accession Codes**

Coordinates and associated diffraction data for complexes of A/NMT with compounds 14 and 43 have been deposited in the Protein Data Bank (PDB) with accession codes 4UWI and 4UWJ, respectively.

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**Notes**

The authors declare no competing financial interest.

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