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Betraying the Parasite's Redox System: Diaryl Sulfide-Based Inhibitors of Trypanothione Reductase: Subversive Substrates and Antitrypanosomal Properties

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Trypanosoma and Leishmania are the causative agents of African sleeping sickness (*Trypanosoma brucei*), Chagas disease (*Trypanosoma cruzi*), and the different forms of leishmaniasis (for example, *Leishmania donovani*). All these tropical diseases cause many thousands of deaths annually, and African sleeping sickness and leishmaniasis are categorized as an emerging or uncontrolled (category 1) disease by the world health organization (WHO).[1] The drugs currently in use show severe side effects, are often difficult to administrate, and are inefficient in the late stages of infection.[2–4] In addition, the parasites show increasing drug resistance.[5,6] This generates the urgent need for new antiparasitic agents.

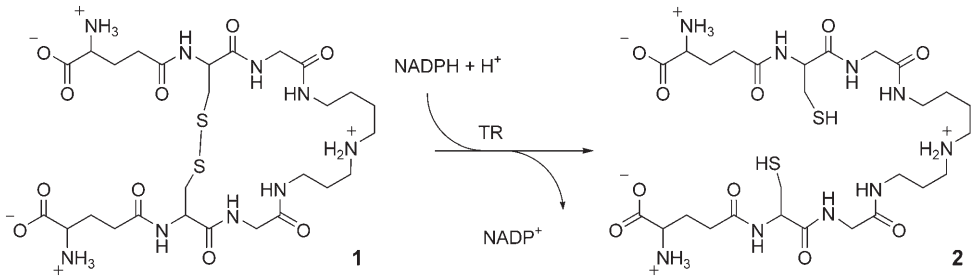
A promising approach in the fight against these diseases is to interfere with the redox metabolism of the parasites. In trypanosomatids, the nearly ubiquitous glutathione system is replaced by a trypanothione system.[7] The key enzyme of the unique thiol metabolism is trypanothione reductase (TR, EC 1.8.1.12) which catalyzes the reduction of trypanothione disulfide (TS_2, **1**) to trypanothione ($T(SH)_2$, **2**) (Scheme 1). The NADPH dependent flavoprotein is the complement of human glutathione reductase (hGR, EC 1.8.1.7) and is essential for the parasites, rendering the enzyme an attractive drug target.[8–10]

An artful option to increase the efficiency of compounds interfering with redox enzymes such as TR is to incorporate structural motives into inhibitors, enabling them to convert the antioxidative disulfide reductase into a pro-oxidative enzyme. Redox-active compounds capable of such transformations have been termed turncoat inhibitors[11] or subversive substrates.[12] Distinguished by a functional group with a low one-electron reduction potential, such a subversive substrate X can be reduced by the flavoprotein to a radical anion $X^{-\bullet}$, which can be reoxidized by molecular oxygen liberating superoxide anion radicals [Equations (1) and (2)]. This enzyme-catalyzed redox cycling process leads to the prodigality of NADPH and molecular oxygen, and the simultaneous release of toxic reactive oxygen species inside the parasites. Such compounds therefore act as catalysts for oxidative stress. The known sensitivity of parasitic protozoa towards reagents promoting free radical damage in cells[13] renders this strategy a promising route to new, potent agents against the pathogens.

$$NADPH + 2X \rightarrow NADP^+ + 2X^{-\bullet} + H^+ \quad (1)$$

$$2X^{-\bullet} + 2O_2 \rightarrow 2O_2^{-\bullet} + 2X \quad (2)$$

Nifurtimox (**3**, Figure 1a), a drug used to treat Chagas disease, is proposed to operate by generation of reactive oxygen species in the *T. cruzi* parasite.[14] It was shown that nifurtimox acts as a weak inhibitor and subversive substrate for TR and other flavoenzymes.[12,15] A thorough study of Blanchard et al.[16] proved that the nitrofuran-containing derivative chinifur (**4**) acts as an inhibitor as well as a subversive substrate of TR. Cenas et al. demonstrated that this compound is also an inhibitor of hGR.[17]



Scheme 1. Reduction of trypanothione disulfide (TS_2, **1**) to trypanothione ($T(SH)_2$, **2**) by trypanothione reductase (TR).

Decoration of a known scaffold described as a good TR ligand with a substituent that is able to subvert the physiological antioxidative function of the enzyme should lead to compounds featuring strong trypanocidal properties. In our attempt to create derivatives acting as inhibitors as well as subversive substrates of this redox enzyme, we selected a 2-aminodiphenyl sulfide core as lead structure. Opening the central ring of tricyclic phenothiazene-based antidepressants, able to inhibit TR,[18] led to piperazine-bearing 2-aminodiphenyl sulfides, which have been described as inhibitors of this enzyme as early as 1995.[19] Subsequent studies revealed that introduction of a permanent positive charge into the inhibitor scaffold increases the inhibition potency. This was accomplished by quaternization of the amine-containing headpiece of a flexible linker that is connected to the aniline nitrogen of the diphenyl

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Supporting information for this article is available on the WWW under http://www.chemmedchem.org or from the author.

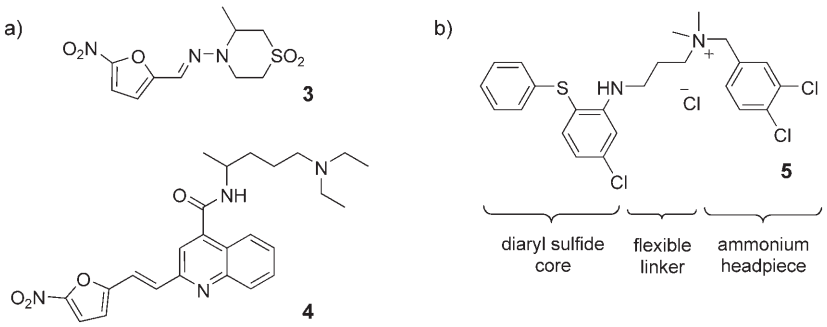


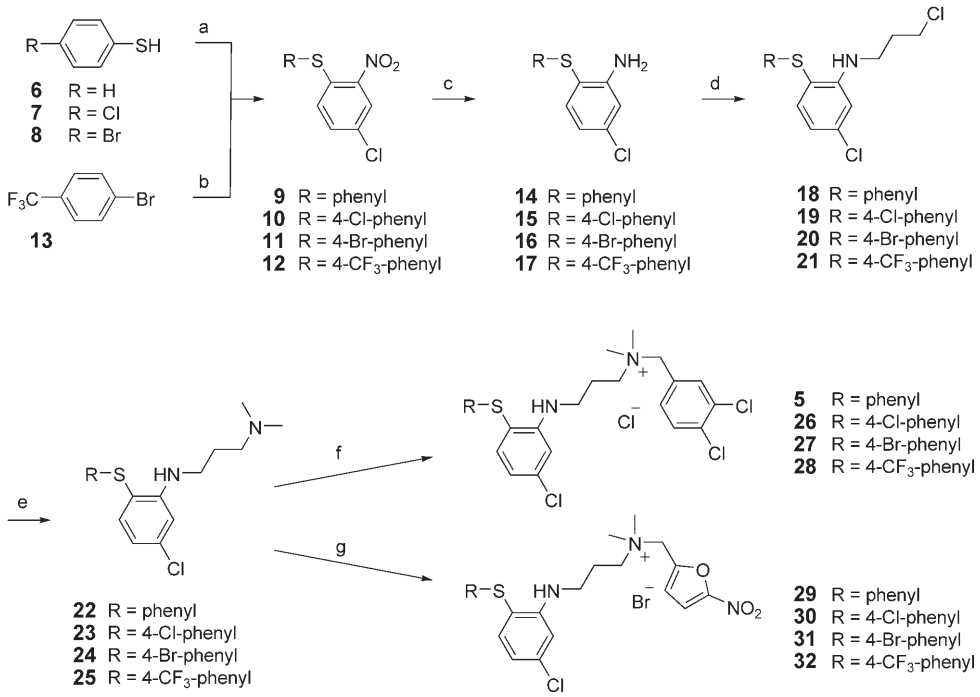
Figure 1. Compounds interacting with trypanothione reductase. a) Nifurtimox (**3**) and chinifur (**4**), both compounds with a 5-nitrofuran moiety, have been shown to act as subversive substrates of the flavoenzyme.[12, 15, 16] b) Assembly of the cationic diphenyl sulfide inhibitor **5** which has been described as competitive TR inhibitor.[18]

sulfide core, as in the benzylammonium derivative **5** (Figure 1 b).[20]

We prepared analogues with a 3,4-dichlorobenzylammonium headpiece and differently *para*-substituted diphenyl sulfide cores. By exchange of the 3,4-dichlorobenzyl against a 5-nitrofurfuryl unit, the desired subversive substrates for the enzyme should be created.

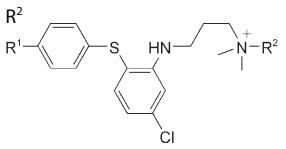
The synthesis of the target molecules is shown in Scheme 2. Reaction of thiophenol (**6**) and the *para*-bromo or *para*-chloro substituted analogues **7** and **8** with 2,5-dichloronitrobenzene yielded the diphenyl sulfide derivatives **9**, **10**, and **11**. For the synthesis of the corresponding trifluoromethyl substituted scaffold **12**, 4-(trifluoromethyl)bromobenzene **13** was treated with *t*BuLi to generate the lithiated species by a bromo-lithium exchange. Sulfur was added to this intermediate leading to the corresponding thiolate, which was treated in situ with 2,5-dichloronitrobenzene to yield diphenyl sulfide **12**. Reduction of the nitro group gave the anilines **14**–**17** that were converted in a two-step procedure with 3-chloropropionyl chloride and subsequent reduction of the resulting amide to the secondary amines **18**–**21**. Microwave-assisted introduction of the dimethylamino substituent, followed by quaternization of the resulting tertiary amines **22**–**25** with 3,4-dichlorobenzyl chloride yielded the benzylammonium inhibitors **5**, **26**, **27**, and **28**. Reaction of the tertiary amines **22**–**25** with (2-bromomethyl)-5-nitrofuran gave the 5-nitrofurfurylammonium inhibitors **29**–**32**.

The biological assays demonstrated fully competitive TR inhibition with respect to the substrate TS_2 for the benzylammonium derivatives **5**, **26**, **27**, and **28** with K_{ic} (competitive inhibition constant) values in the lower micromolar range (Table 1). We were unable to reproduce the published K_{ic} value of 1.69 μM for the phenyl sulfide derivative **5**[20] which was measured in an assay using the artificial substrate (ZCG·dmapa)$_2$ (*N*,*N*′-bis(benzyloxycarbonyl)-L-cysteinylglycyl-3-dimethylamino)propylamide)[21] and a different buffer system containing



Scheme 2. Synthesis of cationic diaryl sulfide-based TR inhibitors. a) Na, 2,5-dichloronitrobenzene, EtOH, 0–78 °C, 15–28 h, 90–95 %; b) 1. *t*BuLi, −78 °C, 10 min, 2. S, −78 °C→25 °C, 30 min, 3. 2,5-dichloronitrobenzene, 5 h, 77 %; c) Zn, NH_4Cl, MeOH, 65 °C, 2–5 h, 73–98 %; d) 1. 3-chloropropionyl chloride, pyridine, THF, 25 °C, 1–4 h; 2. BH_3·THF, THF, 67 °C, 2–4 h, 83–95 % (over 2 steps); e) $NHMe_2$ (purity: 40 %, in H_2O), DMF, 90 °C, 12–15 h, 81–91 %; f) 3,4-dichlorobenzyl chloride, acetone, microwaves, 120 °C, 20 min, 54–74 %; g) 2-(bromomethyl)-5-nitrofuran, Et_2O, acetone, 25 °C, 12–16 h, 63–84 %. DMF = *N*,*N*-dimethylformamide; THF = tetrahydrofuran.

Table 1. Inhibition of TR by benzylammonium- and nitrofurfurylammonium derivatives.



	R^1		K_{ic}[a] [μM]	K_{iu}[a] [μM]	Mode of Inhibition
5	H	3,4-dichlorobenzyl	9±1	–	competitive
26	Cl	3,4-dichlorobenzyl	10±3	–	competitive
27	Br	3,4-dichlorobenzyl	8±4	–	competitive
28	CF_3	3,4-dichlorobenzyl	6±3	–	competitive
29	H	5-nitrofurfuryl	9±0	9±0	mixed comp.–uncomp.
30	Cl	5-nitrofurfuryl	7±1	7±1	mixed comp.–uncomp.
31	Br	5-nitrofurfuryl	7±1	7±1	mixed comp.–uncomp.
32	CF_3	5-nitrofurfuryl	20±1	20±1	mixed comp.–uncomp.

[a] The kinetics (comp: competitive, uncomp: uncompetitive) was measured as described in the Supporting Information. The inhibitory constants were derived from Lineweaver–Burk plots.

0.15 M KCl. In our assay with TS_2 in HEPES-buffer, compound **5** yielded a K_{ic} value of 9 μM.

Whereas the potency for enzyme inhibition of the furfuryl ammonium inhibitors **29**–**32** lies in the low micromolar range, the kinetics changed dramatically upon replacement of the 3,4-dichlorobenzyl moiety by the 5-nitrofurfuryl substituent (Table 1). In contrast to the benzylammonium derivatives **5**, **26**, **27**, and **28**, binding of the corresponding furfuryl derivatives **29**–**32** followed a mixed competitive–uncompetitive mechanism, with the special case where $K_{ic} = K_{uc}$ (uncompetitive inhibition constant), meaning that the binding affinity of the inhibitor to the enzyme is independent of the substrate concentration (Figure 2). This inhibition mechanism is also referred as noncompetitive inhibition in literature.

The structure–activity relationship (SAR) with respect to the substitution pattern of the thiophenyl ring clearly differs among the two cationic inhibitor classes. Whereas exchange of the 4-chloro substituent of the thiophenyl moiety (as in **26**, $K_{ic} = 10$ μM) by a 4-trifluoromethyl group (as in **28**, $K_{ic} = 6$ μM) lowered the inhibitor constant for the competitive benzylammonium inhibitor, the same replacement of chlorine (as in **30**, $K_{ic} = K_{iu} = 7$ μM) by trifluoromethyl (as in **32**, $K_{ic} = K_{iu} = 20$ μM) weakened the binding of the mixed competitive–uncompetitive nitrofurfurylammonium inhibitor. We take this as an indication for the occupation of a second binding site by the nitrofurfurylammonium derivatives, with distinct geometry relative to the TS_2 binding site presumably occupied by the benzylammonium ligands. A cavity at the twofold symmetry axis of the homodimeric enzyme has been identified as a binding site for mixed competitive–uncompetitive tricyclic xanthene inhibitors by X-ray crystallography of the closely related enzyme human glutathione reductase (hGR).[22] The analogous cavity of TR has been postulated to act as a binding site for mixed competitive–uncompetitive naphthoquinone inhibitors.[23] Computer modeling using MOLOC[24] suggests that the furfuryl derivatives **29**–**32** could similarly fit into this cavity of TR (Supporting Information Figure 1SI).

To determine the ability of the 5-nitrofurfuryl inhibitors **29**–**32** to induce the intrinsic oxidase activity of TR, NADPH consumption of the enzyme was monitored in the presence of these compounds, but in the absence of the physiological substrate TS_2.[12] Oxidation of NADPH was increased more than tenfold in the presence of the nitrofurfurylammonium derivatives **29**–**32** (Table 2, Supporting Information Figure 2SI). In contrast, the benzylammonium inhibitor **5** did not affect the intrinsic oxidase activity of TR.

To detect the generation of radical species, the oxidase assay for compound **31** was coupled to the reduction of oxidized cytochrome c (Cytc-Fe^{3+}) that can undergo one-electron reduction to the Fe^{2+} redox state, for instance mediated by

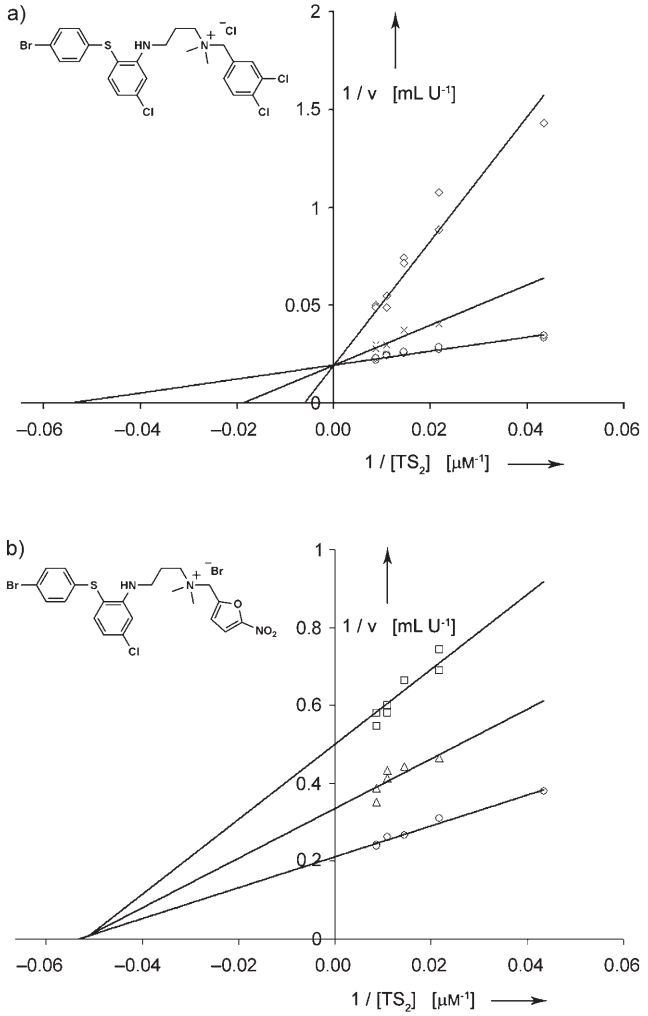


Figure 2. Lineweaver–Burk plots for the inhibition of *T. cruzi* TR by compound **27** and **31**. The concentration of the substrate (TS_2) was varied in the presence of fixed concentrations of inhibitors. a) **27** (○ 0, × 20, ◇ 40 μM); and b) **31** (○ 0, △ 4, □ 10 μM). The kinetics was measured as described in the Supporting Information.

Table 2. Reduction of nitrofuran derivatives by TR and correlation of the induced NADPH oxidase activity by the nitrofurfurylammonium derivatives **29**–**32** with the K_{ic}-values of the corresponding benzylammonium-inhibitors **5**, **26**, **27**, and **28**.

	$K_{ic}=K_{iu}$ [μM]	NADPH ox. [U mg^{-1}][a]	x-fold[b]	K_{ic}(Benz.) [μM]	K_{ic}(Benz.)/NADPH ox. [μM mL U^{-1}]
29	9±0	0.30	11	9±1 (**5**)	17.0
30	7±1	0.40	14	10±3 (**26**)	13.7
31	7±1	0.33	12	8±4 (**27**)	13.3
32	20±1	0.28	10	6±3 (**28**)	12.0

[a] The assays contained in a total volume of 1 mL buffer 100 μM NADPH, 200 μM inhibitor, and 6.5 U TR. NADPH oxidation was followed at 25 °C. [b] Increase in the NADPH oxidase activity of TR as compared to the activity in the absence of any substrate (0.028 U mg^{-1}).

$O_2^{-\bullet}$ [Equation (3)]. By monitoring the absorption increase at 550 nm, the single-electron reduction of the heme protein was observed (Supporting Information Table 1SI).[12] To prove the involvement of superoxide radicals in the reaction, superoxide dismutase (SOD) was added to the assay as described earlier.[15] In the presence of SOD (Equation (4), Supporting Information Figure 2SI b), the rate of Cytc-Fe^{3+} reduction was decreased (as expected if superoxide anions are formed) because the dismutation of superoxide radicals by SOD proceeds much faster than the corresponding reduction to molecular oxygen by Cytc-Fe^{3+} (turnover number for reactions with $2O_2^{-\bullet}$: SOD 1.9×10^9 M^{-1} s^{-1}; Cytc-Fe^{3+}: 1.1×10^5 M^{-1} s^{-1}[25]). No complete quenching of the Cytc-Fe^{3+} reduction was observed. Most probably, a part of Cytc-Fe^{3+} is directly reduced by the furfuryl radicals $X^{-\bullet}$ [Equation (5)]. A similar partial prevention of Cytc-Fe^{3+} reduction by SOD has already been observed for menadione-based subversive substrates of TR[23] and 5-nitrofuran derivatives,[15] demonstrating that direct reduction of Cytc-Fe^{3+} by the respective radicals plays an important role.

$$2O_2^{-\bullet}+2\text{Cytc-}Fe^{3+} \rightarrow 2O_2+2\text{Cytc-}Fe^{2+} \quad (3)$$

$$2O_2^{-\bullet}+\text{SOD} \rightarrow O_2+H_2O_2+\text{SOD} \quad (4)$$

$$X^{-\bullet}+\text{Cytc-}Fe^{3+} \rightarrow X+\text{Cytc-}Fe^{2+} \quad (5)$$

Conspicuously, the rate of NADPH oxidation did not correlate with the observed K_{ic} ($=K_{iu}$) values for the mixed competitive–uncompetitive nitrofurfuryl inhibitors but rather with the K_{ic} values of the corresponding benzylammonium analogues (Table 2, 5th column). Measuring the NADPH consumption of TR in the presence of variable concentrations of the competitive benzylammonium inhibitor **27** and different concentrations of the nitrofurfurylammonium derivative **31** (acting as subversive substrate) showed by approximation a competitive inhibition of the oxidase activity (not shown). As the presence of compound **27** clearly affected the rate of nitrofuran reduction, it is likely that nitrofuran reduction takes place at the active site.

The behavior observed for the nitrofurfurylammonium inhibitors indicates two independent events: on the one hand, they cause mixed competitive–uncompetitive inhibition of TS_2 reduction by binding outside of the trypanothione binding site, likely in the enzyme cavity at the twofold axis. On the other hand, the nitrofuran moiety of the inhibitors is reduced in the active site. Such cycling between two binding sites of the enzyme has already been proposed for the subversive substrate chinifur (**4**).[16]

The in vitro studies with the trypanosomatid flagellates *T. cruzi*, *T. b. rhodesiense*, and *L. donovani* in culture validated the concept of subversive inhibition to gain antitrypanosomal activity. Though none of the compounds showed significant growth inhibition of the intracellularly multiplying *T. cruzi* and *L. donovani*, the nitrofurfurylammonium derivatives **29**–**32** exhibited rather strong activities against *T. b. rhodesiense* with IC_{50} (median inhibitory concentration) values between 0.6 and 1.0 μM (Table 3). The corresponding competitive TR inhibitors **5** and **28**, which are not acting as subversive substrates, showed none or only weak growth inhibition for the parasites. The cytotoxicity on mammalian L6 cells remarkably proved to be lower for the nitrofuran-containing inhibitors compared to the benzylammonium analogues, although nitrofurans have been shown to interact, for instance, with the mammalian selenoprotein thioredoxin reductase (EC 1.6.4.5).[26] This leads to an excellent selectivity index for the subversive substrates **28**–**30**, as the toxicity IC_{50} value on myoblast cells is 100-fold higher compared to the IC_{50} against *T. b. rhodesiense*. The exception was the trifluoromethyl inhibitor **32**.

In summary, we have reported the synthesis and evaluation of diphenyl sulfide-based TR inhibitors. By replacing the 3,4-dichlorophenyl entity by a nitrofuran unit, we discovered a new class of inhibitors with a distinctively changed inhibition mode. For these substances, we demonstrated that they act as subversive substrates for TR. In vitro studies with *T. b. rhodesiense*

Table 3. In vitro antitrypanosomal activity against *T. b. rhodesiense* STIB900 and cytotoxicity of diaryl sulfide-based TR inhibitors.

	K_i[μM]	*T. b. rhodesiense* Subversive Substrate	Growth Inhibition[a]	IC_{50}[μM]	Cytotoxicity[b] IC_{50} [μM]	Selectivity Index[c]
5	9±1	no	0%	–	25.2	–
28	6±3	no	38%	–	8.5	–
29	9±0	yes	99%	0.59	>170	>290
30	7±1	yes	99%	0.77	72.9	95
31	7±1	yes	99%	0.68	86.5	127
32	20±1	yes	99%	1.02	27.6	27

[a] At 0.8 μg mL^{-1} inhibitor concentration. [b] L6 myoblast cells. [c] Selectivity index: [IC_{50}(L6 myoblast cells)/IC_{50}(*T. b. rhodesiense*)].

revealed the importance of the redox-active nitrofuran moiety and validated the concept of subversive inhibition as a promising strategy to interfere with the parasite's redox metabolism and achieve, in future work, potent in vivo growth inhibition.

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Keywords: antiparasitic agents · drug design · inhibitors · subversive substrates · trypanothione reductase

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