Diaryl sulfide-based inhibitors of trypanothione reductase: inhibition potency, revised binding mode and antiprotozoal activities†

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Received 15th April 2008, Accepted 5th August 2008
First published as an Advance Article on the web 9th September 2008
DOI: 10.1039/b806371k

Trypanothione reductase (TR) is an essential enzyme of trypanosomatids and therefore a promising target for the development of new drugs against African sleeping sickness and Chagas′ disease. Diaryl sulfides with a central anilino moiety, decorated with a flexible *N*-alkyl side chain bearing a terminal ammonium ion, are a known class of inhibitors. Using computer modelling, we revised the binding model for this class of TR inhibitors predicting simultaneous interactions of the ammonium ion-terminated *N*-alkyl chain with Glu18 as well as Glu465′/Glu466′ of the second subunit of the homodimer, whereas the hydrophobic substituent of the aniline ring occupies the "mepacrine binding site" near Trp21 and Met113. Systematic alteration of the carboxylate-binding fragments and the diaryl sulfide core of the inhibitor scaffold provided evidence for the proposed binding mode. *In vitro* studies showed IC_{50} values in the low micromolar to submicromolar range against *Trypanosoma brucei rhodesiense* as well as the malaria parasite *Plasmodium falciparum*.

Introduction

African sleeping sickness, Chagas′ disease and the different forms of leishmaniasis are tropical diseases caused by protozoan pathogens of the trypanosomatid family. These infections constitute one of the most serious health problems in the developing world.[1] African sleeping sickness (human African trypanosomiasis) is caused by *Trypanosoma brucei* species and transmitted by tsetse flies. Sleeping sickness threatens 50 million people in ~20 countries of sub-Saharan Africa. The number of infected people is difficult to establish accurately but can be estimated to be between 50 000 and 70 000 cases.[2] The pathogen of Chagas′ disease (American trypanosomiasis), *Trypanosoma cruzi*, is transmitted to humans by blood sucking reduviid bugs. The disease is widespread in central and southern America. Leishmaniasis is caused by various species of Leishmania causing different forms of disease ranging from cutaneous to visceral infections. Millions of people are infected and ~50 000 deaths per year are quoted, mainly due to *L. donovani*.[3]

The causative agent of malaria tropica is *Plasmodium falciparum*, a protozoan parasite that is not a member of the trypanosomatid family. This disease, spread by the bite of infected Anopheles mosquitoes, threatens about 40% of the world's population, mostly those living in the poorest countries in tropical regions and causes over 1 million fatalities annually.[4]

For most of those neglected tropical diseases, there are no safe and efficacious drugs available. In the case of malaria, potent drugs such as chloroquine are losing efficacy more and more due to an increase of drug resistances.[3,5] Chemotherapy of all forms of trypanosomiasis is problematic due to the severe side effects of the few drugs in use, the long duration and high costs of treatment and an increasing number of drug resistant pathogens.[5,6] New and better drugs to fight these diseases are therefore urgently needed.

A promising strategy to develop new compounds active against parasites is targeting enzymes of a unique metabolic pathway that is exclusively present in the pathogens and not in the human hosts. In the case of trypanosomatids, their specific thiol redox metabolism distinguishes them from nearly all other eukaryotes and prokaryotes. Instead of the nearly ubiquitous glutathione system composed of glutathione and the flavoenzyme glutathione reductase (GR, EC 1.6.4.2), the parasites possess trypanothione [N^1,N^8-bis(glutathionyl)spermidine] that is kept in its reduced state by trypanothione reductase (TR, EC 1.6.4.8).[7] The trypanothione system protects the parasites from oxidative damage and delivers the reducing equivalents for DNA synthesis. It has been shown in the past that TR is essential for the parasite that renders this enzyme an attractive target for the development of new drugs against trypanosomiasis and leishmaniasis.[6,8,9]

TR and GR belong to the protein family of FAD disulfide oxidoreductases. The mutually exclusive substrate specificity of TR and GR is considered to rely on their oppositely charged disulfide substrate binding sites. These accommodate either trypanothione disulfide (**1** in Fig. 1) with an overall positive charge due to the protonated spermidine bridge or glutathione disulfide (**2** in Fig. 1), with a twofold negative overall charge.

TR is the most thoroughly studied enzyme of the trypanothione redox metabolism. The crystal structure has been determined in free form,[10–12] in complex with its cofactor NADPH,[12] with glutathionylspermidine,[13] with trypanothione,[14] and with the

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† Electronic supplementary information (ESI) available: Additional figures, full experimental details for the modelling, the biological assays and the preparation of compounds **12**, **13**, **15**, **16**, **17**, **20**, **29–33**, **39–43**, **48–52**, **54–60**, **64–67**, **76**, and **78**. See DOI: 10.1039/b806371k

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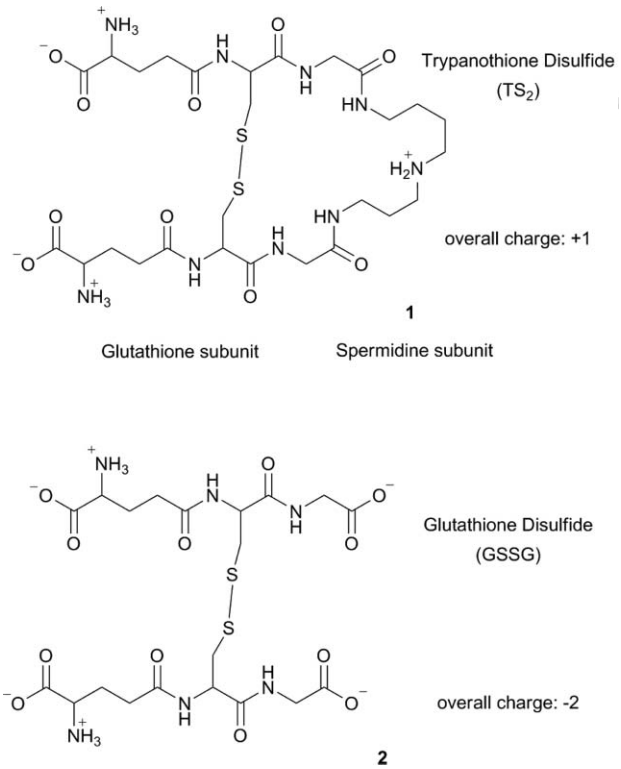

Fig. 1 Above: trypanothione disulfide (**1**), the substrate of trypanothione reductase (TR), found in trypanosomatids. Below: glutathione disulfide (**2**), the substrate of human glutathione reductase (hGR).

competitive inhibitor mepacrine[15] as well as with covalently bound mepacrine derivatives.[16] The homodimeric enzyme has two active sites lying opposed on the interface of the two subunits. With a dimension of approximately $22 \times 20 \times 28$ Å^3, each active site of TR is much wider than those of GR, making it more difficult to develop low molecular weight compounds that bind selectively and with high affinity to the solvent exposed disulfide binding site of the parasite enzyme.

Nevertheless, various compounds have been identified as inhibitors of TR over the last decade.[6,8] Most of them feature, under physiological conditions, a protonated amine or a cationic nitrogen to mimic the positively charged trypanothione. Mepacrine is just one member in the class of tricyclic scaffolds known to interact with TR, among other structurally related compounds such as phenothiazine-based derivatives.[17,18] Opening up the central ring of the tricyclic core of these inhibitors leads to 2-aminodiphenyl sulfide-based derivatives such as the piperazine-decorated compound **3** (Table 1).[19] Upon optimisation of this inhibitor class, two trends emerged: on the one hand, bridging two diphenyl sulfide units *via* an extended amide spacer gave large frameworks such as the dimeric structure **4**[20] or, if an amine spacer was used, diphenyl sulfides as **5**.[21] Both derivatives exhibit IC_{50}-values (concentration of inhibitor resulting in 50% inhibition) in the high nanomolar range under the chosen assay conditions (Table 1). On the other hand, introduction of a permanent positive charge by incorporation of a quaternary ammonium center as the headpiece of a flexible *N*-alkyl chain (compound **6**) increased the potency strongly compared to the piperazine-substituted TR inhibitor **3**. Monomer **6** stood out with a K_{ic} (competitive inhibitor constant) value of 1.7 μM,[22] while the molecular weight of this compound remains moderate compared to the extended dimeric structures of **4** and **5**.

Overall, a clear structure–activity relationship (SAR) has hardly ever been disclosed for TR inhibitors. Computer-aided modelling turned out to be difficult as the huge, solvent exposed active site of the parasite enzyme allows for plentiful binding orientations of the ligands.[23]

Here we present a revised binding model for the class of diaryl sulfide-based TR inhibitors involving the interaction of the ligands with two negatively charged regions of the active site and the occupation of the hydrophobic region near Trp21 and Met113, the "mepacrine binding site". Evidence for this proposed binding mode was found by the SAR derived from a set of newly synthesised TR inhibitors with altered carboxylate ligands and by systematically decorating the phenylthio moiety with hydrophobic substituents. The determination of the antiparasitic properties did not only reveal decent activity against trypanosomes, but also showed potency against the malaria parasite *P. falciparum*.

Results and discussion

Revised binding mode

Apart from mepacrine, no binding mode of any competitive TR inhibitor has been deciphered by protein crystallography to date. Previous molecular dynamics simulations for piperazine **3** predicted two nearly identical binding modes.[24] Each of them predicts interactions of the unsubstituted phenylthio moiety with the hydrophobic groove near Cys52 and Tyr110, whereas the central anilino moiety is oriented towards, but does not occupy, the hydrophobic region near Trp21 and Met113 (Fig. 1 ESI†). The protonated terminal piperazine residue lies in the vicinity of Glu465′ and Glu466′. Both proposed binding modes exclude interactions with the side chain of Glu18. For the rationalisation of the increased binding affinity of cationic benzylammonium phenothiazines compared to non benzylated derivatives, simultaneous binding of the additional benzyl unit to the "Z site" hydrophobic pocket (Fig. 2)—roughly formed by Phe395′, Pro397′ and Leu398′—and ionic interactions of the cationic nitrogen with Glu465′ and Glu466′ were proposed by docking experiments.[18] The same docking procedure applied to cationic diaryl sulfide-based TR inhibitors revealed at least 4 possible binding modes where the cationic nitrogen either interacts with Glu465′/Glu466′ or Ser14.[22]

It could be shown that diaryl sulfide-based compounds act as selective TR inhibitors and do not affect hGR.[19,24] Although the positive charge of TR inhibitors appears to be crucial for the selectivity of these ligands,[25] the previously proposed binding modes consider electrostatical interactions of the diaryl sulfides with Glu465′/Glu466′, residues that are present in TR as well as hGR. In contrast to Glu465′/Glu466′, Glu18 is specific for TR and we therefore assume that this amino acid residue might be of importance for the selective binding of diaryl sulfide-based inhibitors. Further evidence of the role of Glu18 in the recognition of TR inhibitors is provided by the co-crystal structure with mepacrine that undergoes H-bonding to Glu18 mediated by a water molecule.[15] Therefore, we were looking for a binding mode reflecting an interaction of the inhibitor scaffold with this amino acid side chain.

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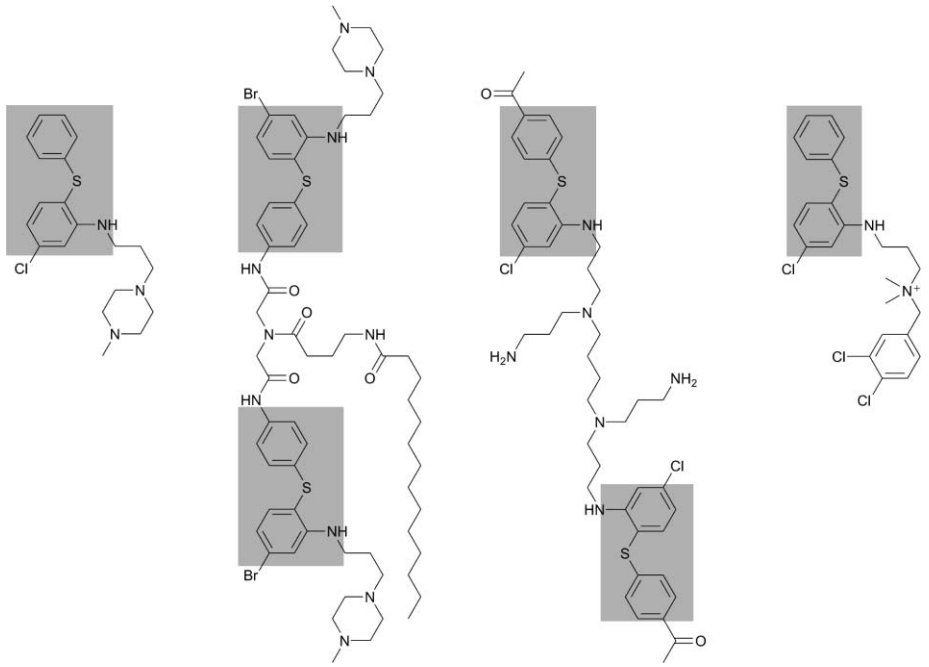
Table 1 Biological activities of previously reported diaryl sulfide derivatives **3**,[19] **4**,[21] **5**,[20] and **6**[22]



	3	**4**	**5**	**6**
IC_{50}[a]	—	0.2 μM	0.3 μM	—
K_{ic}	27 μM[b]	—	0.4 μM[c]	1.7 μM[d]

[a] At 21 °C, KCl-buffer, 57 μM TS_2 as substrate.[19] [b] At 28 °C, KCl-buffer, substrate not indicated.[21] [c] At 21 °C, KCl-buffer, TS_2 as substrate.[20] [d] At 25 °C, KCl-buffer, $(ZCG{\cdot}dmapa)_2$ as substrate.[22] KCl-buffer: 20 mM HEPES, 150 mM KCl, 1 mM EDTA, 0.2 mM NADPH, pH 7.25.

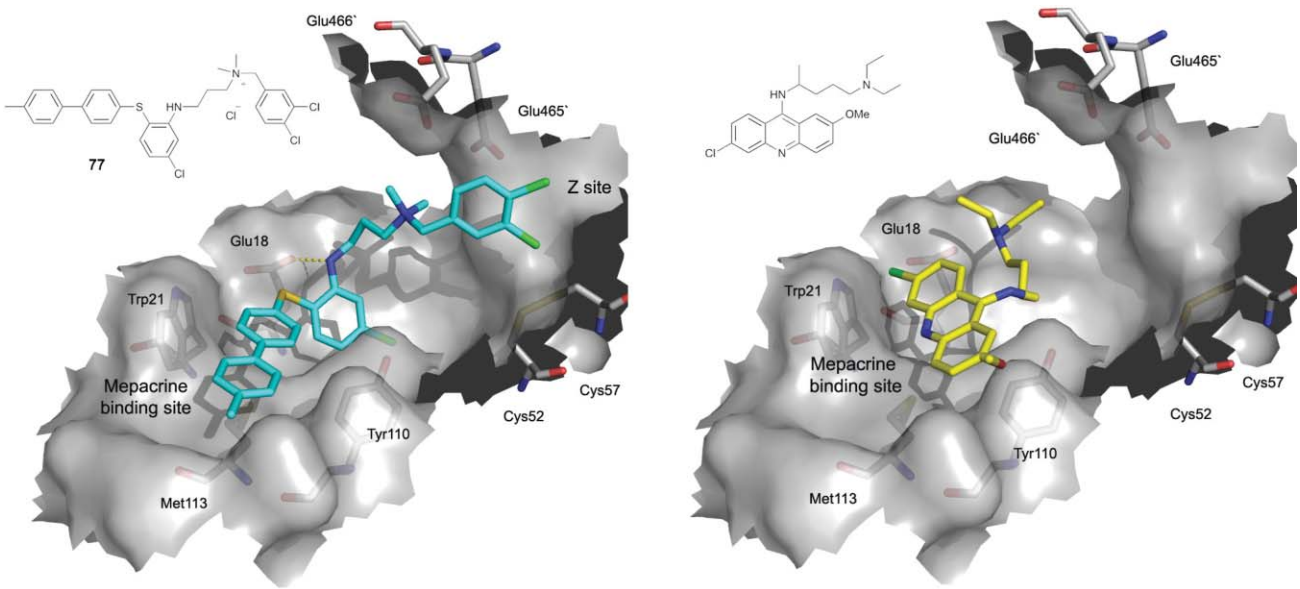


Fig. 2 Left: proposed new binding mode for benzyl ammonium inhibitor **77** with the biphenyl substituent occupying the mepacrine binding site. The aniline NH group H-bonds to Glu18 and the quaternary ammonium center is located in the negative electrostatic field of Glu465′ and Glu466′. Right: crystal structure of mepacrine bound to TR.[15]

By computer modelling using MOLOC,[26] we analyzed the binding mode of diaryl sulfide-based TR inhibitors bearing a flexible piperazine (as in **3**) or onium-ion terminated (as in **6**, Table 1) *N*-alkyl chain. In accordance with the proposal of Sergheraert *et al.*,[19] the amine headpiece of the alkyl chain was placed in the negative electrostatic field of Glu465′ and Glu466′. Rotating the central anilino nucleus by 180 degrees compared to the previously postulated binding mode directs the aniline nitrogen in an ideal position to undergo H-bonding to Glu18 (Fig. 2). As a result of this rotation, the phenylthio moiety is now pointing into the hydrophobic patch near Trp21 and Met113 that is capable to accommodate extended hydrophobic moieties such as the acridine moiety of the antimalarial drug mepacrine, as revealed in the co-crystal structure analysis.[15] The aromatic phenylthio moiety undergoes favourable aromatic edge-to-face interactions with Trp21 and sulfur–arene interactions[27] with Met113. Binding to this hydrophobic area has indeed been proposed for different tricyclic TR inhibitors,[28] but not for diaryl sulfide-based derivatives.

Importance of the simultaneous H-bonding to Glu18 and Glu465′/Glu466′—synthesis and biological properties of piperazine derivatives

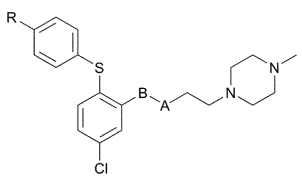
To decide if Glu18 is indeed more important for the binding of 2-aminodiphenyl sulfide-based TR inhibitors than previously postulated, we prepared analogues with altered functional groups in the position of the aromatic amino group of inhibitor **3** in order to modulate the proposed interaction with Glu18.

Starting from 5-chloro-(2-phenylthio)phenylamine[22] (**7**), amide **8** was prepared by acylation of the anilino NH_2 group with 3-chloropropionyl chloride, elimination of the terminal chloride and subsequent 1,4-addition of *N*-methylpiperazine to the obtained acrylamide acceptor (Scheme 1). Amidine **9** was prepared by reacting precursor **7** with the appropriate 3-(4-methylpiperazin-1-yl)propionitrile[29] under harsh conditions. Methylaniline **10** was prepared by reduction of amide **8** and subsequent reductive amination with formaldehyde. Ether **11** was obtained by transformation of aniline **7** to the corresponding phenol **12** under Sandmeyer-type conditions, followed by alkylation with 1-bromo-3-chloropropane and final replacement of the primary chloride of precursor **13** with *N*-methylpiperazine yielding diphenyl sulfide **11**. The potential inhibitor **14** with its all carbon chain was prepared by a Sandmeyer transformation of aniline **7** to the corresponding iodide **15**, followed by Sonogashira-coupling with 1-(but-3-ynyl)-4-methylpiperazine[30] and subsequent reduction of the obtained alkine **16**.

For the synthesis of amide **17**, a convenient synthetic protocol has been worked out avoiding any purification of intermediates. A one-pot two-step procedure starting from 4-bromothiophenol (**18**) delivered aniline **19** that was used without further purification for the amide coupling and subsequent addition of *N*-methylpiperazine to yield the desired 4-bromophenyl sulfide **17** in 87% overall yield after flash chromatography.

To determine if the proposed simultaneous H-bonding to Glu18 and Glu465′/Glu466′ plays a critical role in the binding, the piperazine derivatives **3** and **20** (Scheme 2) as well as **8–11**, **14**, **17** (Scheme 1) were subjected to kinetic analysis (see ESI†). Under the chosen assay conditions, the known inhibitor **3** (concentration: 100 μM) caused 33% inhibition and the corresponding bromo analogue **20** caused 39% inhibition of the TS_2 reduction (Table 2). Introduction of the more basic amidine group in **9** almost doubled the degree of inhibition, as expected if a direct interaction between the side chain of Glu18 and the protonated amidinium moiety takes place. An intramolecular H-bond between the amidine C=NH and the unprotonated proximal piperazine nitrogen is expected to further stabilise the association of the ligand in the active site (Fig. 2a ESI†). In sharp contrast, amide **8** and its bromo analogue **17** does not inhibit *T. cruzi* TR essentially. Modelling suggests that the amide-containing ligands would only be able to undergo a strong H-bond to Glu18 at the expense of losing the second H-bond to Glu465′ or Glu466′: if the terminal, *N*-methylated piperazine nitrogen is protonated and the amide NH is H-bonding to Glu18, the flexible alkyl chain has to move away from Glu465′/Glu466′ to avoid unfavourable C=O···N interactions between the amide carbonyl group and the unprotonated proximal piperazine nitrogen (Fig. 2b ESI, left†). If the piperazine nitrogen connected to the alkyl chain is protonated, the NH can undergo intramolecular H-bonding to the amide C=O group. In this case, without the protonation of the terminal nitrogen, any interaction of the ligand with Glu465′/Glu466′ is unlikely (Fig. 2b ESI, right†). In addition,

Table 2 Influence of the flexible *N*-alkyl chain on *T. cruzi* TR inhibition



	3	8	9	10	11	14	17	20
A	CH_2	C=O	C=NH	CH_2	CH_2	CH_2	C=O	CH_2
B	NH	NH	NH	NCH_3	O	CH_2	NH	NH
R	H	H	H	H	H	H	Br	Br
inh.[a]	33%	0%	64%	26%	15%	—[c]	5%	39%
inh.[b]	17%	0%	30%	9%	4%	2%	0%	16%

[a] 43 μM TS_2, 100 μM inhibitor, *ca.* 4 mU cm^{-3} TR, 25 °C. [b] 43 μM TS_2, 40 μM inhibitor, *ca.* 4 mU cm^{-3} TR, 25 °C. [c] Compound not soluble; inh.: *T. cruzi* TR inhibition.

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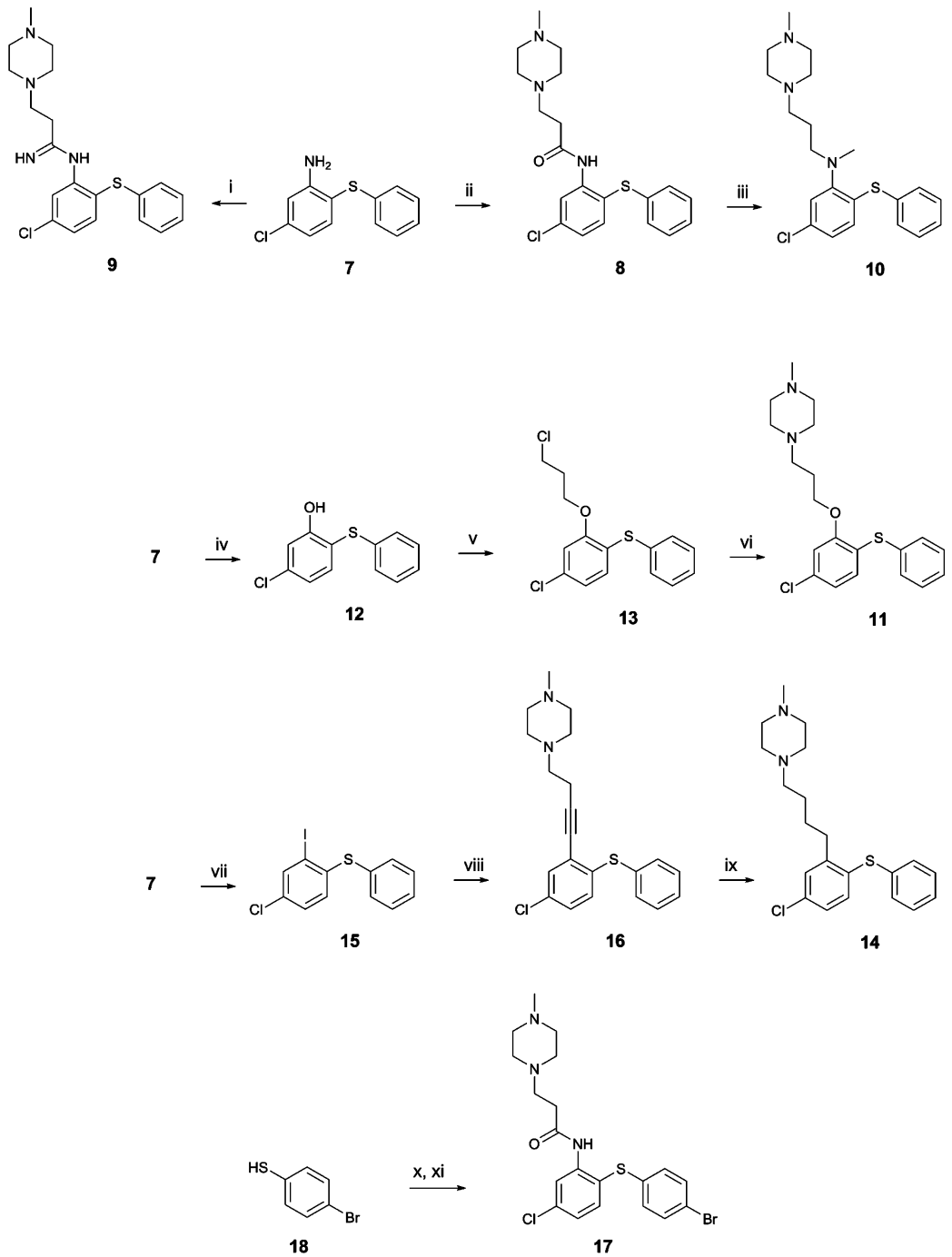
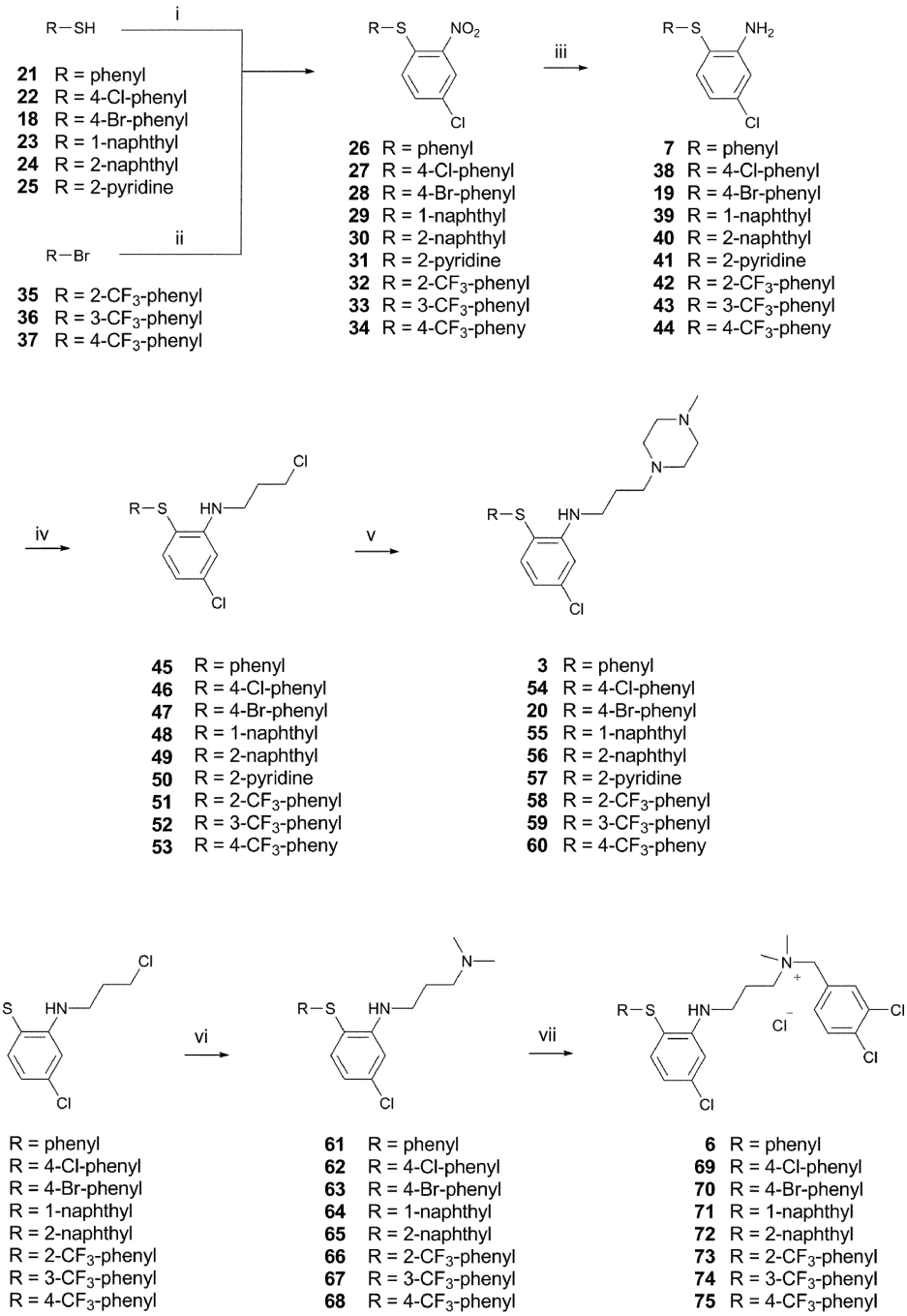


Scheme 1 Synthesis of piperazine-containing TR inhibitors. (i) 1. 3-(4-Methylpiperazin-1-yl)propionitrile,[29] $AlMe_3$, toluene, 100 °C, 2 d, 39%; (ii) 3-chloropropionyl chloride, K_2CO_3, THF, 25 °C, 2 h; 2. *N*-methylpiperazine, 60 °C, 14 h, 91% (over 2 steps); (iii) 1. BH_3·THF, THF, 65 °C, 16 h; 2. H_2SO_4, EtOH, 50 °C, 3 h; 3. HCHO, HCOOH, H_2O, 100 °C, 17 h, 71% (over 3 steps); (iv) 1. $NaNO_2$, H_2SO_4–H_2O, 0 °C, 2 h; 2. H_2SO_4–H_2O, 100 °C, 12 h, 56%; (v) 1-bromo-3-chloropropane, K_2CO_3, acetone, 60 °C, 5 h, 54%; (vi) *N*-methylpiperazine, dioxane, 100 °C, 2 d, 32%; (vii) 1. $NaNO_2$, HCl–H_2O, –10 °C, 90 min; 2. KI, H_2O –10 °C → 25 °C, 2.5 h, 66%; (viii) 1-(but-3-ynyl)-4-methylpiperazine,[30] $PdCl_2(PPh_3)_3$, CuI, NEt_3, THF, 1 d, 60 °C, 36%; (ix) $Pd(OH_2)$/C, H_2, MeOH, 60 °C, 4 d, 94%; (x) 1. 2,5-dichloronitrobenzene, Na, MeOH, 60 °C, 6 h; 2. Zn, NH_4Cl, 65 °C, 4 h; (xi) 1. 3-chloropropionyl chloride, K_2CO_3, THF, 25 °C, 17 h; 2. *N*-methylpiperazine, 50 °C, 5 h, 87% (starting from **18**).

in both cases, the amide group is unfavourably forced out of the plane of the adjacent phenyl ring to undergo H-bonding to Glu18. According to the binding model reflecting an interaction of the inhibitors with Glu18, the reduced TR affinities of amides **8** and **17** can be rationalised by the inability of these ligands to undergo simultaneous interactions with Glu18 and Glu465′/Glu466′.

Replacing the NH functionality (as in **3**) by a NCH_3 (as in **10**), CH_2 (as in **11**) or O (as in **14**) group reduced the *T. cruzi* TR inhibition, in agreement with the expected loss of a hydrogen



Scheme 2 Synthesis of diaryl sulfide derivatives with piperazine (as in **3**) or onium-ion terminated (as in **6**) *N*-alkyl chain. (i) Na, 2,5-dichloronitrobenzene, MeOH, 65 °C, 3–7 h, 71–92%; (ii) 1. *tert*-BuLi, THF, −78 °C, 10 min; 2. sulfur, −78 °C → 25 °C, 30 min; 3. 2,5-dichloronitrobenzene, 3–5 h, 66–71%; (iii) Zn, NH_4Cl, MeOH, 65 °C, 2–5 h, 43–99%; (iv) 1. 3-chloropropionyl chloride, pyridine, THF, 25 °C, 1.5–4 h; 2. BH_3·THF, THF, 67 °C, 2–4 h, 57–95% (over 2 steps); (v) *N*-methylpiperazine, K_2CO_3, NaI, DMF, microwaves, 80 °C → 110 °C, 70 min, 51–82%; (vi) $NHMe_2$ (40%, in H_2O), DMF, microwaves, 150 °C, 5 min, 79–96%; (vii) 3,4-dichlorobenzyl chloride, acetone, microwaves, 120 °C, 20 min, 65–94%. DMF = *N,N*-dimethylformamide.

bond to Glu18 while the overall geometry of the linker is not as disturbed as in the case of the incorporation of an amide group.

Overall, inhibition of *T. cruzi* TR by these piperazine derivatives shows that introduction of a superior carboxylate-binding ligand fragment in the vicinity of the central anilino moiety increases inhibition considerably. This suggests a direct interaction of the anilino NH group of the flexible amine linker in **3** with Glu18, in contrast to the prediction of previous docking studies.[19]

Synthesis and inhibitory activity of ligands with extended diaryl sulfide cores and onium-ion-terminated side chains

Whereas modifications of the flexible piperazine (as in **3**) or onium-ion terminated (as in **6**) *N*-alkyl chain in diaryl sulfide inhibitors have been extensively investigated over the last decade,[20,22,24,31] the central diphenyl sulfide scaffold remained largely unchanged. Our revised model for the binding mode of this class of inhibitors

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suggested that the phenylthio subunit occupies the "mepacrine binding site" roughly formed by Trp21 and Met112, and encouraged the systematic modification of this part of the ligands.

The synthesis of a series of piperazine-decorated target molecules is shown in Scheme 2. Formation of the thioether linkage *via* regioselective nucleophilic aromatic substitution of the *ortho*-chloride of 2,5-dichloronitrobenzene with the thiophenol derivatives **18**, **21–25** provided the diaryl sulfides **26–31**. The synthesis of the corresponding trifluoromethylphenyl analogues **32**, **33** and **34** was achieved by treatment of the (trifluoromethyl)bromobenzene derivatives **35**, **36** and **37**, respectively, with *tert*-BuLi to generate the desired lithiated species. Sulfur was added to yield the thiolates, which were *in situ* reacted with 2,5-dichloronitrobenzene to afford the desired diphenyl sulfides **32**, **33** and **34**. Reduction of the nitro group of the sulfides **26–34** gave anilines **7**, **19** and **38–44**, which where alkylated to yield chlorides **45–53**. Microwave-assisted introduction of the piperazine substituent, as described above, afforded the target compounds **3**, **20** and **54–60**. Unfortunately, unexpectedly low solubility, when compared to **3**, prevented a reliable determination of the TR inhibition caused by this series of new piperazine substituted derivatives.

Inhibitors featuring a permanent positive charge have been described to exhibit increased TR affinity compared to their neutral counterparts.[18] In particular, Douglas *et al.* reported high TR inhibition values for the cationic diphenyl sulfide derivative **6**, bearing a 3,4-dichlorobenzylammonium group as the headpiece of the flexible *N*-alkyl chain.[22] Therefore, replacement of the piperazine moiety, as in derivatives **20** and **54–60**, against a 3,4-dichlorobenzylammonium headpiece should likewise lead to increased TR affinity and also to better solubility of the ligands.

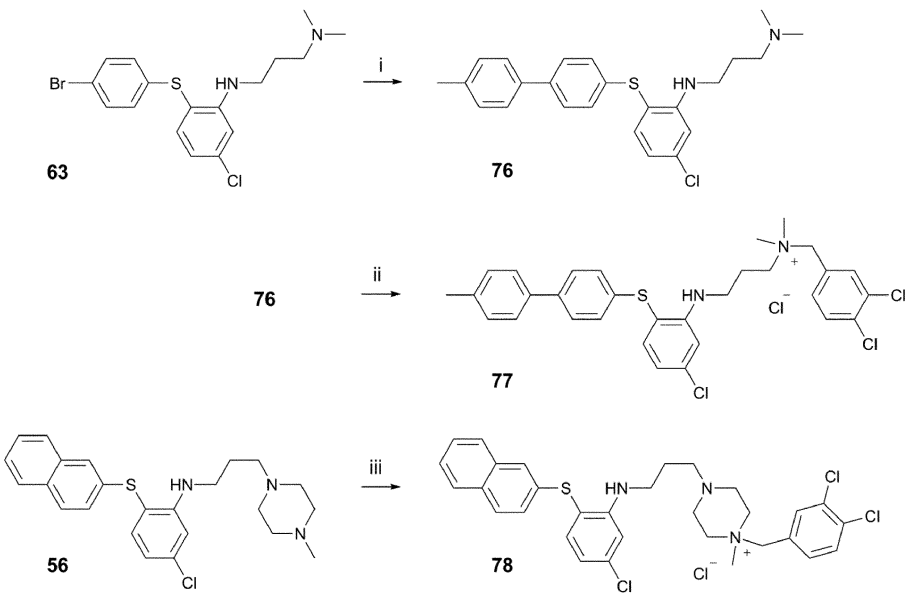
In order to prepare the cationic target molecules, chlorides **45–53** were converted to the corresponding dimethylamino derivatives **61–68** that where quaternized using 3,4-dichlorobenzyl chloride to yield the benzylammonium cations **6** and **69–75** (Scheme 2).

The preparation of additional cationic ligands with extended diaryl sulfide cores is depicted in Scheme 3. Palladium-catalyzed Suzuki–Miyaura cross-coupling[32] of 2-tolylboronic acid with bromide **63** gave biphenyl derivative **76**, which was further converted into benzylammonium derivative **77**, as described above. On the other hand, regioselective alkylation of the less hindered terminal nitrogen of the piperazine moiety of naphthyl derivative **56** with 3,4-dichlorobenzyl chloride yielded cation **78**.

As expected, the inhibitors featuring a permanent positive charge showed better solubility. Both quaternary benzyldimethylammonium and piperazinium ligands displayed a large gain in inhibitory potency, when compared to the respective tertiary amine counterparts (Table 3). Thus, *N*-methylpiperazine **56** showed only weak *T. cruzi* TR inhibition (which could not be accurately determined due to solubility problems), the corresponding cationic species **78** was a much more potent ligand and nearly equally potent than the dimethylammonium cation **6** (Table 3).

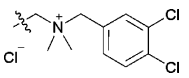
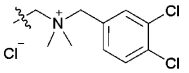
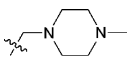
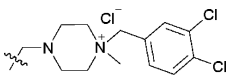
The kinetics of the diaryl sulfide derivatives **6**, **69–71**, **74**, **75** and **77** demonstrated competitive *T. cruzi* TR inhibition, with K_{ic} values in the lower micromolar range (Table 4). A K_{ic} value of 1.69 μM has been reported for phenyl derivative **6**,[22] measured in a TR buffer system containing 0.02 M HEPES, 0.15 M KCl, 1 mM EDTA at pH 7.25 with $(ZCG{\cdot}dmapa)_2$ (*N,N*-bis-(benzyloxycarbonyl)-L-cysteinylglycyl-3-dimethylamino)propylamide as an artificial TR substrate.[33] In our TR assay containing 0.04 M HEPES, 1 mM EDTA at pH 7.50 with the natural substrate TS_2,[34] we measured a higher K_{ic} value for **6** (9 μM).

Derivatisation of the phenylthio substituent by introducing Cl, Br or CF_3 substituents in the *para*-position as in **69**, **70** and **75** or a CF_3 group in the *ortho*-position as in **74** essentially did not affect the binding (Table 4). Interestingly, the inhibition constants for the naphthyl **71** and the biphenyl derivative **77** are also in the same



Scheme 3 Synthesis of cationic ligands with extended diaryl sulfide core and piperazinium headpiece, respectively. (i) 4-Tolylboronic acid, Cs_2CO_3, $[Pd(PPh_3)_4]$, DME, H_2O, 60 °C, 2.5 d, 79%; (ii) 3,4-dichlorobenzyl chloride, acetone, microwaves, 120 °C, 20 min, 65%; (iii) 3,4-dichlorobenzyl chloride, acetone, 45 °C, 1 d, 60%. DME = 1,2-dimethoxyethane.

Table 3 Inhibition of *T. cruzi* TR by diaryl sulfide-based inhibitors with different amine-headpieces of the flexible *N*-alkyl chain

Compound	R^1	R^2	inh.[a]
6	Phenyl		*ca.* 60%
72	2-Naphthyl		*ca.* 60%
56	2-Naphthyl		≤ 5%
78	2-Naphthyl		*ca.* 70%

[a] 40 μM Inhibitor, 105 μM TS_2, 100 μM NADPH, 25 °C. inh.: *T. cruzi* TR inhibition.

Table 4 *T. cruzi* TR inhibition of benzylammonium compounds

Compound	R	K_{ic}[a]/μM
6	Phenyl	9 ± 1
69	4-Chlorophenyl	10 ± 3
70	4-Bromophenyl	8 ± 4
71	1-Naphthyl	9 ± 5
74	3-(Trifluoromethyl)phenyl	5 ± 2
75	4-(Trifluoromethyl)phenyl	6 ± 3
77	4′-Methylbiphenyl	8 ± 1

[a] 5–10 mU cm^{-3} TR, 25 °C.

range as for the phenyl derivative **6**. According to the previously proposed binding mode of **3**,[19] the additional tolyl residue should clash with residues in the vicinity of the catalytic Cys52/57. This would result in a substantial loss in ligand affinity, which, however, is not observed.

The results from the variation of the anilino nitrogen, connecting the flexible alkyl side chain with the diaryl sulfide core, support that two negatively charged regions of the TR active site, formed by Glu18 and Glu465′/Glu466′, interact with the bound ligands. The anilino NH undergoes H-bonding to the side chain of Glu18 and the protonated terminal piperazine nitrogen (as in **3**), as well as the permanently charged quaternary onium ions (as in **6** and the new derivatives described in this paper), interact with the side chains of Glu465′/Glu466′.

Coupled with the establishment of these interactions is a different orientation of the diaryl sulfide moiety (Fig. 2 and Fig. 3 ESI†). This proposal is supported by the maintained activity of the ligands bearing bulky hydrophobic residues (such as **77**) attached

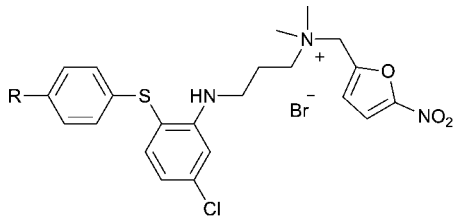


Fig. 3 Structures of nitrofuran-derivatized mixed competitive–uncompetitive TR inhibitors displaying antitrypanosomal and antiplasmodial *in vitro* activity.[35]

to the phenylthio moiety of the core, as all derivatized phenylthio moieties of the inhibitors **69–71**, **75** and **77** can be modeled into the "mepacrine binding site" using the revised binding model (Fig. 3 ESI†).

Parasitology

The newly synthesised TR inhibitors were tested for their potency against the trypanosomatids *T. cruzi*, *T. b. rhodesiense* and *L. donovani* as well as against the haemosporida parasite *Plasmodium falciparum in vitro.* Though no significant influence on the growth of the intracellular parasites *T. cruzi* and *L. donovani* could be detected, a good correlation between TR inhibition and activity against *T. b. rhodesiense* emerged for the derivatisation of the flexible *N*-alkyl linker of the piperazine derivatives. Amines **3** and **20** as well as amidine **9** showed *T.b.r.*-IC_{50} values between 1.0 and 1.6 μM (Table 5). In contrast, the amide derivatives **8** and **17**, causing no detectable TR inhibition, were also inactive in *in vitro* studies against *T. b. rhodesiense.*

Although *P. falciparum* does not have the unique trypanothione metabolism, simultaneous activities against *P. falciparum* and *T. b. rhodesiense* have been reported previously for tricyclic and diphenyl sulfide-based TR inhibitors bearing a permanent positive charge.[22] Therefore, the newly synthesised compounds were also tested for activity against the malaria parasite. In contrast to the activities against *T. b. rhodesiense*, all three piperazines exhibited remarkable activities against *P. falciparum* (Table 5). Introduction of the amide bond even appears beneficially for the antiplasmodial properties, as the activity of the acylated aniline **8** (IC_{50} = 0.86 μM) was slightly increased compared to the alkylated aniline **3** (IC_{50} = 1.55 μM), while the cytotoxicity was tenfold lower (L-6-IC_{50} = 10.48 μM for **3**, 107.50 μM for **8**) leading to a selectivity index higher than 100 for amide **8**.

Simultaneous activities against *T. b. rhodesiense* as well as against *P. falciparum* likewise emerged for the dimethylamine- and piperazine-bearing derivatives **3**, **20**, **54–60** and **61–68**, respectively. For the piperazine derivatives **3**, **20** and **54–60**, altering the phenylthio moiety preserved the activity against *P. falciparum* (*P.f.*-IC_{50} between 0.86 and 1.55 μM), whereas the replacement of a derivatized phenylthio moiety (as in **3**, **20**, **54–56**, **58–60**, *T.b.r.*-IC_{50} between 0.70 and 2.03 μM) by a pyridinethio moiety (as in **57**, not active) annihilates the activity against *T. b. rhodesiense* (Table 6a). As a drawback, all these diaryl sulfide derivatives bearing a tertiary nitrogen as the headpiece of the flexible alkyl chain showed considerable cytotoxicity towards L-6 myoblast cells. In the case of the derivatives with a dimethylamine headpiece, only the phenyl- and the *meta*-trifluoromethylphenyl sulfide **61** and **67**, respectively, showed no effect on the growth of *T. b. rhodesiense.*

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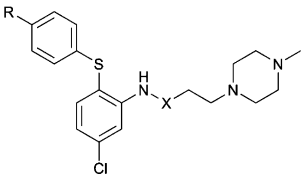
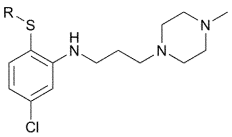
Table 5 Influence of altering the flexible *N*-alkyl chain of diaryl sulfide-based TR inhibitors on their *in vitro* antiprotozoal activities against *T. b. rhodesiense* and *P. falciparum*



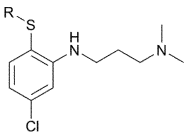
				T. brucei rhodesiense[a]		*P. falciparum*[b]		L-6 cells[c]
	R	X	TR inhibitor	IC_{50}/μM	SI	IC_{50}/μM	SI	IC_{50}/μM
3	H	CH_2	Yes	0.98	10.7	1.55	6.8	10.5
8	H	C=O	No	Not active[d]		0.86	125.0	107.5
9	H	C=NH	Yes	1.56	50.3	0.65	120.7	78.5
17	Br	C=O	No	Not active[d]		1.01	26.7	26.9
20	Br	CH_2	Yes	1.32	6.9	1.31	7.0	9.1

Assays were run in duplicate and repeated once.[a] STIB900 *T. b. rhodesiense* strain, trypomastigote stage. [b] K1 strain, intra-erythrocytic form (IEF). [c] Rat myoblast cells to assess cytotoxicity. [d] Less than 50% growth inhibition at 0.8 μg inhibitor per cm^3; IC_{50}: inhibitory concentration 50%; SI: selectivity index: IC_{50} for L-6/IC_{50} for parasite.

Table 6 *In vitro* activities of piperazine- and dimethylamine-bearing diaryl sulfides against *T. b. rhodesiense* and *P. falciparum*



		T. brucei rhodesiense[a]		*P. falciparum*[b]		Cytotoxicity[c]
	R	IC_{50}/μM	SI	IC_{50}/μM	SI	IC_{50}/μM
3	Phenyl	0.98	10.7	1.55	6.8	10.5
20	4-Br-phenyl	1.32	6.9	1.31	7.0	9.1
54	4-Cl-phenyl	0.97	9.4	1.33	6.8	9.1
55	1-Naphthyl	0.70	12.1	1.31	6.5	8.5
56	2-Naphthyl	0.70	12.4	0.92	9.5	8.7
57	2-Pyridinyl	Not active[d]	0.89	71.8	63.9	
58	2-CF_3-phenyl	0.90	10.4	0.86	10.9	9.4
59	3-CF_3-phenyl	1.06	9.2	1.35	7.2	9.7
60	4-CF_3-phenyl	2.03	4.8	1.23	7.9	9.7



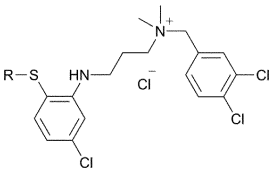
61	Phenyl	Not active[d]		1.05	12.2	12.8
62	4-Cl-phenyl	1.44	5.3	1.52	5.0	7.6
63	4-Br-phenyl	2.33	4.1	0.99	6.3	9.5
64	1-Naphthyl	1.89	4.6	1.51	5.8	8.7
65	2-Naphthyl	1.62	5.7	1.25	7.4	9.2
66	2-CF_3-phenyl	2.06	5.9	1.68	7.3	12.2
67	3-CF_3-phenyl	Not active[d]		1.12	9.1	10.2
68	4-CF_3-phenyl	1.16	8.5	1.72	5.7	9.8

Assays were run in duplicate and repeated once.[a] STIB900 *T. b. rhodesiense* strain, trypomastigote stage. [b] K1 strain, intra-erythrocytic form (IEF). [c] Rat myoblast cells to assess cytotoxicity. [d] Less than 50% growth inhibition at 0.8 μg inhibitor per cm^3; IC_{50}: inhibitory concentration 50%; SI: selectivity index: IC_{50} for L-6/IC_{50} for parasite.

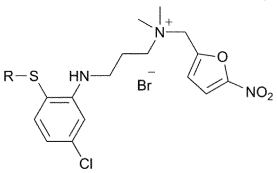
All other dimethylamines exhibited *T.b.r.*-IC_{50}s between 1.16 and 2.33 μM and all compounds affected the growth of *P. falciparum* with IC_{50}-values in the range of 0.99 to 1.72 μM (Table 6a).

In vitro activities against *T. b. rhodesiense* of the cationic, 5-nitrofurfuryl substituted diaryl sulfide-based TR inhibitors **79–82** (Fig. 3) with IC_{50}-values between 0.59 and 1.02 μM have been reported recently.[35] *In vitro* studies with the malaria parasite now revealed, in addition, rather strong antiplasmodial activities with IC_{50}-values between 0.58 and 0.85 μM (Table 6b). The replacement of the 5-nitrofurfuryl substituent as in **79–82** (IC_{50}-values from 0.58 to 0.85 μM) by a 3,4-dichlorobenzyl unit as in **6** and **69–75** did not substantially affect the antiplasmodial activity (IC_{50}-values from 0.32 to 1.13 μM), but completely abolished any activity on *T. b. rhodesiense* growth.

Table 7 *In vitro* activities against *T. b. rhodesiense* and *P. falciparum* of cationic diaryl sulfides



	R	*T. brucei rhodesiense*[a] IC$_{50}$/μM	SI	*P. falciparum*[b] IC$_{50}$/μM	SI	Cytotoxicity[c] IC$_{50}$/μM
6	Phenyl	Not active[d]		1.13	42.2	47.7
69	4-Cl-phenyl	Not active[d]		0.37	32.4	12.0
70	4-Br-phenyl	Not active[d]		0.45	29.1	13.1
71	1-Naphthyl	Not active[d]		0.55	37.3	20.5
72	2-Naphthyl	Not active[d]		0.67	33.7	22.6
73	2-CF_3-phenyl	Not active[d]		0.34	29.7	10.1
74	3-CF_3-phenyl	Not active[d]		0.38	29.2	11.1
75	4-CF_3-phenyl	Not active[d]		0.32	45.3	14.5



79	Phenyl	0.59	> 288	0.67	> 254	> 170
80	4-Cl-phenyl	0.77	94.7	0.59	123.6	72.9
81	4-Br-phenyl	0.68	127.2	0.58	149.1	86.5
82	4-CF_3-phenyl	1.02	27.1	0.85	32.5	27.6

Assays were run in duplicate and repeated once.[a] STIB900 *T. b. rhodesiense* strain, trypomastigote stage. [b] K1 strain, intra- erythrocytic form (IEF). [c] Rat myoblast cells to assess cytotoxicity. [d] Less than 50% growth inhibition at 0.8 μg inhibitor per cm^3; IC$_{50}$: inhibitory concentration 50%; SI: selectivity index: IC$_{50}$ for L-6/IC$_{50}$ for parasite.

Conclusions

We report experimental evidence for a newly proposed, modelling-based binding mode for the class of diaryl sulfide-based *T. cruzi* TR inhibitors. Altering the nature of the flexible *N*-alkyl chain supported the importance of two carboxylate recognition sites in the inhibitor framework. Modelling rationalizes that these ligands interact with the enzyme by simultaneous binding to Glu18 and the electrostatically strongly negatively charged region of Glu465′ and Glu466′. By adding additional substituents to the phenylthio unit of the diaryl sulfide core, the inhibition properties remained remarkably unaffected, with K_{ic} values in the low micromolar range. The similar inhibition values for phenylthio and biphenylthio derivatives supports that these ligand parts occupy the large "mepacrine binding site" of TR. The revised binding geometry, with simultaneous interactions of the flexible *N*-alkyl linker with Glu18 as well as Glu465′/Glu466′ and the occupation of the mepacrine binding site by the arylthio substituent of the aniline moiety, is highly probable for all members of this class of TR inhibitors.

The proposed binding mode opens up the way to the rational design of new diaryl sulfide inhibitors with thioaryl substituents that fulfil even better the steric and electronic requirements of the spacious hydrophobic patch that is occupied by the acridine moiety of mepacrine. By benefiting from additional hydrophobic contacts or by H-bonding to Ser109, an enhanced binding affinity should be achieved in future work.

In vitro studies with the trypanosomatids *T. b. rhodesiense, T. cruzi* and *L. donovani* revealed activity of the tertiary amines against the causative agent of African sleeping sickness. Interestingly, the derivatives exhibited even stronger activities against *P. falciparum.* Whereas the potency of the diaryl sulfide-based derivatives against *T. b. rhodesiense* was annihilated upon interaction of a cationic benzylammonium substituent and could only be retained by exchange of the benzyl- against a nitrofurfuryl headpiece, all cationic derivatives were shown to be active against *P. falciparum.*

Experimental

General details and procedures

See ESI.†

***N*-[5-Chloro-2-(phenylthio)phenyl]-3-(4-methylpiperazin-1-yl)-propanamide (8).** Aniline **7** (2.00 g, 8.48 mmol) and K_2CO_3 (3.52 g, 25.45 mmol) were suspended in THF (100 cm^3), 3-chloropropionyl chloride (0.97 cm^3, 10.18 mmol) was added and the mixture was stirred for 2 h, when *N*-methylpiperazine (4.71 cm^3, 42.42 mmol) was added to the solution. The mixture was stirred for 14 h at 60 °C. Dilution (H_2O, then EtOAc), washing (saturated aqueous NaCl solution), extraction (EtOAc), drying of the combined organic layers ($MgSO_4$), filtration and concentration *in vacuo* followed by purification by column chromatography (CC) (SiO_2; CH_2Cl_2–MeOH 95 : 5) delivered amide **8** (3.01 g, 91%)

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as a white solid; mp: 90 °C; ν_{max}(neat)/cm^{-1} 3116, 2946, 2821, 2775, 1683, 1563, 1514, 1397, 1249, 1138, 1009, 811, 736, 687; δ_H(300 MHz, $CDCl_3$) 2.22 (s, 3 H), 2.44–2.57 (m, 12 H), 7.03–7.07 (m, 3 H), 7.13–7.19 (m, 1 H), 7.22–7.27 (m, 2 H), 7.38 (d, J = 8.4, 1 H), 8.44 (d, J = 2.1, 1 H) 10.46 (br s, 1 H); δ_C(75 MHz, $CDCl_3$) 33.4, 45.7, 52.5, 53.6, 54.3, 120.0, 122.5, 124.7, 126.2, 127.3, 129.2, 135.7, 135.9, 136.3, 141.1, 170.8; MALDI-HR-MS: calcd for $C_{20}H_{25}ClN_3OS^+$ ([M + H]$^+$): 390.1401; found: 390.1394.

***N*-[5-Chloro-2-(phenylthio)phenyl]-3-(4-methylpiperazine-1-yl)-propanamidine (9).** 3-(4-Methylpiperazine-1-yl)-propionitrile[29] (115 mg, 0.75 mmol) and $AlMe_3$ (2 M in hexane, 0.38 cm^3, 0.75 mmol) were added to aniline **7** (93 mg, 0.40 mmol) in toluene (1 cm^3), and the mixture was stirred for 2 d at 100 °C. Dilution (H_2O and EtOAc), washing (saturated aqueous Na_2CO_3 solution), extraction of the aqueous phases (EtOAc), drying of the combined organic layers ($MgSO_4$), filtration and concentration *in vacuo* followed by purification by CC (SiO_2; CH_2Cl_2–MeOH 90 : 10) yielded amidine **9** (60 mg, 39%) as a pale yellow oil; ν_{max}(neat)/cm^{-1} 3162, 2938, 2796, 1635, 1566, 1456, 1371, 1283, 1162, 1086, 1011, 908, 731, 690; δ_H(300 MHz, MeOD) 2.30 (s, 3 H), 2.35–2.78 (m, 12 H), 6.88 (dd, J = 8.4, 2.1, 1 H), 6.98 (d, J = 8.4, 1 H), 7.27–7.39 (m, 6 H); δ_C(75 MHz, MeOD) 31.1, 45.9, 52.4, 54.9, 55.0, 114.9, 118.7, 123.8, 125.7, 126.5, 127.5, 129.1, 129.3, 131.7, 132.3, 138.4; MALDI-HR-MS: calcd for $C_{20}H_{26}ClN_4S^+$ ([M + H]$^+$): 389.1561; found: 389.1554.

5-Chloro-*N*-methyl-*N*-(3-(4-methylpiperazin-1-yl)propyl)-2-(phenylthio)aniline (10). BH_3·THF (1 M in THF, 2.5 cm^3, 2.5 mmol) was added to a solution of amide **8** (105 mg, 0.27 mmol) in THF (5 cm^3). The mixture was left to stir for 16 h at 65 °C and concentrated *in vacuo*. The residue was dissolved in EtOH (10 cm^3), conc. sulfuric acid (0.2 cm^3) was added and the mixture was stirred for 3 h at 50 °C. Dilution (saturated aqueous $NaHCO_3$ solution), extraction (EtOAc), washing (saturated aqueous NaCl solution), extraction of the aqueous phases (CH_2Cl_2), drying of the combined organic phases ($MgSO_4$) and filtration followed by concentration *in vacuo* delivered a yellow oil that was redissolved in formic acid (0.062 cm^3) and H_2O (0.5 cm^3). HCOH (37% in H_2O, 0.043 cm^3, 1.62 mmol) was added and the reaction mixture was left to stir overnight at 100 °C. The mixture was cooled to 25 °C, HCl (12 M aqueous solution) was added, the solvent was evaporated and the residue was made alkaline by addition of 5% aqueous NaOH solution. Extraction (CH_2Cl_2), drying of the combined organic phases ($MgSO_4$), filtration and concentration *in vacuo*, followed by purification by CC (SiO_2; CH_2Cl_2–MeOH 98 : 2 → 90 : 10) delivered methylamine **10** (75 mg, 71%) as a light yellow oil; ν_{max}(neat)/cm^{-1} 3446, 2793, 1572, 1458, 1390, 1281, 1165, 1013, 803, 691; δ_H(300 MHz, $CDCl_3$) 1.73 (quint, J = 7.5, 2 H), 2.27 (s, 3 H), 2.35 (t, J = 7.5, 2 H), 2.35–2.56 (m, 8 H), 3.02 (t, J = 7.8, 2 H), 6.80 (d, J = 8.0, 1 H), 6.85 (dd, J = 8.4, 2.1, 1 H), 7.03 (d, J = 2.1, 1 H), 7.30–7.36 (m, 5 H); δ_C(100 MHz, $CDCl_3$) 24.4, 41.6, 45.3, 52.2, 54.0, 54.3, 55.6, 121.3, 123.9, 127.8, 129.4, 130.9, 131.8, 132.3, 132.7, 134.0, 152.1; MALDI-HR-MS: calcd for $C_{21}H_{29}ClN_3S^+$ ([M + H]$^+$): 390.1765; found: 390.1758.

1-(3-(5-Chloro-2-(phenylthio)phenoxy)propyl)-4-methylpiperazine (11). *N*-Methylpiperazine (0.11 cm^3, 0.99 mmol) was added to a solution of chloride **13** (31 mg, 0.099 mmol) in dioxane (3 cm^3). The mixture was left to stir for 2 d at 100 °C, cooled to 25 °C, diluted (saturated aqueous $NaHCO_3$ solution), extracted (CH_2Cl_2), dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by CC (SiO_2; CH_2Cl_2–MeOH 98 : 2 → 90 : 10) yielded the desired product **11** (12 mg, 32%); ν_{max}(neat)/cm^{-1} 2921, 2793, 1574, 1461, 1385, 1250, 1164, 1093, 885, 690; δ_H(300 MHz, $CDCl_3$) 1.88 (quint, J = 6.9, 2 H), 2.29 (s, 3 H), 2.34–2.56 (m, 8 H), 2.37 (t, J = 6.9, 2 H), 4.02 (t, J = 6.0, 2 H), 6.85 (dd, J = 8.1, 2.1, 1 H), 6.89 (d, J = 2.1, 1 H), 7.05 (d, J = 8.1, 1 H), 7.26–7.30 (m, 5 H); δ_C(100 MHz, $CDCl_3$) 26.4, 46.0, 53.0, 54.7, 60.1, 67.2, 112.7, 121.1, 122.8, 127.0, 129.1, 130.9, 132.7, 134.0, 134.6, 157.4; MALDI-HR-MS: calcd for $C_{20}H_{26}ClN_2OS^+$ ([M + H]$^+$): 377.1449; found: 377.1455.

1-(4-(5-Chloro-2-(phenylthio)phenyl)butyl)-4-methylpiperazine (14). A mixture of alkyne **16** (20 mg, 0.054 mmol) and $Pd(OH)_2$/C (20% wt dry basis, 2 mg) in MeOH (10 cm^3) was stirred under a hydrogen atmosphere for 4 d at 60 °C. Filtration over celite, concentration *in vacuo*, followed by purification by CC (SiO_2; CH_2Cl_2–MeOH 99 : 1 → 92 : 8) delivered **14** (19 mg, 94%) as a colourless oil; ν_{max}(neat)/cm^{-1} 2924, 2793, 1581, 1460, 1163, 1085, 1014, 879, 736, 701; δ_H(300 MHz, $CDCl_3$) 1.48–1.66 (m, 4 H), 2.32 (s, 3 H), 2.42–2.60 (m, 8 H), 2.56 (t, J = 7.4, 2 H), 2.75 (t, J = 6.0, 2 H), 7.09 (dd, J = 8.4, 2.1, 1 H), 7.15–7.27 (m, 8 H); δ_C(100 MHz, $CDCl_3$) 27.3, 30.9, 32.9, 44.7, 51.7, 53.8, 57.1, 125.6, 126.0, 127.5, 128.2, 128.7, 128.8, 132.8, 133.7, 135.2, 144.8; MALDI-HR-MS: calcd for $C_{21}H_{28}ClN_2S^+$ ([M + H]$^+$): 375.1656; found: 375.1662.

General procedure for the quaternisation of amines using 3,4-dichlorobenzyl chloride

A mixture of the dimethylamine derivative (1 eq.) and 3,4-dichlorobenzyl chloride (10 eq.), dissolved in acetone (*ca.* 2 cm^3), was stirred in a closed vessel in a microwave reactor for 20 min at 120 °C before it was concentrated *in vacuo*. The residue was suspended in hexane, the solvent decanted and the residue dried *in vacuo* and purified by reversed phase HPLC (CH_3CN–H_2O (0.1% TFA) 50 : 0 → 100 : 0 in 50 min).

3-({5-Chloro-2-[1-naphthylthio]phenyl}amino)-*N*-(3,4-dichlorobenzyl)-*N*,*N*-dimethyl-propan-1-ammonium chloride (71). Inhibitor **71** (37 mg, 97%) was obtained as a colourless oil starting from amine **64** (25 mg, 67 μmol) and 3,4-dichlorobenzyl chloride (0.093 cm^3, 0.67 mmol); ν_{max}(neat)/cm^{-1} 3029, 2961, 2839, 1677, 1582, 1504, 1473, 1201, 1174, 1122, 1036, 831, 799, 771, 720; δ_H(300 MHz, $CDCl_3$) 1.79–1.86 (m, 2 H), 2.71 (s, 6 H), 2.81–2.87 (m, 2 H), 3.11–3.13 (m, 2 H), 4.30 (s, 2 H), 4.81 (t, J = 5.7, 1 H), 6.60 (d, J = 1.8, 1 H), 6.72 (dd, J = 8.1, 1.8, 1 H), 6.86 (d, J = 7.5, 1 H), 7.01 (dd, J = 8.4, 1.8, 1 H), 7.16 (t, J = 7.5, 1 H), 7.28–7.33 (m, 2 H), 7.40 (d, J = 8.1, 1 H), 7.47–7.60 (m, 3 H), 7.79 (dd, J = 7.5, 1.5, 1 H), 8.28 (d, J = 6.0, 1 H); δ_C(75 MHz, $CDCl_3$) 22.7, 39.6, 49.6, 61.5, 66.2, 110.4, 113.4, 117.9, 123.7, 124.2, 125.7, 126.3, 126.7, 128.5, 131.1, 131.9, 133.0, 133.3, 133.5, 134.0, 135.5, 137.4, 138.2, 148.7 (three signals not visible); MALDI-HR-MS: calcd for $C_{28}H_{28}Cl_3N_2S^+$ ([M – Cl]$^+$): 529.1033; found: 529.1025.

3-({5-Chloro-2-[2-naphthylthio]phenyl}amino)-*N*-(3,4-dichlorobenzyl)-*N*,*N*-dimethyl-propan-1-ammonium chloride (72). Inhibitor **72** (87 mg, 73%) was obtained as a white solid starting from amine **65** (44 mg, 0.12 mmol) and 3,4-dichlorobenzyl chloride (0.16 cm^3, 1.19 mmol); mp: 156 °C; ν_{max}(neat)/cm^{-1} 3362, 2964,

1581, 1505, 1475, 1420, 1264, 1139, 1092, 1037, 825, 789, 668; δ_H(300 MHz, $CDCl_3$) 1.85–2.00 (m, 2 H), 2.67 (s, 6 H), 3.02–3.07 (m, 2 H), 3.25–3.29 (m, 2 H), 4.53 (s, 2 H), 5.71 (t, $J = 6.0$, 1 H), 6.64 (d, $J = 2.1$, 1 H), 6.77 (dd, $J = 8.1, 2.1$, 1 H), 7.20 (dd, $J = 8.7$, 2.0, 1 H), 7.34–7.44 (m, 6 H), 7.50 (d, $J = 8.4$, 1 H), 7.60–7.73 (m, 3 H); δ_C(75 MHz, MeOD) 21.8, 39.1, 49.0, 62.5, 65.6, 110.0, 112.9, 116.7, 124.3, 125.1, 125.6, 126.6, 126.7, 127.4, 127.4, 128.3, 131.0, 131.6, 132.1, 132.9, 133.6, 133.9, 134.3, 135.0, 137.5, 138.5, 149.6; MALDI-HR-MS: calcd for $C_{28}H_{28}Cl_3N_2S^+$ ($[M - Cl]^+$): 529.1033; found: 529.1033.

3-[(5-Chloro-2-{[2-(trifluoromethyl)phenyl]thio}phenyl)amino]-*N*-(3,4-dichlorobenzyl)-*N*,*N*-dimethylpropan-1-ammonium chloride (73). Inhibitor **73** (28 mg, 74%) was obtained as a white solid starting from amine **66** (25 mg, 64 μmol) and 3,4-dichlorobenzyl chloride (0.098 cm^3, 0.64 mmol); mp: 138 °C; ν_{max}(neat)/cm^{-1} 3348, 3261, 3093, 3021, 2966, 2864, 1590, 1464, 1228, 1137, 1117, 1031, 731, 617; δ_H(300 MHz, $CDCl_3$) 2.01–2.12 (m, 2 H), 3.19 (s, 6 H), 3.25–3.26 (m, 2 H), 3.48–3.54 (m, 2 H), 4.92 (br s, 1 H), 5.13 (s, 2 H), 6.66 (d, $J = 2.1$, 1 H), 6.79 (dd, $J = 7.5, 2.1$, 1 H), 6.87 (d, $J = 7.5$, 1 H), 7.21–7.27 (m, 2 H), 7.42–7.47 (m, 2 H), 7.55–7.68 (m, 3 H); δ_C(75 MHz, $CDCl_3$) 22.8, 40.2, 49.7, 61.9, 66.3, 111.0, 112.0, 118.3, 125.8, 126.8 (q, $J = 5.7$), 127.3, 127.8 (q, $J = 30.7$), 128.3, 131.4, 132.3, 132.7, 133.7, 134.6, 135.9, 136.1, 138.6, 139.3 (CF_3-signal not visible); δ_F(282 MHz, $CDCl_3$) –60.8 (s, 3 F); MALDI-HR-MS: calcd for $C_{25}H_{25}Cl_3F_3N_2S^+$ ($[M - Cl]^+$): 547.0751; found: 547.0742.

3-[(5-Chloro-2-{[3-(trifluoromethyl)phenyl]thio}phenyl)amino]-*N*-(3,4-dichlorobenzyl)-*N*,*N*-dimethylpropan-1-ammonium chloride (74). Inhibitor **74** (20 mg, 74%) was obtained as a white solid starting from amine **67** (18 mg, 46 μmol) and 3,4-dichlorobenzyl chloride (0.064 cm^3, 0.46 mmol); mp: 168 °C; ν_{max}(neat)/cm^{-1} 3282, 3002, 2976, 2962, 2818, 1582, 1471, 1320, 1165, 1115, 1098, 1072, 796, 695; δ_H(300 MHz, $CDCl_3$) 2.01–2.15 (m, 2 H), 3.16 (s, 6 H), 3.27–3.31 (m, 2 H), 3.56–3.61 (m, 2 H), 5.01–5.09 (m, 3 H), 6.67 (d, $J = 2.0$, 1 H), 6.72 (dd, $J = 8.4, 2.0$, 1 H), 7.14–7.44 (m, 6 H), 7.53 (dd, $J = 8.4, 1.5$, 1 H), 7.71 (d, $J = 1.5$, 1 H); δ_C(75 MHz, $CDCl_3$) 22.7, 40.2, 49.7, 62.2, 66.1, 110.7, 111.6, 118.1, 122.4 (q, $J = 3.9$), 122.7 (q, $J = 3.9$), 123.7 (q, $J = 272.8$), 127.3, 129.6, 131.3, 131.4 (q, $J = 31.1$), 132.6, 133.6, 134.6, 135.8, 138.1, 138.4, 138.9, 149.3 (one signal not visible); δ_F(282 MHz, $CDCl_3$) –62.5 (s, 3 F); MALDI-HR-MS: calcd for $C_{25}H_{25}Cl_3F_3N_2S^+$ ($[M - Cl]^+$): 547.0751; found: 547.0757.

3-({5-Chloro-2-[(4′-methylbiphenyl-4-yl)thio]phenyl}amino)-*N*-(3,4-dichlorobenzyl)-*N*,*N*-dimethylpropan-1-ammonium chloride (77). Inhibitor **77** (30 mg, 65%) was obtained as a pale yellow oil starting from amine **76** (33 mg, 80 μmol) and 3,4-dichlorobenzyl chloride (0.11 cm^3, 0.16 mmol); ν_{max}(neat)/cm^{-1} 3404, 2924, 2854, 1678, 1583, 1506, 1443, 1207, 1133, 1036, 845, 803, 725; δ_H(300 MHz, $CDCl_3$) 1.97–2.04 (m, 2 H), 2.35 (s, 3 H), 2.87 (s, 6 H), 3.36–3.40 (m, 4 H), 5.59 (s, 2 H), 5.03–5.05 (m, 1 H), 6.65 (d, $J = 2.2$, 1 H), 6.77 (dd, $J = 8.1, 2.2$, 1 H), 7.11 (d, $J = 8.3$, 2 H), 7.18 (d, $J = 8.3$, 2 H), 7.24–7.50 (m, 8 H); δ_C(75 MHz, $CDCl_3$) 21.2, 27.2, 42.2, 45.4, 49.9, 58.1, 111.3, 111.6, 116.4, 117.3, 120.3, 124.2, 127.4, 127.6, 128.1, 128.4, 128.6, 130.6, 130.8, 136.5, 138.4, 138.7, 139.8, 151.6, 162.9, 163.4; MALDI-HR-MS: calcd for $C_{31}H_{32}Cl_3N_2S^+$ ($[M - Cl]^+$): 569.1346; found: 569.1350.

Acknowledgements

This research was supported by a graduate student fellowship from Novartis (to B. S.), the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) (to M. K. and R. B.) and by the Deutsche Forschungsgemeinschaft (SFB 544, project B3, L. K.-S.). We thank *Jörg Klein* for the synthesis of piperazine derivatives and *Edith Röckel* and *Natalie Dirdjaja* for help in measuring TR assays.

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