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# Bistacrines as potential antitrypanosomal agents

Ines Schmidt [a], Sarah Göllner [e], Antje Fuß [b], August Stich [b], Anna Kucharski [a], Tanja Schirmeister [c], Elena Katzowitsch [d], Heike Bruhn [d], Alexandra Miliu [e], R. Luise Krauth-Siegel [e], Ulrike Holzgrabe [a,*]

[a] Institute for Pharmacy and Food Chemistry, Julius-Maximilians-University of Würzburg, Am Hubland, 97074 Würzburg, Germany
[b] Medical Mission Institute, Hermann-Schell-Strasse 7, 97074 Würzburg, Germany
[c] Institute for Pharmacy and Biochemistry, Johannes-Gutenberg-University of Mainz, Staudinger Weg 5, 55128 Mainz, Germany
[d] Institute for Molecular Infection Biology, Julius-Maximilians-University of Würzburg, Josef-Schneider-Strasse 2, 97080 Würzburg, Germany
[e] Biochemistry Center (BZH), Heidelberg University, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany

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ABSTRACT

Human African Trypanosomiasis (HAT) is caused by two subspecies of the genus *Trypanosoma*, namely *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*. The disease is fatal if left untreated and therapy is limited due to only five non-adequate drugs currently available. In preliminary studies, dimeric tacrine derivatives were found to inhibit parasite growth with $IC_{50}$-values in the nanomolar concentration range. This prompted the synthesis of a small, but smart library of monomeric and dimeric tacrine-type compounds and their evaluation of antiprotozoal activity. Rhodesain, a lysosomal cathepsin-L like cysteine protease of *T. brucei rhodesiense* is essential for parasite survival and likely target of the tacrine derivatives. In addition, the inhibition of trypanothione reductase by bistacrines was found. This flavoprotein oxidoreductase is the main defense against oxidative stress in the thiol redox system unique for protozoa.



## 1. Introduction

Human African Trypanosomiasis (HAT), a parasitic disease caused by protozoa of the genus *Trypanosoma*, imperils more than 60 million people living in 36 sub-Saharan countries. The pathogens are transmitted by the bite of the tsetse fly.[1] The disease occurs in two manifestations depending on the species of the pathogen. *Trypanosoma brucei rhodesiense* initiates an acute infection and is primarily found in eastern and southern Africa, whereas *Trypanosoma brucei gambiense* causes the protracted form mainly in central and western Africa. HAT, also called sleeping sickness, is fatal if left untreated. Treatment options are very limited to only five drugs currently available. However, increasing resistance and marked host cytotoxicity are related to these drugs. Pentamidine and suramine are predominantly used for treatment of the hemolymphatic stage of the disease.[2] During the second stage of the disease, after the parasites having crossed the blood brain barrier, only melarsoprol, eflornithine, and the recently introduced combination therapy of nifurtimox and eflornithine (NECT) proved impact. This medication is expensive, painful, and associated with severe side effects. Therefore, the development of new and affordable drugs is urgently needed.[3]

Preliminary studies showed the antitrypanosomal activity of tacrine (1,2,3,4-tetrahydroacridin-9-amine, THA, see Fig. 1) in the micromolar concentration range ($IC_{50}$ (cell line TC221) = 14.6 μM). Linking of two tacrine moieties with a hexamethylene chain led to a significant increase of the activity being in the submicromolar concentration range. This bistacrine was originally synthesized because of its activity against *Plasmodium falciparum*.[4]

These results prompted us to synthesize monomeric and dimeric tacrine congeners and to evaluate their antitrypanosomal activity and putative mode of action. Since a couple of tacrine-type compounds has already shown inhibitory activity towards falcipain,[4] which is closely related to rhodesain,[5] a cysteine protease being a cathepsin-L like enzyme of the papain family expressed by bloodstream form of *Trypanosoma brucei brucei* (Tbb), the new library of compounds was tested for the inhibitory activity towards rhodesain. Of note, rhodesain is essential for the development and growth of parasites and facilitates the crossing of the blood brain barrier to enter the central nervous system.[6]

Additionally, for representative compounds the inhibition of *trypanothione reductase* (TryR) was tested, because a couple of similar tricyclic systems, such as phenothiazines, xanthenes, bis-acridines, and 4-aminoquinoline-type compounds[7] showed inhibition of the NADPH-dependent flavoenzyme.[8–10] The TryR is responsible for keeping the intracellular reducing environment for defence against oxidative stress and thus constitutes a promising target.[11]

* Corresponding author.
*E-mail address:* ulrike.holzgrabe@uni-wuerzburg.de (U. Holzgrabe).

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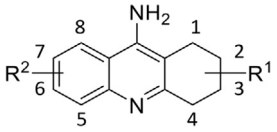


**Fig. 1.** Monomeric tacrine derivatives, $R^1$ and $R^2$ are outlined in Table 1.

## 2. Results and discussion

### 2.1. Synthesis pathways

Monomeric tacrine derivatives **1** are easily available by condensation and cyclization of correspondingly substituted 2-aminobenzonitriles and cyclohexanones with catalytic amounts of *p*-toluene sulfonic acid in toluene as solvent. Some members of the library (**1a**, **1d**, **1e**, **1f**, **1j**, **1m**, and **1n**) have already been reported in a previous paper.[3]

For the synthesis of the dimeric tacrine-type compounds **2** and **3**, 9-chloro-1,2,3,4-tetrahydroacridine derivatives were produced by condensation of correspondingly substituted 2-aminobenzonitriles and cyclohexanones using an excess of $POCl_3$. Two of the intermediates were linked by conversion with 1,ω-dibromoalkanes to give the compounds **2** and **3**. **2a**–**2i**, and **3a**, **3b**, **3c**, **3g**, and **3f** were already reported.[4]

The synthesis of dimeric $N^1$,$N^9$-bis(3,4-dihydroacridin-9-yl) alkanes **4** started off with the condensation of 2-aminobenzonitrile and 1,3-cyclohexanedione to give the 1-oxotacrine **1q**, which was dimerized by means of the corresponding dibromoalkane in presence of KOH and tetrabutylammonium iodide. The oxotacrine dimers **5a-e** were reduced with sodium borohydride to give the corresponding alcohols **6a-e** whose hydroxyl groups were eliminated by usage of HCl in isopropanol to achieve the compounds **4a-e** (see Scheme 1).

### 2.2. Biology

#### 2.2.1. In vitro activity against Trypanosoma brucei brucei

Monomeric and dimeric tacrine derivatives were tested against *Trypanosoma brucei brucei*, cell line TC221, using the cell proliferation alamarBlue® assay. Living cells are able to reduce the redox indicator resazurine to resorufine, resulting in a color change, whereas dead cell reduce this reaction. $IC_{50}$ values were determined according to the method published by Räz et al.[12] Cytotoxicity was measured against murine macrophages, cell line J774.1 using the alamarBlue®-based assay.[13] The data are summarized in Tables 1 and 2. Of note, the oxotacrine dimers **5** were not screened because of their very low aqueous solubility, and the hydroxytacrine dimers **6** were not tested because they are prone to dehydratisation which results in compounds **4**.

The comparison of the series **1a**–**1d**, **1f**–**1i**, and **1j**–**1m** with either hydrogen, methyl, ethyl or propyl substituents in position 2 indicates a stepwise increase of activity with enlargement of this residue. Though cytotoxicity increases with an additional chlorine substituent in the aromatic moiety, this does not affect activity. Thus, **1d** shows a good activity with acceptable toxicity resulting in a selectivity index of 16.5. An additional nitro group in position 7 (**1n**-**p**) is also advantageous in leading to a slight improvement in both antitrypanosomal activity and cytotoxicity.

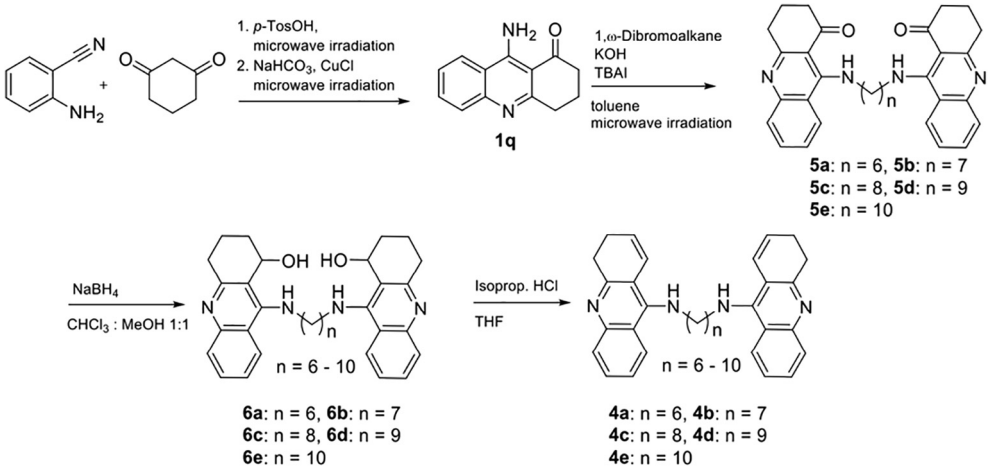
Next, the influence of the linker length between two tacrine-type molecules was studied for the compounds **2a-2i**. The dimerisation shifts both antitrypanosomal activity and cytotoxicity to lower $IC_{50}$ values resulting in almost constant selectivity indices. The compounds **2h** and **2i** with nine- and ten-membered linkers exhibit the best antitrypanosomal activities ($IC_{50}$ = 130 nM and 120 nM) and selectivity index (SI) values (i.e., 13.8 and 15.0, respectively).

Interestingly, the different substitution pattern of compound **3** has as a minor influence on the inhibition of parasite growth which holds also true for the cytotoxicity, with **3d** and **3g** being the most active antitrypanosomal derivatives (Fig. 2).

Of note, the compounds **4a**–**4e** having an additional double bond in the saturated region of the tacrine moiety (Fig. 3) showed a very good activity against *Trypanosoma*. As can be seen in Table 3, especially compounds **4b**, **4c**, and **4d** with a linker length of seven, eight, and nine methylene units, respectively, are active in the two-digit nanomolar range of concentration. Although being toxic to macrophages in low micromolar concentrations, these derivatives displayed the best SIs in the entire series of bistacrines. The most active compound **4b** shows a SI of about 150, which makes it an interesting lead compound. All compounds **4** are stable throughout the assays.

#### 2.2.2. Inhibition of rhodesain

The cysteine protease rhodesain is essential as a digestive enzyme[14] and enables African trypanosomes to cross the blood brain barrier.[15] Dimeric tacrine derivatives **2a**–**2i** and **3a**–**3g** were evaluated *in vitro* for their ability to inhibit rhodesain using a fixed concentration of 20 μM (see Table 4). In this assay, an equivalent volume of DMSO was used as negative control. Since fluorogenic




**Scheme 1.**

**Table 1**
Antitrypanosomal activities, cytotoxicities, and selectivity indices of monomeric tacrine derivatives; all compounds were tested in duplicate; values represent the mean of the two measurements. Selectivity indices were calculated by $IC_{50}$ (J774.1)/$IC_{50}$ (*Tbb*).

| Compound | | | *T. brucei brucei* $IC_{50}$ [μM] | | Cytotoxicity J774.1 | SI |
|---|---|---|---|---|---|---|
| | $R^1$ | $R^2$ | 48 h | 72 h | $IC_{50}$ [μM] | |
| **1a** | H | H | 14.6 ± 7.8 | 18.4 ± 1.2 | >100 | >6.8 |
| **1b** | 2-Me | H | 15.8 ± 1.1 | 18.1 ± 0.14 | 44.9 | 2.8 |
| **1c** | 2-Et | H | 12.3 ± 0.88 | 17.2 ± 0.85 | 43.6 | 3.5 |
| **1d** | 2-Pr | H | 2.6 ± 0.74 | 3.5 ± 0.46 | 43.0 | 16.5 |
| **1e** | 3-Me | H | 14.5 ± 1.6 | 17.6 ± 0.42 | 45.0 | 3.1 |
| **1f** | H | 6-Cl | 12.6 ± 0.59 | 17.1 ± 0.48 | 41.0 | 3.3 |
| **1** g | 2-Me | 6-Cl | 6.0 ± 1.2 | 13.4 ± 0.39 | 41.2 | 6.9 |
| **1** h | 2-Et | 6-Cl | 2.8 ± 0.02 | 3.5 ± 0.07 | 24.4 | 8.7 |
| **1i** | 2-Pr | 6-Cl | 2.6 ± 0.13 | 3.4 ± 0.12 | 8.4 | 3.2 |
| **1j** | H | 7-Cl | 12.0 ± 1.0 | 16.5 ± 0.50 | 43.2 | 3.6 |
| **1k** | 2-Me | 7-Cl | 3.9 ± 0.71 | 10.3 ± 4.8 | 43.8 | 11.2 |
| **1l** | 2-Et | 7-Cl | 2.7 ± 0.15 | 3.7 ± 0.42 | 8.3 | 3.1 |
| **1m** | 2-Pr | 7-Cl | 2.7 ± 0.53 | 3.4 ± 0.11 | 8.4 | 3.1 |
| **1n** | H | 7-$NO_2$ | 8.6 ± 0.23 | 15.1 ± 0.06 | 44.6 | 5.2 |
| **1o** | 2-Me | 7-$NO_2$ | 3.2 ± 0.14 | 4.8 ± 0.48 | 46.4 | 14.5 |
| **1p** | 3-Me | 7-$NO_2$ | 6.1 ± 3.5 | 3.7 ± 0.58 | 43.3 | 7.1 |
| **1q*** | H | H | 14.6 ± 1.38 | 18.0 ± 0.89 | >100 | >6.8 |
| Pentamidine | | | 5.3 nM | | 38.6 | 7283 |

* Oxotacrine.

**Table 2**
Antitrypanosomal activities, cytotoxicities and selectivity indices of dimeric tacrine derivatives; all compounds were tested in duplicate; values represent the mean of the two measurements. SIs were calculated by $IC_{50}$ (J774.1)/$IC_{50}$ (Tbb).

| Compound | Linker (methylene units) | *T. brucei brucei* $IC_{50}$ [μM] | | Cytotoxicity J774.1 $IC_{50}$ [μM] | Selectivity index |
|---|---|---|---|---|---|
| | | 48 h | 72 h | | |
| **2a** | 2 | 3.6 ± 0.53 | 5.1 ± 1.1 | 45.2 | 12.6 |
| **2b** | 3 | 3.3 ± 0.15 | 5.6 ± 0.30 | 42.6 | 12.9 |
| **2c** | 4 | 0.75 ± 0.01 | 2.1 ± 0.36 | 8.3 | 11.1 |
| **2d** | 5 | 5.2 ± 0.42 | 6.4 ± 2.5 | 1.8 | 0.35 |
| **2e** | 6 | 0.26 ± 0.09 | 0.33 ± 0.13 | 3.0 | 11.5 |
| **2f** | 7 | 0.16 ± 0.05 | 0.59 ± 0.01 | 1.9 | 11.9 |
| **2g** | 8 | 0.40 ± 0.02 | 0.66 ± 0.05 | 1.9 | 4.8 |
| **2h** | 9 | 0.13 ± 0.02 | 0.16 ± 0.01 | 1.8 | 13.8 |
| **2i** | 10 | 0.12 ± 0.01 | 0.15 ± 0.01 | 1.8 | 15.0 |
| **3a** | 6 | 0.64 ± 0.04 | 0.71 ± 0.04 | 2.8 | 4.4 |
| **3b** | 6 | 0.64 ± 0.01 | 0.72 ± 0.03 | 1.7 | 2.7 |
| **3c** | 6 | 0.62 ± 0.05 | 0.68 ± 0.03 | 25.0 | 38.5 |
| **3d** | 9 | 0.16 ± 0.01 | 0.21 ± 0.07 | 1.8 | 11.3 |
| **3e** | 9 | 0.43 ± 0.38 | 0.42 ± 0.21 | 8.6 | 20.0 |
| **3f** | 9 | 0.63 ± 0.08 | 0.71 ± 0.01 | 1.7 | 2.8 |
| **3g** | 9 | 0.12 ± 0.01 | 0.14 ± 0.01 | 1.7 | 10.6 |

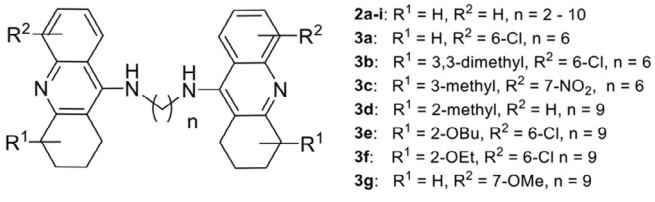



**Fig. 2.** Chemical structures of compounds **2a**–**2i** and **3a**–**3g**.

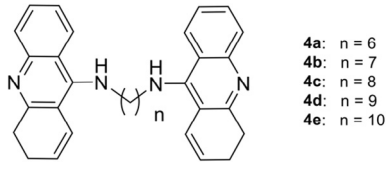



**Fig. 3.** Chemical structures of compounds **4a**–**4e.**

7-amino-4-methylcoumarin (AMC) is released from the substrate Cbz-Phe-Arg-AMC by the enzyme, fluorescence of the reaction can be monitored and allows conclusions of enzyme activity and inhibition, respectively. Compounds which showed an inhibition superior than >50% at 20 μM concentration were selected for determination of their $IC_{50}$-values by varying the inhibitor concentration between 0 and 100 μM. Progress curves which were recorded over a period of 10 min confirmed a time-independent inhibition for all tested compounds.

Compounds **2g**, **2h**, **3c**, **3f**, and **3g** seem to be reasonable inhibitors, all of them showing $IC_{50}$-values below 30 μM whereby **3a** and **3c** have values being in the one-digit micromolar range. This supports rhodesain as a possible target of dimeric bistacrines. Of note, these compounds show similar activities against falcipain-2,[4] an enzyme related to rhodesain expressed by the malarial parasite *Plasmodium falciparum*. However, the $IC_{50}$-values (rhodesain) are some 10-times higher than the antitrypanosomal activity towards *Tbb*, indicating that the compounds may be enriched in the parasite or hit an additional target. Thus, no other compounds of the library were tested.

### 2.2.3. Inhibition of trypanothione reductase

The antitrypanosomal activity of tricyclic, hydrophobic compounds such as phenothiazines, xanthenes,[8] acridines,[9,7] and heterodimeric 4-aminoquinoline-type compounds[7] is well known

**Table 3**
Antitrypanosomal activities, cytotoxicities, and selectivity indices of compounds **4a**–**4e**; all compounds were tested in duplicates. Values represent the mean of the two measurements. SIs were calculated by $IC_{50}$ (J774.1)/$IC_{50}$ (*Tbb*).

| Compound | Linker (methylene units) | *T. brucei brucei* $IC_{50}$ [μM] | | Cytotoxicity J774.1 $IC_{50}$ [μM] | SI |
|---|---|---|---|---|---|
| | | 48 h | 72 h | | |
| **4a** | 6 | 0.51 ± 0.09 | 0.70 ± 0.01 | 1.8 | 3.5 |
| **4b** | 7 | 0.01 ± 0.01 | 0.01 ± 0.01 | 1.5 | 152 |
| **4c** | 8 | 0.08 ± 0.01 | 0.25 ± 0.18 | 1.7 | 21.3 |
| **4d** | 9 | 0.09 ± 0.03 | 0.25 ± 0.18 | 1.6 | 16.7 |
| **4e** | 10 | 0.29 ± 0.37 | 0.88 ± 0.23 | 9.7 | 33.4 |

**Table 4**
Inhibition of rhodesain by compounds **2d**–**2h**, **3a**–**3g**; all compounds were tested in duplicate. Values represent the mean of the two measurements with a deviation less than 10%; nd = not determined.*

| Compound | $R^1$ | $R^2$ | Linker (methylene units) | % Inhibition (at 20 μM) | $IC_{50}$ [μM] |
|---|---|---|---|---|---|
| **2d** | H | H | 5 | 9 | nd |
| **2e** | H | H | 6 | 25 | nd |
| **2f** | H | H | 7 | 44 | nd |
| **2g** | H | H | 8 | 87 | 16.9 |
| **2h** | H | H | 9 | 82 | 19.4 |
| **3a** | H | 6-Cl | 6 | 61 | 7.4 |
| **3b** | 3-$(CH_3)_2$ | 6-Cl | 6 | 64 | 26.6 |
| **3c** | 3-Me | 7-$NO_2$ | 6 | 82 | 5.8 |
| **3d** | 2-Me | H | 9 | 68 | 26.6 |
| **3e** | 2-OBu | 6-Cl | 9 | 26 | nd |
| **3f** | 2-OEt | 6-Cl | 9 | 89 | 13.3 |
| **3g** | H | 7-OMe | 9 | 82 | 18.1 |

* Reference inhibitor K11777: $k_{2nd}$ = 555,000 [1/(M·s)][30]; $k_{2nd}$ (measured here) = 500,000 [1/(M·s)].

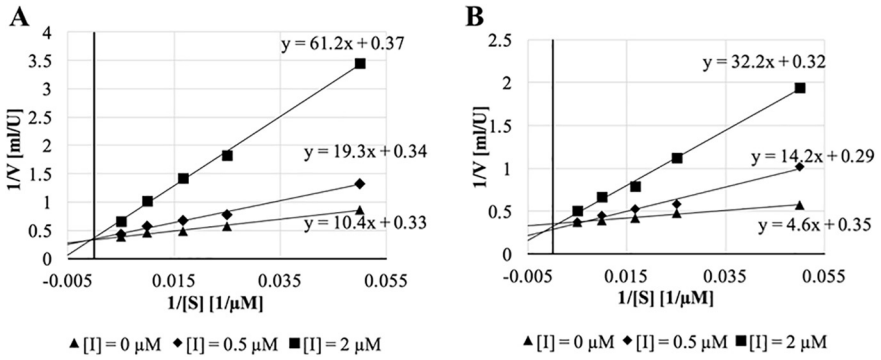



**Fig. 4.** Lineweaver-Burk-Plots for compound **2h** inhibition ofTcTryR (A) and TbTryR (B); 0 μM = control DMSO.

and attributed to the large, hydrophobic active site of TryR. This essential enzyme is unique to trypanosomatid parasites and represents the main weapon against oxidative stress, rendering it an attractive drug target. Hence, a representative selection of compounds was tested as putative inhibitors of the reductase.

First, compounds **2e**–**2h** showing the lowest $IC_{50}$ values *in vitro*, were studied at two fixed concentrations each (**2e**–**2g**: 2 μM and 5 μM, **2h**: 1 μM and 3 μM, respectively) and two concentrations of trypanothione disulfide ($TS_2$). 40 and 100 μM $TS_2$ correspond to about twice and five-times the $K_m$-value for this substrate (18 μM in the case of *Trypanosoma cruzi* (*Tc*) TryR.[16] A decrease of the substrate concentration resulted in an increase of inhibition suggesting a competitive type of inhibition. In the presence of 40 μM substrate, 2 μM **2e**, **2f**, and **2g** resulted in 28, 36, and 46% inhibition of *Tc*TryR, respectively. In comparison, 1 μM **2h** yielded 51% inhibition (for details, see Table S1).

Thus, compound **2h** was subjected to a detailed kinetic analysis using TryR species from both *Tb* and *Tc*. Lineweaver-Burk-Plots confirmed the competitive type of inhibition (Fig. 4). The $K_i$-values were calculated from the direct plots giving a value of 0.4 μM for both *Tc*TryR and *Tb*TryR.

Third, since compound **4b** had the highest antitrypanosomal activity and the highest selectivity index, the inhibitory activity of this compound was tested towards *Tb*TR. At a concentration of 5 μM and a substrate concentration of 100 and 40 μM $TS_2$, **4b** resulted in 53 and 47% inhibition (see Fig. 5). **4b** is a mixed-type

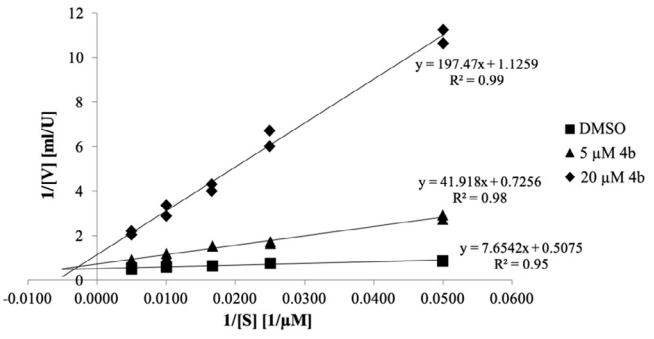



**Fig. 5.** Lineweaver-Burk-Plot for compound **4b** and TbTryR.

inhibitor with $K_i$ = 1.2 μM and $K_{i'}$ = 12 μM, indicating a higher affinity for the free enzyme than for the enzyme-substrate complex.

Comparing the inhibitory potency of 5 μM **2f** and **4b**, both having a heptane linker, in the presence of 100 and 40 μM of $TS_2$ revealed a stronger inhibition for **2f** (53 and 66%) and a slightly greater difference. This might be due to the different mode of inhibition, i.e. competitive *versus* mixed type.

Since TryR and *human* glutathione reductase (hGR) share almost 40% of the residues and thus have a similar structure and mechanism of action,[17] compound **2h** was tested for its inhibitory activity towards hGR as well. At 100 μM GSSG, 2 and 50 μM **2h** only weakly inhibited the host enzyme by 2% and 19%, respectively. Thus, the compounds are likely to be selective for the protozoal TyrR.

## 3. Conclusion

Substitution variations of both ends of the bistacrines and the length of the linker led to compounds of inhibitory activities against *Trypanosoma brucei brucei* covering three orders of magnitude which allow to establish pronounced structure-activity relationships (SARs). SARs point to a specific interaction with a defined target. Whereas some compounds showed inhibition of rhodesain in micromolar range of concentration, the inhibition of TryR was partially found in submicromolar concentrations, which is in line with the *in vitro* activity. Interestingly, some compounds are competitive (series **2**) and some are mixed-mode inhibitors. This may suggest TryR being a more important target of these compounds compared to rhodesain. Of note, preliminary investigations showed that the bistacrine compounds show antileishmanial activity and inhibit the corresponding TryR of *Leishmania infantum* (data not shown).

However, the compounds were found to have a high lipophilicity[4] which might affect the toxicity adversely.[18] It might be speculated that a decrease of log P – e.g. by introducing carbonyl groups or polyoxyethylene moieties into the linker – may lead to reduced promiscuity and thus, to reduced toxicity. Anyway, the bistacrine moiety and especially compound **4b** is a promising lead structure for further development of an antitrypanosomal drug.

## 4. Experimental

### 4.1. Chemistry

Tacrine (**1a**)[19] and compounds **1d**,[4] **1e**,[20] **1f**,[4,21] **1j**,[22] **1m**,[4] **1n**,[22] **2a–i**,[4,23–25] **3a–c**,[4] **3f**,[4] and **3g**[4] were prepared as previously described. The new compounds were synthesized accordingly. Details and the synthetic pathway for compounds **4a–4e** can be found in the Supporting information.

### 4.2. Biological assays

#### 4.2.1. Trypanosoma and macrophage assay

AlamarBlue® assays for investigating the activity against *T. brucei brucei* laboratory strain TC221 and J774.1 macrophages were conducted as previously reported.[12,26]

#### 4.2.2. Enzyme assays

*4.2.2.1. Rhodesain assay.* The assay was performed as described previously.[27] In brief: The assays were performed twice in a Varian Cary Eclipse spectrofluorometer in 96-well plates using a microplate reader (excitation wavelength 380 nm, emission wavelength 460 nm) and a total volume of 200 μl. Cbz-Phe-Arg-AMC was used as substrate. To 180 μl of sodium acetate buffer (50 mM, pH 5.5, containing 5 mM DDT, 5 mM EDTA and 200 mM NaCl) 5 μl enzyme solution, 10 μl inhibitor stock solution (400 μM in DMSO), and 5 μl substrate stock solution (400 μM in DMSO) were added and the increase of the fluorescence was observed over 5 min. The enzyme activity was calculated from the slopes of the curves obtained (ΔF vs. time). Compounds having an inhibitory effect greater than 50% at 20 μM were chosen for detailed assays. Therefore, 185 μl buffer, 5 μl enzyme solution, 5 μl inhibitor solution, and 5 μl substrate solution were mixed and the fluorescence intensity was recorded over 10 min. Inhibitor concentrations were in the range of 0–100 μM in case of compounds **3b**, **3d**, and **3g**; 0–50 μM in case of compounds **2h**; 0–40 μM in case of compounds **2g**, **3a**, and **3f**; and 0–20 μM in case of compounds **3c**. $IC_{50}$-values were obtained by nonlinear regression analysis using Origin® Pro 2015 software.

*4.2.2.2. Trypanothione reductase assay.* Trypanothione disulfide ($TS_2$) was synthesized enzymatically.[28] TryR activity was measured in a total volume of 1 ml of 40 mM of HEPES buffer, 1 mM EDTA, pH 7.5, containing 100 μM NADPH and the enzyme. The reaction was started by adding $TS_2$; the absorbance decrease at 340 nm was followed at 25 °C using a Jasco V-650 spectrophotometer. The consumption of NADPH ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$) was used to calculate the volume activity of the enzyme. The amount of TryR in the assay was chosen to obtain an absorbance decrease of −0.02 to $-0.08\ min^{-1}$ (corresponding to 10 mU per 1 mL assay solution, i.e. 2 nM).

Millimolar stock solutions of the inhibitors were prepared in DMSO. Each assay contained a total volume of 50 μl of DMSO, either of inhibitor solution or DMSO as control. The preliminary screening was performed with inhibitor concentrations from 1 to 5 μM and a fixed concentration of either 40 or 100 μM $TS_2$. For the detailed kinetic analyses, the $TS_2$ concentration was varied from 20 to 200 μM. The inhibitor constants were calculated by means of Graphpad Prism 7 (GraphPad Software, Inc.La Jolla, CA, USA).

*4.2.2.3. Glutathione reductase assay.* The assay was performed according to 29. In brief, the activity of hGR was measured in 47 mM potassium phosphate, 200 mM KCl, 1 mM EDTA, pH 6.9. In analogy to the TR assay, the assays contained in a total volume of 1 ml buffer 100 μM NADPH and 6–30 mU of hGR (i.e. 1–2 nM), and the activity was measured at 25 °C. The reaction was started by addition of glutathione disulfide (GSSG) and the decrease of absorbance was followed at 340 nm.

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## A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2017.06.051.

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