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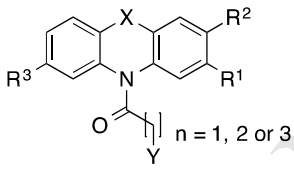

X = S, O, CH_2–O, CH_2–CH_2, or CH=CH
Y = H, Cl, Br or CN
R = H, Cl or CF_3

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Abbreviations

L. major: Leishmania major, MDR: multiple drug resistance, ND: not determined, Px: nonselenium glutathione peroxidase type tryparedoxin peroxidase, SAR: structure–activity relationship, SRB: sulforhodamine B, TBAB: tetra-n-butylammonium bromide, TR: trypanthione reductase, Tpx: tryparedoxin, $T(SH)_2$: trypanothione [bis(glutathionyl)spermidine], TS_2: trypanothione [bis(glutathionyl)spermidine] disulfide, NADPH: nicotinamide adenine dinucleotide phosphate, T. brucei: Trypanosoma brucei

Key words
Phenoxazines, phenothiazines, antileishmanial activity, chloroacetamides, trypanothione reductase

Abstract

A number of phenothiazine-, phenoxazine- and related tricyclics-derived chloroacetamides were synthesized and evaluated *in vitro* for antiprotozoal activities against *Leishmania major* (*L. major*) promastigotes. Several analogs were remarkably potent inhibitors, with antileishmanial activities being comparable or superior to those of the reference antiprotozoal drugs. Furthermore, we explored the structure–activity relationships of N-10 haloacetamides that influence the potency of such analogs toward inhibition of *L. major* promastigote growth in vitro. With respect to the mechanism of action, selected compounds were evaluated for time-dependent inactivation of *T. brucei* trypanothione reductase. Our results are indicative of a covalent interaction which could account for potent antiprotozoal activities.

1. Introduction

Protozoan parasites of the order Kinetoplastida are causative to Chagas disease (*Trypanosoma cruzi*), sleeping sickness (*Trypanosoma brucei* subspp.) and the different manifestations of leishmaniasis (*Leishmania* spp.). Although the latter can be treated, limited therapeutic options including pentavalent antimonials, miltefosine or amphotericin B need a long-term parenteral administration and show severe side effects and toxicity. Additionally, these drugs are expensive and require adequate medical care which is not readily available in the most affected regions of India or Africa. Clearly more efficient and affordable antileishmanial therapeutics are needed. In our search strategy for novel, potent antileishmanial compounds we extended our studies to tricyclic heterocycles, which are attractive scaffolds in medicinal chemistry and part of numerous important therapeutic agents (Chart 1).[1]

Phenothiazine (**1**, Chart 1), phenoxazine (**2**) and related structures are building blocks for a large number of drugs that have shown diverse biological activities including antipsychotic, anticancer, antihelminthic and other pharmacological properties.[2]

Chart 1 should be inserted here

Chlorpromazine (**3**), prepared in 1950, is the prototypical phenothiazine antipsychotic drug and perhaps the best known of the phenothiazine drugs for treatment of neurological disorders. Many other 10-substituted phenothiazines and related structures such as clomipramine (**4**) are also well-known neuroleptic and antidepressant drugs. The phenothiazine dye methylene blue (**5**) is employed as a fast-acting antidote for the treatment of methemoglobinemia[3]. The antileishmanial activity of chlorpromazine against both promastigotes and amastigotes of *L. donovani* has been demonstrated by Pearson et al.[4] A trypanocidal effect of neuroleptic phenothiazines such as chlorpromazine and thioridazine on *Trypanosoma brucei* was described by Seebeck et al., most likely due to an interaction of the

drugs with pellicular microtubules.[5] Recently, Fauro et al. found clomipramine to be a good candidate for Chagas disease in the chronic cardiac form of the infection.[6] Notably, no single component of the molecular structure of the tricyclic neuroleptics and antidepressants appears to be critical for activity.[7]

Moreover, antifungal activities of α-chloro-*N*-acetyl derivatives of phenoxazine, carbazole and diphenylamine (**13a**, **17** and **18**, see Scheme 1**)** have been described by Sarmiento et al.[8] We were able to identify a panel of leishmanicidal phenothiazine-, phenoxazine- and related tricyclics-derived chloroacetamides. Parent compounds were originally obtained during synthetic studies on phenothiazine– and phenoxazine–based tubulin inhibitors,[9] when subjecting selected analogs to a *L. major* promastigote screening. *L. major* may cause cutaneous leishmaniasis with symptoms ranging from skin sores, cutaneous and muco-cutaneous lesions, leading to the formation of disfiguring scars after wound healing. Though the leishmanicidal activities of phenothiazines and related compounds have been intensively investigated,[10-12] structure–activity relationships of their central nitrogen N-10 haloacetamides have never, to our knowledge, been reported systematically.

Some of the compounds described herein were remarkably potent inhibitors, with antileishmanial activities of the most active compounds being comparable or superior to those of the reference compounds, such as miltefosine, paromomycine and pentamidine. To get an insight in the specifity, the cytotoxicity of selected compounds was assayed against human leukemia K562 cells. To further elucidate the mechanism of action, time-dependent inactivation of *T. brucei* TR by chloroacetamides was investigated.

2. Results and discussion

2.1 Chemistry

Synthetic precursors (Chart 2) were available from commercial sources or prepared according to literature protocols.[13]

Chart 2 should be inserted here

The synthesis of haloacetamides was accomplished by chloroacylation of the respective tricyclics and related structures with appropriately substituted acyl chlorides under reflux in toluene (Scheme 1). Compound **20** was prepared by alkylation with 1-bromo-2-chloroethane using catalytic tetrabutylammonium bromide (TBAB).

Scheme 1 should be inserted here.

2.2 Biological evaluation

2.2.1 Growth inhibition of *L. major* promastigotes

Although the *Leishmania* parasite exists in the human host in its intra-macrophage amastigote form, the extracellular insect-infective promastigote form of *Leishmania* is addressed in many drug screening assays against *Leishmania*, which can be performed readily, thus being more suitable for automation. Detailed aspects of promastigote versus amastigote screening have recently been excellently reviewed by De Muylder et al.[14]

Table 1 displays the inhibition data of the novel compounds against promastigotes of *L. major*, which were assesssed by colorimetric AlamarBlue® assay.[15] These data explore the effect of altering not only the nature of the tricyclic nucleus but also substitutions on the nitrogen atom. The most potent promastigote inhibitors within the phenothiazine- and phenoxazine series displayed IC_{50} values in the range of 10 µM and include a tricyclic 6-6-6 (**12a, 12b, 13b, 13f, 13i**) or 6-7-6 scaffold (**14**) and – with the exception of compound **13l** – the *N*-chloroacetamido moiety.

The comparison of the IC_{50} values obtained for **13a** bearing an acetamide moiety with those obtained for chloracetamides **12a, 12b, 13b, 13f** and **13i** clearly suggests the importance of

the intact haloacetamide for potent promastigote inhibition. The mere presence of an amide functional group is apparently not sufficient to impart antileishmanial activity, as seen with **13a** (*L. major* IC_{50} > 100 µM, Table 1), and did not induce antiproliferative potency. Other evidence that the chloroacetamido provides an important partial structure comes from the synthetic precursor 2-chloro-10*H*-phenoxazine (**2a**), which does not show substantial activity. In particular the replacement of the carbonyl functional group (**20**, Scheme 1, Table 1) by a methylene spacer or substitution of the terminal chloro atom by a bromo- or a cyano group (**13g**, **13h,** Table 1) caused a dramatic decrease in the IC_{50} value, again pointing to the particular importance of the α-chloroacetamido as a main determinant for inhibiton of parasite proliferation. In this connection it is interesting to note that chain elongation by inserting methylene spacers (**13c–13e**) between the amide and the terminal chloro did not cause an activity enhancement, but even resulted in a reduced antipromastigote potency. Thus, the spatial disposition of the terminal chloro atom and the heterocycle is an influential factor in determining antileishmanial activity. Ring-opened variants **18** and **19**, both bearing the chloroacetamide, proved considerably less effective (**18**, IC_{50} 40.30 µM and **19**, IC_{50} 42.60 µM *vs.* **12a**, IC_{50} 9.30 µM and **13b**, IC_{50} 7.60 µM) compared to the most active analogs, indicating that rigidifying the diphenylamine or the diphenyl ether is important for target binding. This is supported by similar observations made by Richardson et al. with diphenylmethane analogs.[16]

On the one hand, efficiency against promastigotes could be attained by 7-chloro-5,11-dihydrodibenzo[*b*,*e*][1,4]oxazepine–derived **14**, leading to one of the most active compounds of the series (IC_{50} 7.20 µM). This finding indicates that compared with **13b** the ring-enlarged tricyclic 6-7-6 oxazepine is also a favored scaffold. On the other hand, 5*H*-dibenzo[*b*,*f*]azepines **15** and **16** are only moderate inhibitors of promastigote growth (**15**, IC_{50} 43.30 µM and **16**, IC_{50} 48.10 µM). Interestingly, decreasing the ring size to obtain the

carbazole **17** completely abolished activity against *L. major*. In general, introduction of different substituents into the phenothiazine- or phenoxazine, such as chloro (**12b**), dichloro (**13f**), trifluoromethyl (**13i)** or cyano (not shown) had no significant impact on the antiparasitic activities. Based on the broad range of potencies, inhibition of *L. major* promastigote growth by the chloroacetamides cannot be considered unselective. Fig. 1 summarizes the SAR on promastigote inhibition.

Fig. 1 should be inserted here

2.2.2 Growth inhibition of K562 cells

Additionally, we preliminarily evaluated the putative cytotoxicity of the compounds towards K562 cells (human chronic myelogenous leukemia, DSMZ ACC-10).[17] Cell proliferation was determined directly by counting the cells with a hemocytometer after 48 h of treatment.

Table 1 summarizes the data for inhibition of K562 cell growth. The most active chloroacetamides displayed IC_{50} values in the range ≤ 5 µM, indicating strong cytotoxic potential. The chloro acetamides such as **15**, **16** and especially **17**, which were not or only weakly active against promastigotes showed antiproliferative potencies in the range of 2-5 µM. Obviously, the chloroacetamide is crucial for the cytotoxicity against K562 cells with cytotoxic potencies being more or less similar. These finding suggests a nonselective growth inhibition in the K562 cell-based assay and is in contrast with the results from the promastigote assay, where the analogs may act specifically toward a target-protein with the nature of the tricyclic ring playing a critical role for target interaction.

Table 1 should be inserted here

2.2.3 Studies on the mechanism of antiprotozoal action

Earlier, molecular modeling approaches as well as kinetic analyses and crystallographic analysis revealed phenothiazine (**1**), imipramine and related tricyclic antidepressants are competitive inhibitors of TR.[10-12]

The flavoenzyme trypanothione reductase (TR), which does not occur in the mammalian host, is a prominent target for antitrypanosomal and -leishmanial drug development approaches.[18] TR catalyzes the NADPH–dependent reduction of trypanothione [bis(glutathionyl)spermidine] disulfide TS_2 to trypanothione [$T(SH)_2$], the main low molecular mass thiol in trypanosomatids (Equation 1).[19]

Equation 1 should be inserted here

The tricyclic structures are proposed to bind to TR with the tricyclic ring lodged against the hydrophobic wall of TR active site formed by Trp21 and Met 113 and the aminopropyl side chain extending towards Glu466´and Glu467` residues.[10, 11] By inhibiting TR, the parasite should become susceptible to oxidative stress induced by drugs or the host defence system (Fig. 1). Notably, N-substituted **12a** (Scheme 1), has been noted previously as a moderate inhibitor of *T. cruzi* TR.[11]

We suggest that an irreversible inhibition by alkylation of biologically important nucleophiles such as susceptible SH- or NH-groups on the active site of distinct proteins could possibly be involved in the modes of action of the chloroacetamides against the *L. major* promastigotes, as described for other compounds.[20, 21] Several drugs, such as the 2-chloroethyl nitrosourea carmustine[22], and quinacrine analogs[23] are known to inhibit trypanothione reductase irreversibly. Basically, the reactive α-haloacetamide moiety itself is prone to form covalent bonds by attacking nucleophiles with concomitant displacement of the halogen.[24] However, since numerous enzymes contain SH-groups vital to the activity of the enzyme, it

would seem obvious that a large variety of enzymes might be nonselectively inhibited by chloroacetamides.

Trypanosoma and *Leishmania* are protozoan parasites which have a unique trypanothione-based thiol redox metabolism (Fig. 2). In order to get more insight into the mechanism of action of the chloroacetamides, we performed additional studies using recombinant *T. brucei* TR. TR is common to all parasites of the Trypanosomatidae family and it has been documented, that kinetic and physical properties of *T. brucei* TR are consistent with trypanothione reductases from other trypanosomatids such as *T. cruzi* and *L. donovani*.[25] None of the compounds proved to be a reversible inhibitor of the enzyme (data not shown). Thus, in the next step, selected derivatives were studied for their ability to interfere with the unique peroxidase cascade of the parasites. All enzymes establishing the detoxification system, namely TR, tryparedoxin (Tpx) and the tryparedoxin peroxidases have been shown to be essential for *T. brucei*.

Fig. 2 should be inserted here

In a first set of experiments, to identify a distinct target protein, selected compounds were characterized in a time-dependent peroxidase. For this purpose, a reaction mixture composed of NADPH, *T. brucei* TR, Tpx, Px, $T(SH)_2$ and inhibitor was incubated for 10 min and then the reaction started by adding H_2O_2. Compounds **12a** and **14** showed remarkable time-dependent inhibition (data not shown).

In the next set of experiments, selected compounds were evaluated for their activity toward *T. brucei* TR. TR was preincubated with the compounds in the presence or absence of NADPH. After different times, an aliquot of the reaction mixture was subjected to a TR standard assay, starting the reaction by adding TS_2.

Fig. 3 should be inserted here

Our cuvette-based assays showed a time-dependent inactivation of TR for the most active compounds of the *Leishmania* assay. It has to be pointed out that preincubation mixtures that lacked either NADPH or the potential inhibitor showed no decrease of enzyme activities with time. Moreover, when the assay was started directly, no inactivation became visible (data not shown).

Examination of time-dependent TR inhibition revealed that all tested analogs (Fig. 2) with potent antileishmanial activities were also active as *T. brucei* TR inhibitors.

3. Conclusion

We have shown that phenothiazine-, phenoxazine- and related tricyclics-derived haloacetamides are highly potent inhibitors of the promastigote form of *L. major*. Structural comparisons of the compounds hints at a selective mechanism of action. Those analogs with the highest antiprotozoal activities in the promastigote assay displayed strong time-dependent inhibition of recombinant TR. The most active analogs described herein showed two essential moieties. One is the tricyclic (6-6-6 or 6-7-6) scaffold which possibly interacts with hydrophobic regions of the target enzyme active site, in a way that has been suggested for several tricyclic, mainly competitive TR inhibitors.[10-12] The second is the chloroacetamido moiety. Since alkylation of biologically important nucleophiles such as susceptible SH- or NH-groups of active sites could contribute to antileishmanial and antitrypanosomal potencies, we investigated the time-dependent effects of the chloroacetamides on inhibition of *T. brucei* TR. Preincubation of TR with selected compounds in the presence – but not in the absence - of NADPH resulted in a time-dependent inactivation of TR, pointing to a covalent modification of Cys52, part of the redox active dithiol/disulfide. Our findings strongly suggest that TR is a target of the chloroacetamides. This is strongly supported by literature findings

for structurally related TR-inhibiting tricyclic systems.[10-12, 23] However, it cannot yet be completely ruled out that putative drug targets of the peroxidase cascade such as Tpx are also affected by our inhibitors.[24]

Taken together, our results are indicative of a covalent mechanism of action of the haloacetamides and the effects of the compounds toward the peroxidase cascade. To our knowledge this is the first report on covalent inhibitors derived from phenothiazines or phenoxazines. Scope of future experiments will be expanded to the selectivity for TR over human glutathione reductase, and the correlation of TR inhibition with antiparasitic growth-inhibitory activity. Activities against *L. major* amastigotes have yet to be determined.

4. Experimental

4.1 Chemistry

4.1.1 General

Melting points were determined with a Kofler melting point apparatus and are uncorrected. Spectra were obtained as follows: ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz) spectra were recorded with a Varian Gemini 2000 or Varian Mercury 400 plus (400 MHz) spectrometers, respectively. NMR signals were referenced to TMS (δ = 0 ppm) or solvent signals and recalculated relative to TMS. Fourier-transform IR spectra were recorded on a Bio-Rad laboratories Typ FTS 135 spectrometer and analysis was performed with WIN-IR Foundation software. Mass spectra were obtained on Finnigan GCQ and LCQ apparatuses applying electron beam ionization (EI) and electrospray ionization (ESI). Atmospheric pressure chemical ionization (APCI) method was performed with a microTOF-QII apparatus (Bruker). The purity of all target compounds was determined either by elemental analyses or by reversed phase HPLC at 254 nm. Compound purity for all target compounds is $\geq$ 95%. Elemental analyses were performed at the Münster microanalysis laboratory, using a Vario EL elemental analyzer from Elementar Analysensysteme GmbH Hanau, and all values were

within ± 0.4% of the calculated composition. The HPLC system applied a C18 phase (Nucleosil, 3 μm, 3.0 × 125 mm, CS-Chromatographie Service GmbH, Langerwehe, Germany) eluting the compounds with an acetonitrile/H_2O gradient at a flow rate of 0.40 mL/min. All organic solvents were appropriately dried or purified prior to use. Acid chlorides were obtained from commercial sources or prepared to literature protocols. Purification by chromatography refers to column chromatography on silica gel (Macherey-Nagel, 70-230 mesh). In most cases, the concentrated pure fractions obtained by chromatography using the indicated eluants were treated with a small amount of hexane to induce precipitation. All new compounds displayed ^{1}H NMR and MS spectra consistent with the assigned structure. Yields have not been optimized. Analytical TLC was done on Merck silica 60 F_{254} alumina coated plates (E. Merck, Darmstadt).

2-Chloro-10*H*-phenoxazine (2a).[13]

The title compound was prepared according to the literature protocol.

2-Chloro-1-(10*H*-phenothiazin-10-yl)ethan-1-one (12a).[8]

See SI for details.

2-Chloro-1-(2-chloro-10*H*-phenothiazin-10-yl)ethan-1-one (12b).[11]

See SI for details.

1-(2-Chloro-10*H*-phenoxazin-10-yl)ethan-1-one (13a). [9]

The title compound was prepared as recently described.[9]

2-Chloro-1-(2-chloro-10*H*-phenoxazin-10-yl)ethan-1-one (13b).[26]

The title compound was prepared from **2a** (2.12 g, 9.74 mmol) and chloroacetyl chloride (1.22 g, 10.8 mmol) in toluene (60 mL) in a similar manner according to a literature protocol.[26] Purification of the residue by chromatography (CH_2Cl_2) afforded **13b** as white crystals (2.21 g, 94 %). mp 97 °C (Lit. [26] 96 °C); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.71 – 7.67 (m, 1H), 7.50 (d, *J* = 7.9 Hz, 1H), 7.31 – 7.25 (m, 1H), 7.24 – 7.14 (m, 3H), 7.08 (dd, *J* = 8.3, 0.6 Hz, 1H), 4.32 (s, 2H).

3-Chloro-1-(10*H*-phenoxazin-10-yl)propan-1-one (13c).[27]

See SI for details.

3-Chloro-1-(2-chloro-10*H*-phenoxazin-10-yl)propan-1-one (13d).

See SI for details.

4-Chloro-1-(2-chloro-10*H*-phenoxazin-10-yl)butan-1-one (13e).

See SI for details.

2-Chloro-1-(2,8-dichloro-10*H*-phenoxazin-10-yl)ethan-1-one (13f).

See SI for details.

2-Bromo-1-(2-chloro-10*H*-phenoxazin-10-yl)ethan-1-one (13g).

See SI for details.

3-Oxo-3-(10*H*-phenoxazin-10-yl)propanenitrile (13h).

See SI for details.

2-Chloro-1-(2-(trifluoromethyl)-10*H*-phenoxazin-10-yl)ethan-1-one (13i).[28]

See SI for details.

3-Chloro-1-(10*H*-phenoxazin-10-yl)butan-1-one (13j).

See SI for details.

3,3-Dichloro-1-(10*H*-phenoxazin-10-yl)propan-1-one (13k).

See SI for details.

2,3-Dichloro-1-(10*H*-phenoxazin-10-yl)propan-1-one (13l).

See SI for details.

2-Chloro-1-(7-chlorodibenzo[*b*,*e*][1,4]oxazepin-5(11*H*)-yl)ethan-1-one (14).

In a typical procedure, to a solution of **6** (0.59 g, 3 mmol) in toluene (30 mL), chloroacetyl chloride (0.34 g, 3 mmol) was added. The mixture was stirred at reflux for 4 h. After that, the solvent was evaporated in vacuo. Purification of the residue by chromatography (CH_2Cl_2) afforded **14** as white crystals (0.54 g, 58%). mp 142-143 °C; FTIR 1685 cm^{-1}; ^1H NMR (400 MHz, Chloroform-*d*) δ 7.58 – 7.28 (m, 5H), 7.13 (s, 1H), 6.81 (s, 1H), 5.70 (d, *J* = 12.5 Hz,

1H), 4.87 (d, *J* = 12.5 Hz, 1H), 4.40 – 3.83 (m, 2H); MS (APCI) calcd for $C_{15}H_{11}Cl_2NO_2$ $[M]^+$ 307.02; found 308.03; purity (HPLC): 99.66%.

2-Chloro-1-(10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepin-5-yl)ethan-1-one (15).

See SI for details.

2-Chloro-1-(5*H*-dibenzo[*b*,*f*]azepin-5-yl)ethan-1-one (16).

See SI for details.

1-(9*H*-Carbazol-9-yl)-2-chloroethan-1-one (17).[8]

See SI for details.

2-Chloro-*N*-(3-chlorophenyl)-*N*-phenylacetamide (18).[8]

See SI for details.

2-Chloro-*N*-(5-chloro-2-phenoxyphenyl)acetamide (19).

See SI for details.

2-Chloro-10-(2-chloroethyl)-10*H*-phenoxazine (20). To a solution of 2-chloro-10*H*-phenoxazine (**2a,** 2.0 g**,** 9.22 mmol) in CH_2Cl_2 (20 mL) were added KOH (6N, 30 mL), tetra-*n*-butylammonium bromide (TBAB, 1.5 g, 4.65 mmol) and 1-bromo-2-chloroethane (3.17 g, 22.34 mmol, 1.84 mL). The reaction mixture was stirred at room temperature until the reaction was completed, then treated with water (50 mL) and subsequently extracted with CH_2Cl_2 (2 × 25 mL). The combined organic phases were washed with water, dried over Na_2SO_4, and concentrated in vacuo. Purification by chromatography (CH_2Cl_2/hexane 1/1) afforded **20** as a fine white powder (0.14 g, 20%): mp 84-85 °C; FTIR 1458, 1423 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.67-6.63 (m, 1H), 6.56-6.52 (m, 1H), 6.48-6.44 (m, 2H), 6.37 (d, 1H, *J* = 8.22 Hz), 6.34 (dd, 1H, *J* = 6.65 Hz, *J* = 1.18 Hz), 6.28 (d, 1H, J = 2.35 Hz), 3.65 (t, 2H, *J* = 7.43 Hz), 3.45 (t, 2H, *J* = 7.04 Hz); MS *m/z* 353 (<1), 277 (100); Purity (HPLC), 96.67%.

4.2 Biological evaluation

Materials and methods for the biological assays have been described before by the authors.

AlamarBlue assay for investigation of antileishmanial activities against *L. major* promastigotes was conducted as previously reported.[15]

4.2.1 Assay of Cell Growth.

K562 cells were plated at 2×10^5 cells/mL in 24 well dishes (Costar, Cambridge, MA). Untreated control wells were assigned a value of 100%. Test compounds were made soluble in DMSO/methanol 1:1 (stock solution 3 mg/mL), and control wells received equal volumes (0.5%) of vehicle alone. We demonstrated, that 0.5 % DMSO (final DMSO concentration in all samples including the controls) did not affect K562 cell growth. To each well 5 µL of compound were added and the final volume in the well was 500 µL. Cell numbers were counted with a Neubauer counting chamber (improved, double grid) after 48-h compound exposure. Each assay was prepared in triplicate, and the experiments were carried out three times. IC_{50} values were obtained by nonlinear regression (GraphPad Prism™) and represent the concentration at which cell growth was inhibited by 50%. The adjusted cell number was calculated as a percentage of the control, which was the number of cells in wells without the test compound.

4.2.2 Time-dependent TR inactivation assay.

In a total volume of 200 µL TR assay buffer, 400 µM NADPH, 1 µM *T. brucei* TR[18], and either 25 (**2a**), 50 (**13b**) or 100 µM (**12a**, **13a**, **14**) compound or DMSO (5%) were preincubated at 25 °C. After 10 min, 30 min, 60 min, 120 min and 180 min, respectively, a 5 µL aliquot was subjected to a 1 mL TR standard assay,[22] containing 100 µM NADPH and the reaction was started by addition of 100 µM (5 µL) trypanothione disulfide (TS_2). The absorption decrease at 340 nm was monitored at 25 °C. The volume activity (U/mL) was plotted against the pre-incubation time.

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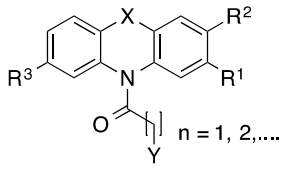
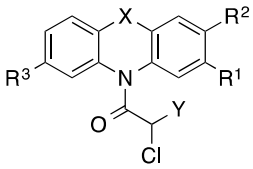
Table 1. EC_{50} values of selected compounds towards L. major in comparison to reference compounds as well as mammalian cells



12a-12b, 13a13-i



13j-13l

cmpd	**X**	**Y**	**n**	**R^1**	**R^2**	**R^3**	***L. major*[a] IC_{50} [µM]**	**K562[b] EC_{50} [µM]**
2a							69.20	
12a	S	Cl	1	H	H	H	**9.30**	5.00
12b	S	Cl	1	Cl	H	H	**10.4**	3.20
13a	O	H	1	Cl	H	H	> 100	> 30
13b	O	Cl	1	Cl	H	H	**7.60**	3.00
13c	O	Cl	2	H	H	H	39.10	15.23
13d	O	CH_2Cl	1	Cl	H	H	15.70	10.22
13e	O	CH_2CH_2Cl	2	Cl	H	H	> 100	> 90
13f	O	Cl	1	Cl	H	Cl	**9.50**	2.03
13g	O	Br	1	Cl	H	H	> 100	6.15
13h	O	CN	1	H	H	H	> 100	23
13i	O	Cl	1	CF_3	H	H	**9.60**	ND
13j	O	CH_3		H	H	H	> 100	> 30
13k	O	Cl		H	H	H	82	> 30
13l	O	CH_2Cl		H	H	H	**9.60**	ND
14	CH_2-O	Cl		H	H		**7.20**	3.30

15	CH_2-CH_2	Cl		H	H		43.30	4.80
16	CH=CH	Cl		H	H		48.10	6.20
17*							> 100	6.44
18*							40.30	ND
19*							42.60	ND
20*							> 100	ND
amphotericine B							0.33	ND
miltefosine							36	ND
pentamidine isethionate							84	ND
paromomycine							> 100	ND

[a]The drug concentrations resulting in 50% cell growth (EC_{50}). Cell viability was determined after 48 h by the AlamarBlue® assay. [b]EC_{50} K562, concentration of drug required for 50% inhibition of cell growth. Cells were treated with drugs for two days. EC_{50} values are the means of at least three independent determinations (SD < 10%) . * See Scheme 1 for chemical structure.

Figure Legends

Chart 1 Phenothiazine (**1)**, phenoxazine (**2)** and related drugs **3**, **4** and **5**.

Chart 2 Synthetic precursors

Scheme 1 R^1-R^3 are defined in Table 1. Reagents and conditions: (a) haloacyl chloride, toluene, 90 °C, N_2; b) TBAB, CH_2Cl_2, 6N KOH, 1-bromo-2-chloroethane, room temperature;

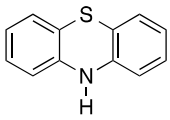
Equation 1

Fig. 1 SAR summary

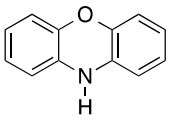
Fig. 2 Detoxification of hydroperoxides (ROOH) by the trypanothione cascade.

Fig. 3 Time-dependent inactivation of *T. brucei* trypanothione reductase. The enzyme was incubated with the compounds in the presence or absence of NADPH. After different times, an aliquot of the reaction mixture was subjected to a standard assay as outlined in section 4.2.2. . Compounds **2a** (A) and **13a** (B) represent negative controls, which show no inhibition over time. On the other hand compounds **13b** (C), **12a** (D) and **14** (E) show a strong time-dependent inhibition, suggesting a covalent mechanism of action. The EC_{50} refers to the *L. major* promastigote assay.

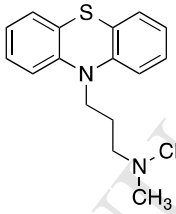
Chart 1



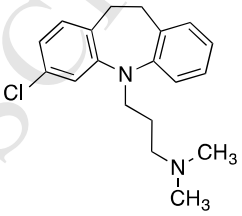
1, 10*H*-phenothiazine



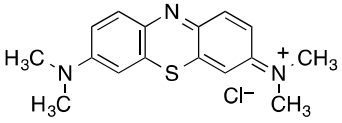
2, 10*H*-phenoxazine



3, chlorpromazine

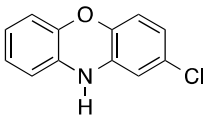


4, clomipramine

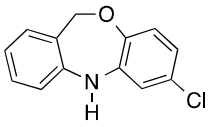


5, methylene blue

Chart 2.



2a



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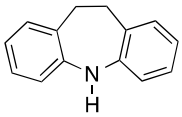

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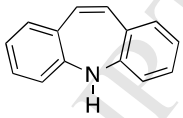

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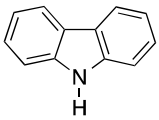

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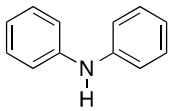

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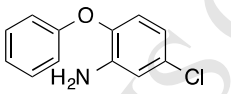
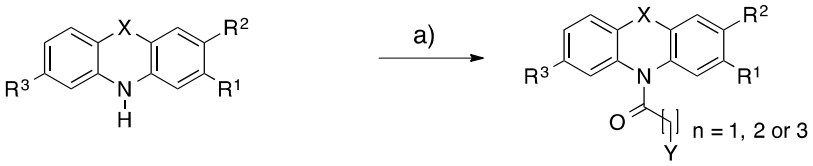


11

Scheme 1.

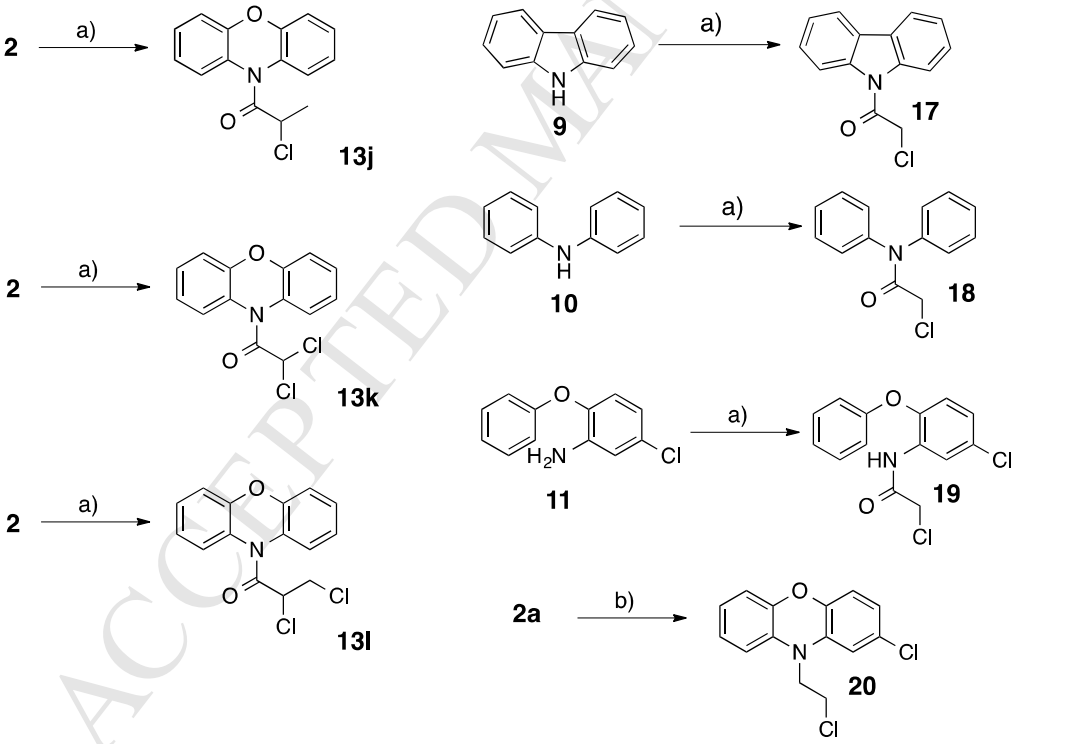


1, X = S, $R^1 = R^2 = R^3 = H$
1a, X = S, R^1 = Cl, $R^2 = R^3 = H$
2, X = O, $R^1 = R^2 = R^3 = H$
2a, X = O, R^1 = Cl, $R^2 = R^3 = H$
2b, X = O, $R^1 = CF_3$, $R^2 = R^3 = H$
2c, X = O, R^1 = Cl, R^2 = H, R^3 = Cl
6, X = CH_2–O, R^1 = Cl, $R^2 = R^3 = H$
7, X = CH_2–CH_2, $R^1 = R^2 = R^3 = H$
8, X = CH=CH, $R^1 = R^2 = R^3 = H$

12a, X = S, Y = Cl, n = 1, $R^1 = R^2 = R^3 = H$
12b, X = S, Y = Cl, n = 1, R^1 = Cl, $R^2 = R^3 = H$

13a, X = O, Y = H, n = 1, $R^1 = R^2 = R^3 = H$
13b, X = O, Y = Cl, n = 1, R^1 = Cl, $R^2 = R^3 = H$
13c, X = O, Y = Cl, n = 2, R^1 = Cl, $R^2 = R^3 = H$
13d, X = O, Y = Cl, n = 3, R^1 = Cl, $R^2 = R^3 = H$
13e, X = O, Y = Cl, n = 2, R^1 = Cl, $R^2 = R^3 = H$
13f, X = O, Y = Cl, n = 1, R^1 = Cl, R^2 = H, R^3 = Cl
13g, X = O, Y = Br, n = 1, R^1 = Cl, $R^2 = R^3 = H$
13h, X = O, Y = CN, n = 1, $R^1 = R^2 = R^3 = H$
13i, X = O, Y = Cl, n = 1, $R^1 = CF_3$, $R^2 = R^3 = H$

14, X = CH_2–O, Y = Cl, n = 1, $R^1 = R^2 = R^3 = H$
15, X = CH_2–CH_2, Y = Cl, n = 1, $R^1 = R^2 = R^3 = H$
16, X = CH=CH, Y = Cl, n = 1, $R^1 = R^2 = R^3 = H$



Equation 1

$$TS_2 + NADPH + H^+ \longrightarrow T(SH)_2 + NADP^+$$

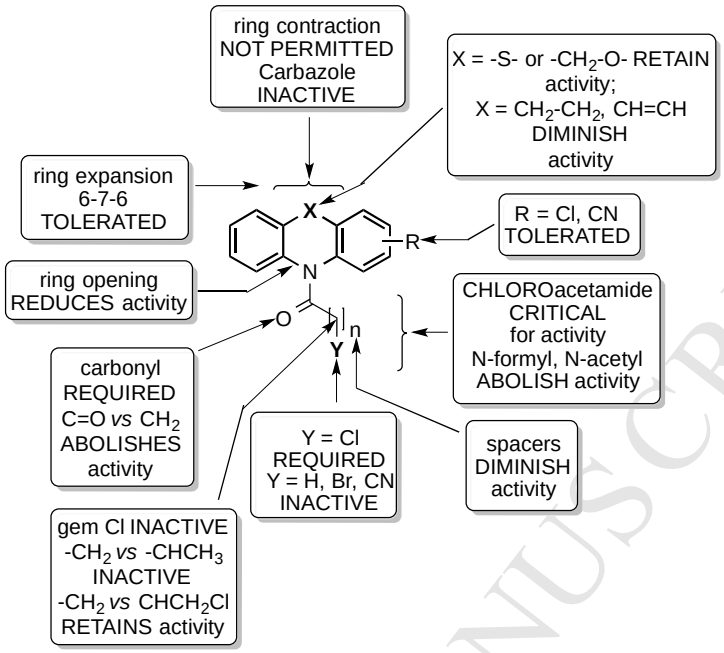


Fig. 1 SAR summary

Fig. 2

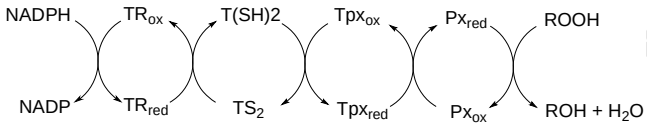
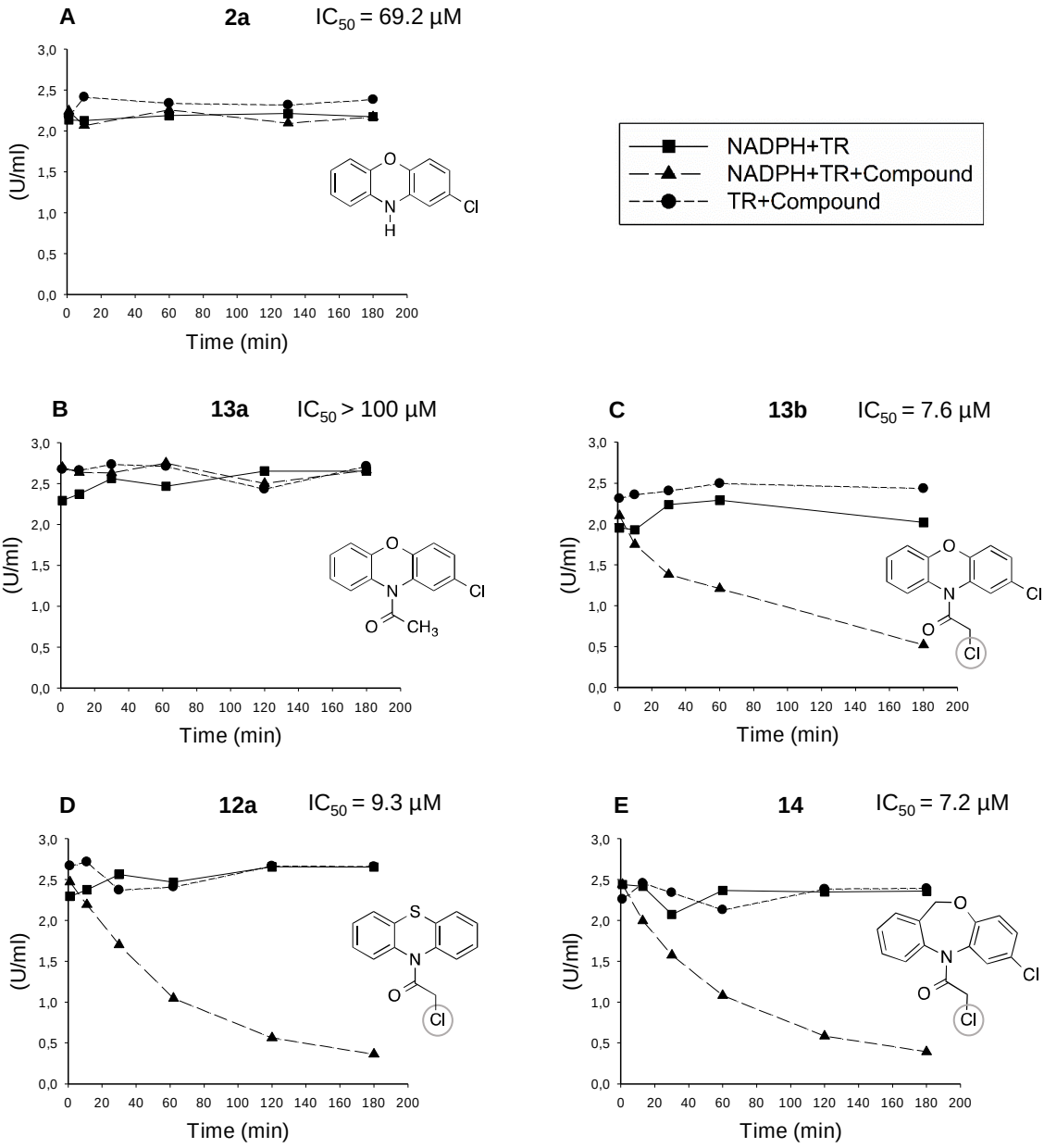


Fig. 3



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Highlights

- We describe phenothiazine-, phenoxazine- and related tricyclics-derived chloroacetamides as potent inhibitors of the promastigote form of *L. major*.
- Several analogs display time-dependent inhibition of Trypanosoma brucei trypanothione reductase.
- A covalent interaction is suggested to account for antiprotozoal activities.