

1 **Evaluating 5-nitrothiazoles as trypanocidal agents**

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24 **Running title: *Anti-T. brucei* activity of a nitrothiazole series**

27 **Abstract**

28 The growth inhibitory properties of a 5-nitrothiazole series was evaluated against
29 *Trypanosoma brucei*. A subset of related compounds displayed the greatest potency towards
30 the parasite while exhibiting little cytotoxic effect on mammalian cells, with this anti-
31 parasitic activity being dependent on expression of a type I nitroreductase by the
32 trypanosome. We conclude that the 5-nitrothiazole class of nitroheterocycle may represent
33 new leads in the treatment of human African trypanosomiasis.

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35 Spread *via* the blood feeding habits of tsetse flies, parasites belonging to the *Trypanosoma*
36 *brucei* complex are responsible for human African trypanosomiasis (HAT) (1). Drugs
37 represent the only option to combat this infection but their use is often problematic (2). One
38 treatment that targets the cerebral stage of this disease is a nifurtimox-eflornithine
39 combination therapy (3, 4). In this medication eflornithine acts as an inhibitor of ornithine
40 decarboxylase, blocking polyamine biosynthesis (5, 6) while nifurtimox is converted to a
41 toxic metabolite following activation by a type I nitroreductase (NTR) (7, 8). As type I NTRs
42 are expressed by some unicellular eukaryotes but not by metazoan organisms, the
43 bioreductive activity of this enzyme has been exploited to develop a series of novel anti-
44 parasitic agents that often exhibit little or no toxicity towards cultured mammalian cells (9 &
45 10).

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47 The 5-nitrothiazoles represent a class heterocyclic compounds of which niridazole and
48 nitazoxanide display potent antimicrobial and antihelminthic activities (11, 12). The mode(s)
49 of action of these agents is unclear with both structures shown to inhibit of key enzymes
50 involved in energy metabolism (13, 14) and able to function as prodrugs, undergoing
51 reduction to form adduct forming metabolites (15-17). To date only niridazole and its
52 derivatives have been screened for trypanocidal activity against *T. brucei* with this in
53 combination with suramin able to cure mice of trypanosomiasis (18). However, concerns over
54 its carcinogenic properties resulted in trials using niridazole being suspended (19). Here, we
55 assessed a 2-amide 5-nitrothiazole series for growth inhibitory activity against bloodstream
56 form (BSF) *T. brucei* (Table 1). Out of the fifteen compounds tested, seven had no effect on
57 trypanosomal growth at a concentration of 30 μ M. For the remaining chemicals, detailed
58 inhibition assays were conducted generating dose response curves from which IC₅₀s were
59 determined (Table 1). For NT2, NT4, NT6, NT7 and NT11 an appreciable trypanocidal

60 activity (IC_{50} 's $>10.0 \mu\text{M}$) equivalent to the potency exhibited by nifurtimox was noted with
61 the other agents being less effective (IC_{50} s $\sim 17 \mu\text{M}$). Screening against two mammalian lines
62 (Table 1) revealed that NT2, NT10, NT12 and NT15 displayed toxicity towards THP-1 or
63 SK-N-SH cells with NT10 and NT12 having growth inhibitory effects against both lines. For
64 the remaining agents no growth inhibitory effect at concentrations up to $100 \mu\text{M}$ was
65 observed.

66

67 Before mediating its trypanocidal effects nifurtimox must undergo activation in a reaction
68 catalysed by a type I NTR (7). Using purified HIS-tagged TbNTR (Fig. 1A) we evaluated
69 whether the 2-amide 5-nitrothiazoles series could serve as substrates for this enzyme (Fig.
70 1B). Five compounds were shown to be “good” NTR substrates, generating a specific activity
71 ~ 3 -fold greater than that noted for nifurtimox (Fig. 1B). Of these structures, NT2, NT4, NT6
72 and NT7 are related in that they contained a saturated unbranched hydrocarbon chain.
73 However, the number of carbon atoms in this sequence and the associated increase in
74 lipophilicity did not affecting the specific activity displayed by TbNTR towards a given
75 substrate. Of the remaining compounds, three yielded activities similar to that observed for
76 nifurtimox while the others were not metabolised by TbNTR at an appreciable rate under the
77 conditions used here (Fig. 1B).

78

79 To investigate whether NTR plays a role in prodrug activation within the parasite itself the
80 susceptibility of BSF *T. brucei* engineered to over express this enzyme was evaluated (Table
81 1; Fig. 2) (8). Cells having elevated levels of TbNTR were up to 10-fold more sensitive to
82 NT2, NT4, NT6 or NT7 than controls. This effect was NTR specific as recombinant and wild
83 type parasite lines displayed similar sensitivities to the non-nitroaromatic compound G418
84 ($IC_{50} \sim 0.6 \mu\text{M}$). When these studies were extended to test other trypanocidal nitrothiazoles, a

85 lower (~2) fold or no difference in IC₅₀ was observed (Table 1; Fig. 2). This implies that for
86 these less effective trypanocidal compounds, NTR plays little or no role in the metabolism of
87 these structures within the parasite itself.

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89 By comparing the specific activity values and growth inhibitory effects of each compound, a
90 number of structure activity relationships (SARs) were identified. In contrast to their non-
91 substituent counterparts' addition of a methyl or *tert*-butyl group at the 4-position on the
92 thiazole ring generated compounds that were not TbNTR substrates and did not exhibit
93 trypanocidal activities: compare NT2 with NT3 and NT4 with NT5. This lack of activity
94 could be due to steric hindrance with the 4-alkyl side chain blocking the trypanosomal
95 enzyme from gain access to the adjacent 5-nitro grouping or could reflect an inductive effect
96 with the alkyl substituent on the thiazole backbone rendering nitroreduction energetically
97 unfavourable. Extending the SAR studies to investigate grouping attached to the thiazole ring
98 *via* a 2-amide linker revealed that compounds containing an unbranched, saturated
99 hydrocarbon chain (NT2, NT4, NT6, NT7) were efficiently metabolised by TbNTR with this
100 translating to a trypanocidal effect equivalent to that of the reference nitrofurans.
101 Encouragingly, these structures displayed also little/no *in vitro* toxicity to mammalian cells
102 suggesting that they warrant *in vivo* analysis. Modification of this saturated linear
103 hydrocarbon chain (incorporation of an unsaturated bond (NT8) or an ether linkage (NT13),
104 inclusion of halogen substituents (NT10-12) or its replacement with a hydrogen atom (NT1)
105 or a benzyl-containing grouping (NT9, NT15)) generated structures that displayed lower
106 TbNTR activity and/or had reduced potency towards BSF trypanosomes. Presumably, such
107 alterations to the saturated alkyl chain alter the affinity these variants have for the parasite
108 oxidoreductase. As the broad spectrum ant-infective agent nitazoxanide is structurally related
109 to NT9 and NT15 (all contain a phenyl group attached to the amide linker) we predict that

110 this particular antimicrobial agent is unlikely to function as an effective TbNTR substrate
111 and/or display activity against BSF *T. brucei*. Intriguingly, despite being screened against a
112 wide range of microbial infectious agents including *Trypanosoma cruzi* and *Leishmania* the
113 potency of this particular nitrothiazole against *T. brucei* has not been reported.

114

115 There has been renewed interest in the use of nitroheterocyclic prodrugs for the treatment of
116 trypanosomatid infections with nifurtimox in combination with eflornithine now being used
117 to treat the form of HAT prevalent in West and Central Africa while the nitroimidazole
118 fexinidazole is under clinical evaluation against HAT, Chagas disease and visceral
119 leishmaniasis. In both cases these nitroheterocycles are converted to toxic metabolites by a
120 type I NTR activity (7, 20). Here, we have identified several trypanocidal nitrothiazoles,
121 including some that are activated by the type I NTR, as being potent against BSF *T. brucei* as
122 nifurtimox. Promisingly the most effective structures exhibited little or no toxicity to cultured
123 mammalian cells with trypanosomal expression of the type I NTR underlying their
124 selectivity. As such, these compounds warrant further attention in terms of developing novel
125 therapies targeting HAT and could potentially represent one component of a new
126 combinatorial treatment against this disease.

127

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130

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134 comments on the manuscript.

135

136 **Abbreviations.**

137 BSF, bloodstream form; HAT, human African trypanosomiasis; NECT, nifurtimox-

138 eflornithine combination therapy; NTR, nitroreductase; SAR, structure activity relationship;

139 TbNTR, *Trypanosoma brucei* type I nitroreductase

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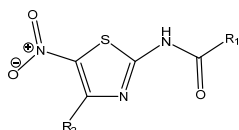
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200 **Table 1. Structure and growth inhibitory properties of nitrothiazole compounds.** All compounds tested satisfy the Lipinski's Rule of 5 (see
 201 PubChem database (<http://pubchem.ncbi.nlm.nih.gov/>)). Susceptibility of parasites and mammalian cells to nitrothiazole compounds was
 202 assessed as previously described (7). Average IC₅₀ values ± standard deviations were calculated from dose response curves performed in
 203 triplicate. TbNTR^{ox} represents the *T. brucei* cell line overexpressing the type I nitroreductase. The figures in parenthesis correspond to the fold
 204 difference in IC₅₀ values of the TbNTR^{ox}, SK-N-SH and THP-1 cell lines when compared against wild type.
 205



compound	Structure		IC ₅₀ (μM)			
	R ₁	R ₂	<i>T. brucei</i>		Mammalian line	
			wild type	TbNTR ^{ox}	SK-N-SH	THP-1
NT1	H	H	>30.00			
NT2	CH ₃	H	4.67 ± 0.34	0.58 ± 0.11 (8)	>100.00 (>21)	86.97 ± 0.99 (19)
NT3	CH ₃	CH ₃	>30.00			
NT4	CH ₂ CH ₃	H	3.67 ± 0.50	0.51 ± 0.09 (7)	>100.00 (>27)	>100.00 (>27)
NT5	CH ₂ CH ₃	C(CH ₃) ₃	>30.00			
NT6	CH ₂ CH ₂ CH ₃	H	6.47 ± 0.06	0.64 ± 0.13 (10)	>100.00 (>15)	>100.00 (>15)
NT7	CH ₂ CH ₂ CH ₂ CH ₃	H	4.32 ± 0.90	0.57 ± 0.02 (8)	>100.00 (>23)	>100.00 (>23)
NT8	CH ₂ CHCH ₂	H	>30.00			
NT9	benzyl	H	>30.00			
NT10	CH ₂ Cl	H	16.12 ± 0.97	13.53 ± 0.50 (1)	18.33 ± 0.68 (1)	7.68 ± 0.56 (<1)
NT11	C(F) ₃	H	8.87 ± 0.70	11.24 ± 0.58 (1)	>100.00 (>11)	>100.00 (>11)
NT12	CH(Br)CH ₃	H	17.41 ± 1.08	8.37 ± 1.84 (2)	19.83 ± 0.44 (1)	21.53 ± 0.19 (1)
NT13	CH ₂ OCH ₃	H	>30.00			
NT14	C(CH ₃) ₂ CH ₃	CH ₃	>30.00			
NT15	3,5 dichlorobenzyl	H	16.26 ± 1.24	8.17 ± 0.61 (2)	>100.00 (>6)	6.75 ± 1.08 (<1)
NFX			4.12 ± 0.13	0.31 ± 0.06 (13)	>100.00 (>24)	64.80 ± 1.50 (16)

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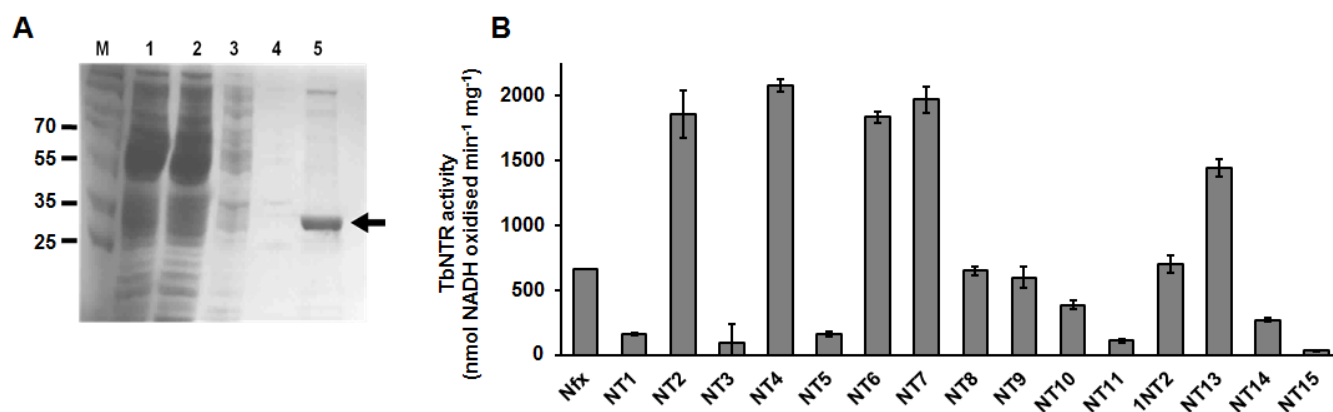


Figure 1. Activity of TbNTR toward different nitrothiazoles. (A) Samples obtained during purification of recombinant TbNTR were analysed on by SDS-PAGE (10 %) stained with Coomassie blue. *E. coli* crude extract (lane 1) was loaded onto a Ni-NTA column and the flow through (lane 2) collected. The column was washed with 50 mM imidazole (lane 3) and 100 mM imidazole (lane 4) containing buffers. Recombinant protein was eluted in a buffer containing 500 mM imidazole; 0.5 % Triton X-100 (lane 5). Markers (M) are in kiloDaltons. The ~30 kDa band corresponding to recombinant TbNTR is indicated. (B) Activity of purified recombinant TbNTR was assessed by using nitrothiazoles (NT1-15) as substrate (100 μ M) at a fixed concentration of NADH (100 μ M). Enzyme activity, expressed in nmoles of NADH oxidised per minute per mg TbNTR, was then calculated using an ϵ value of 6,220 $M^{-1} cm^{-1}$. Nfx (nifurtimox) was used as control and enzyme activity determined as previously described (7). The enzyme activity values are the means of data from 3 assays \pm standard deviations.

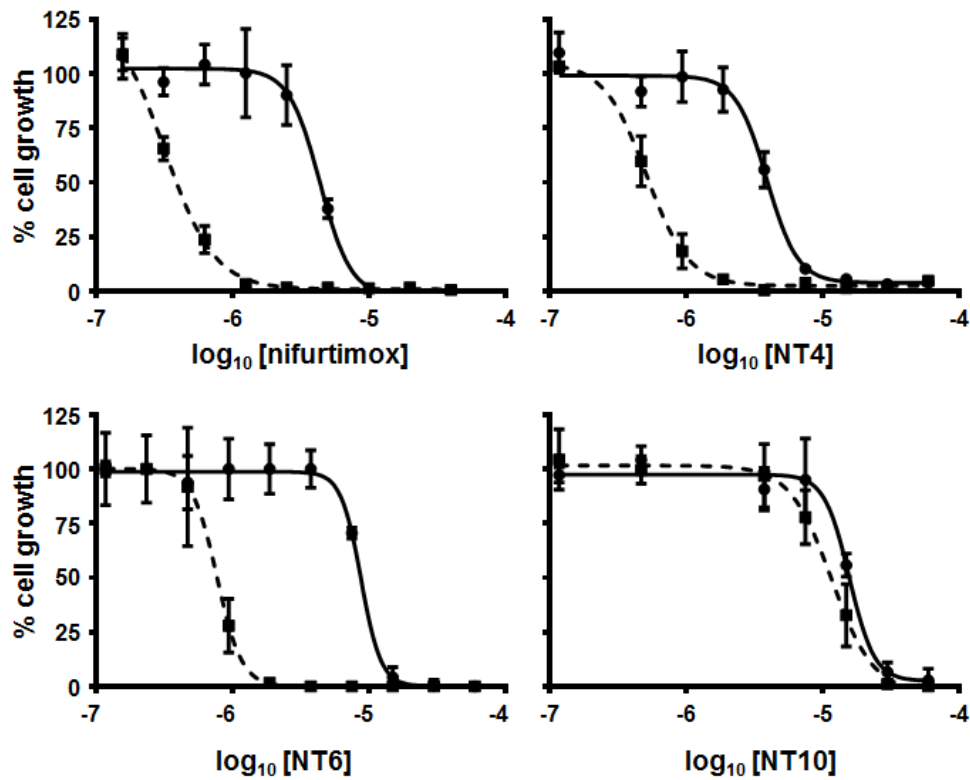


Figure 2. Susceptibility of bloodstream form *T. brucei* over expressing TbNTR to nitrothiazoles. Dose-response curves of *T. brucei* (solid line) and parasites expressing an ectopic copy of *Tbntnr* (dashed line) towards representative nitrothiazoles. The growth inhibitory effect expressed as IC_{50} values was determined (see Table 2). All data points are mean values \pm standard deviations from experiments performed in quadruplicate. Nifurtimox was used as drug control.