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Evaluating 5-nitrothiazoles as trypanocidal agents

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

Abstract

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

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

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

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

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

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

lower (~2) fold or no difference in IC_{50} was observed (Table 1; Fig. 2). This implies that for these less effective trypanocidal compounds, NTR plays little or no role in the metabolism of these structures within the parasite itself.

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

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

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

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Abbreviations.

BSF, bloodstream form; HAT, human African trypanosomiasis; NECT, nifurtimox-eflornithine combination therapy; NTR, nitroreductase; SAR, structure activity relationship; TbNTR, *Trypanosoma brucei* type I nitroreductase

References

1. **Brun R, Blum J, Chappuis F, Burri C.** 2010. Human African trypanosomiasis. Lancet **375**:148-159.
2. **Wilkinson SR, Kelly JM.** 2009. Trypanocidal drugs: mechanisms, resistance and new targets. Expert Rev Mol Med. **11**:e31.
3. **Priotto G, Kasparian S, Mutombo W, Ngouama D, Ghorashian S, Arnold U, Ghabri S, Baudin E, Buard V, Kazadi-Kyanza S, Ilunga M, Mutangala W, Pohlig G, Schmid C, Karunakara U, Torreele E, Kande V.** 2009. Nifurtimox-eflornithine combination therapy for second-stage African *Trypanosoma brucei* gambiense trypanosomiasis: a multicentre, randomised, phase III, non-inferiority trial. Lancet. **374**:56-64.
4. **Yun O, Priotto G, Tong J, Flevaud L, Chappuis F.** 2010. NECT is next: implementing the new drug combination therapy for *Trypanosoma brucei gambiense* sleeping sickness. PLoS Negl. Trop. Dis. **4**:e720.
5. **Bacchi CJ, Nathan HC, Hutner SH, McCann PP, Sjoerdsma A.** 1980. Polyamine metabolism: a potential therapeutic target in trypanosomes. Science. **210**:332-334.
6. **Willert EK, Phillips MA.** 2008. Regulated expression of an essential allosteric activator of polyamine biosynthesis in African trypanosomes. PLoS Pathog. **4**:e1000183.
7. **Hall BS, Bot C, Wilkinson SR.** 2011. Nifurtimox activation by trypanosomal type I nitroreductases generates cytotoxic nitrile metabolites. J. Biol. Chem. **286**:13088-13095.
8. **Wilkinson SR, Taylor MC, Horn D, Kelly JM, Cheeseman I.** 2008. A mechanism for cross-resistance to nifurtimox and benznidazole in trypanosomes. Proc. Natl. Acad. Sci. USA. **105**:5022-5027.

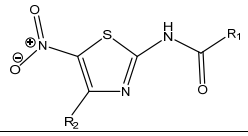
9. **Wilkinson SR, Bot C, Kelly JM, Hall BS**. 2011. Trypanocidal activity of nitroaromatic prodrugs: current treatments and future perspectives. Curr Top Med Chem. **11**:2072-2084.
10. **Patterson S, Wyllie S.** 2014. Nitro drugs for the treatment of trypanosomatid diseases: past, present, and future prospects. Trends Parasitol. **30**:289-98
11. **Archer S.** 1985. The chemotherapy of schistosomiasis. Annu. Rev. Pharmacol. Toxicol. **25**:485-508.
12. **Hemphill A, Mueller J, Esposito M.** 2006. Nitazoxanide, a broad-spectrum thiazolide anti-infective agent for the treatment of gastrointestinal infections. Expert Opin. Pharmacother. **7**:953-964.
13. **Bueding E, Fisher J.** 1970. Biochemical effects of niridazole on *Schistosoma mansoni*. Mol Pharmacol. **6**:532-9.
14. **Hoffman PS, Sisson G, Croxen MA, Welch K, Harman WD, Cremades N, Morash MG.** 2007.. Antiparasitic drug nitazoxanide inhibits the pyruvate oxidoreductases of *Helicobacter pylori*, selected anaerobic bacteria and parasites, and *Campylobacter jejuni*. Antimicrob. Agents Chemother. **51**:868-876.
15. **Blumer JL, Friedman A, Meyer LW, Fairchild E, Webster LT, Jr., Speck WT.** 1980. Relative importance of bacterial and mammalian nitroreductases for niridazole mutagenesis. Cancer Res. **40**:4599-4605.
16. **Tracy JW, Catto BA, Webster LT, Jr.** 1983. Reductive metabolism of niridazole by adult *Schistosoma mansoni*. Correlation with covalent drug binding to parasite macromolecules. Mol. Pharmacol. **24**:291-299.
17. **Muller J, Wastling J, Sanderson S, Muller N, Hemphill A.** 2007. A novel *Giardia lamblia* nitroreductase, GlNR1, interacts with nitazoxanide and other thiazolides. Antimicrob. Agents Chemother. **51**:1979-1986.

18. **Jennings FW.** 1987. Chemotherapy of late-stage trypanosomiasis: the effect of the nitrothiazole compounds. Trans. R. Soc. Trop. Med. Hyg. **81**:616.

19. **Bulay O, Urman H, Clayson DB, Shubik P.** 1977. Carcinogenic effects of niridazole on rodents infected with *Schistosoma mansoni*. J. Natl. Cancer Inst. **59**:1625-1630.

20. **Wyllie S, Patterson S, Stojanovski L, Simeons FR, Norval S, Kime R, Read KD, Fairlamb AH.** 2012. The anti-trypanosome drug fexinidazole shows potential for treating visceral leishmaniasis. Sci. Transl. Med. **4**:119re1.

Table 1. Structure and growth inhibitory properties of nitrothiazole compounds. All compounds tested satisfy the Lipinski's Rule of 5 (see PubChem database (http://pubchem.ncbi.nlm.nih.gov/)). Susceptibility of parasites and mammalian cells to nitrothiazole compounds was assessed as previously described (7). Average IC_{50} values ± standard deviations were calculated from dose response curves performed in triplicate. $TbNTR^{ox}$ represents the *T. brucei* cell line overexpressing the type I nitroreductase. The figures in parenthesis correspond to the fold difference in IC_{50} values of the $TbNTR^{ox}$, SK-N-SH and THP-1 cell lines when compared against wild type.



compound	Structure		IC_{50} (µM)			
			T. brucei		Mammalian line	
	R_1	R_2	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_2CH_3	H	3.67 ± 0.50	0.51 ± 0.09 (7)	>100.00 (>27)	>100.00 (>27)
NT5	CH_2CH_3	$C(CH_3)_3$	>30.00			
NT6	$CH_2CH_2CH_3$	H	6.47 ± 0.06	0.64 ± 0.13 (10)	>100.00 (>15)	>100.00 (>15)
NT7	$CH_2CH_2CH_2CH_3$	H	4.32 ± 0.90	0.57 ± 0.02 (8)	>100.00 (>23)	>100.00 (>23)
NT8	CH_2CHCH_2	H	>30.00			
NT9	benzyl	H	>30.00			
NT10	CH_2Cl	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_2OCH_3	H	>30.00			
NT14	$C(CH_3)_2CH_3$	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)

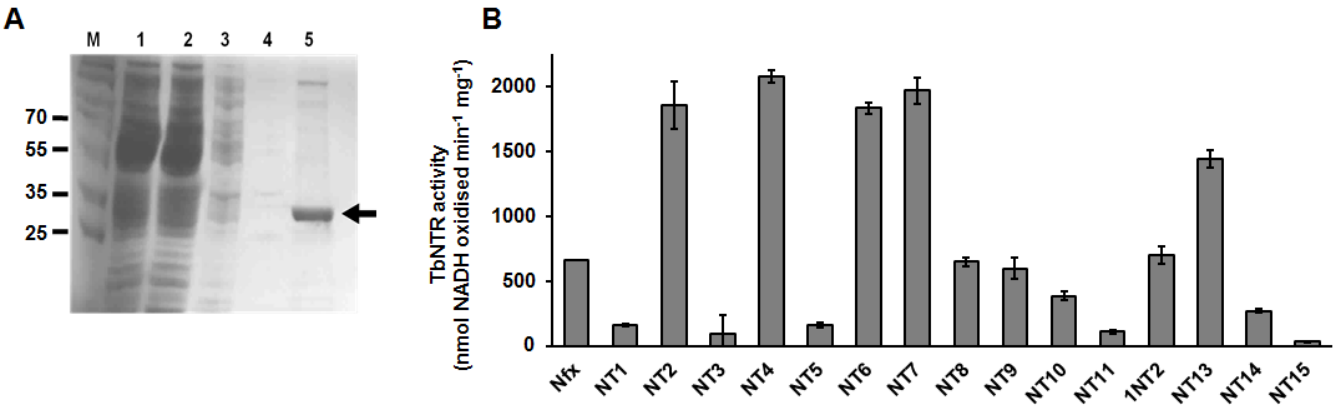


Figure 1. Activity of TbNTR toward different nitrothiazoles. (A) Samples obtained during purification of recombinant TbNTR were analysed on by SDS-PAGE (10 %) stained with Coomassie blue. *E. coli* crude extract (lane 1) was loaded onto a Ni-NTA column and the flow through (lane 2) collected. The column 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 μM) at a fixed concentration of NADH (100 μM). 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 ± standard deviations.

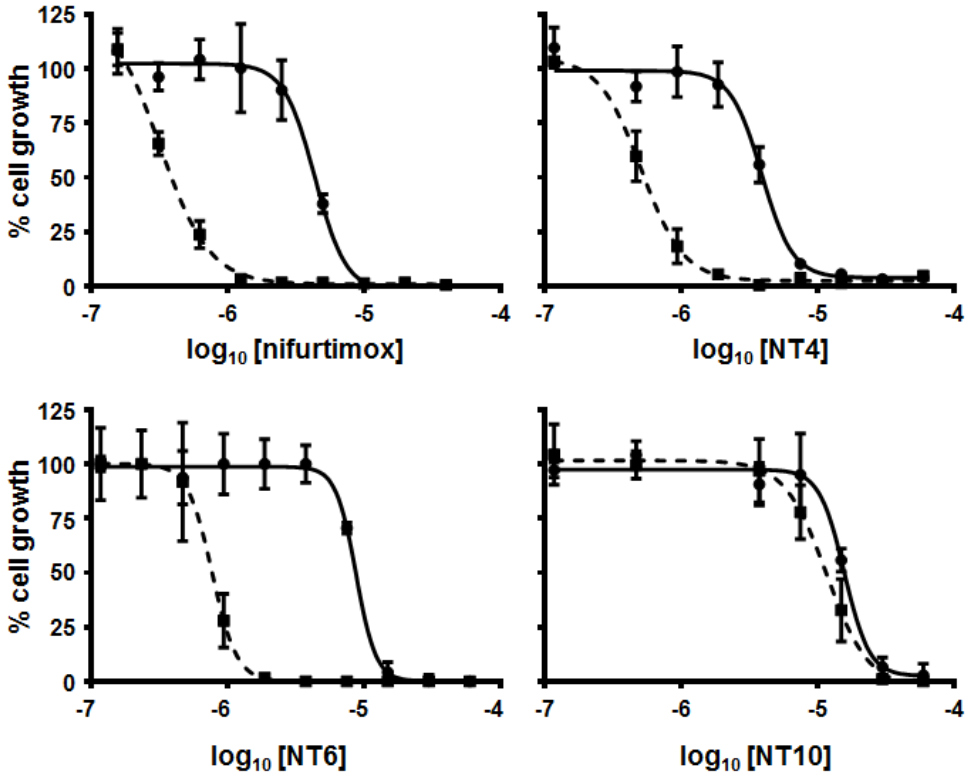


Figure 2. Susceptibility of bloodstream form *T. brucei* over expressing TbNTR to nitrothiazoles. Dose-response curves of *T. brucei* (solid line) and parasites expressing an ectopic copy of Tb*ntr* (dashed line) towards representative nitrothiazoles. The growth inhibitory effect expressed as IC_{50}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