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Synthesis and Uptake of Nitric Oxide-Releasing Drugs by the P2 Nucleoside Transporter in *Trypanosoma equiperdum*

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Abstract—A series of *S*-nitrosothiols, structurally related to the NO•-donor *S*-nitroso-*N*-acetylpenicillamine, and of organic nitrate esters that contain amidine groups which specify a recognition via the trypanosomal purine transporter P2, were synthesized and tested for their ability to inhibit the uptake of [2-^{3}H]adenosine on *Trypanosoma equiperdum*.

Introduction

The radical nitric oxide (NO•) is involved in numerous biological processes[1] including vasodilatation or neurotransmission, and also plays a critical role in protection against parasitic infections as a regulatory molecule and cytotoxic mediator of the immune system.[2] Although few physiological-target molecules of nitric oxide have been clearly identified, its role in protective mechanisms is believed to occur through inactivation of critical enzymes and nitrosation of nucleophilic residues.[3] NO• can also react rapidly with a variety of radical species, such as superoxide radical anion $O_2^{\bullet -}$ to produce the potent toxic agent peroxynitrite anion $ONOO^-$ which is able to oxidize a great variety of biomolecules, and that could act as a source of toxic hydroxyl radicals.[4] Peroxynitrite anion, like NO•, seems to play a major role in the protective mechanisms of the host against parasitic infections. For instance, it has been shown to be highly cytotoxic against *T. cruzi* epimastigotes, the causative agents of Chagas' disease, inactivating two key enzymes, succinate dehydrogenase and NADH-fumarate reductase.[5] This high vulnerability of trypanosomes towards active oxygen and nitrogen species is due to a weak enzymatic antioxidant system which is, in the absence of catalase, essentially assured by an iron-superoxide dismutase and a spermidine-glutathion conjugate, called trypanothione, associated with the enzyme trypanothione reductase.[6] A strategy of development for the treatment of typanosomiasis could be considered by combining both specific targeted nitric oxide generation and parasite iron superoxide dismutase inhibition for an accumulation of superoxide anion whose dismutation into hydrogen peroxide and oxygen represents a mechanism for evasion of the host immune attack. As a consequence of the combination of these two radicals, peroxynitrite should be produced that may cause the killing of trypanosomes. This present work is focused on the first component of this strategy, outlining how nitric oxide can be selectively delivered to the parasite using specific transporters. Most of the parasitic protozoa are unable to biosynthesize purines de novo, and consequently must use active transporters to salvage them from the hosts. The transport of adenosine in the African trypanosomes *T. brucei* and *T. equiperdum* is ensured by two transporter systems: a P1 type, which also transports inosine, and a P2 type, which also enables adenine uptake. The P2 transporter has been shown to be implicated in the selective uptake of some melaminophenyl arsenicals[7] **1**, or pentamidine[8] **2**, and probably of the nitroheterocyclic compound megazol[9] **3** (Fig. 1) that has been shown to be active against many micro-organisms, including *T. cruzi*.

Recently, the gene that encodes the *T. brucei* P2-transporter activity has been identified, cloned and expressed

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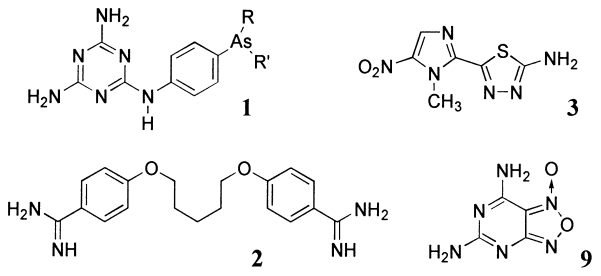


Figure 1.

in yeast, thus opening up prospects for the therapy of sleeping sickness.[10] In view of the analogies between adenine (or adenosine), benzamidine and melamine, it has been suggested that a specific P2-recognition may be related to the presence of the amidine motif (N=C-NH_2).[11]

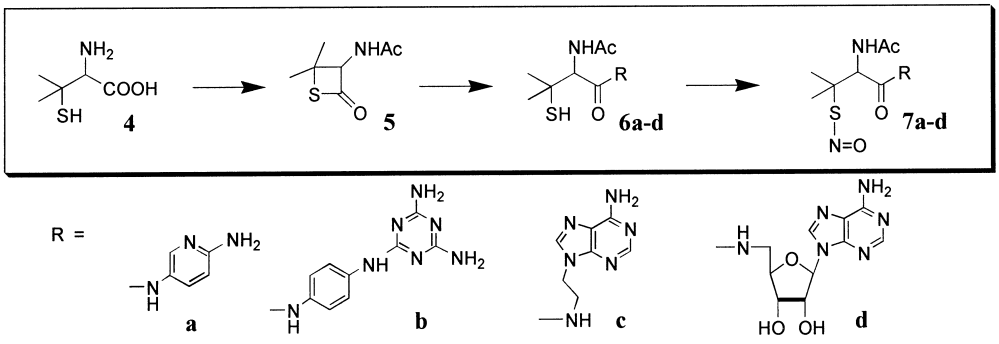
In this paper we report the synthesis and the uptake studies on adenosine transport in *T. equiperdum* of a number of drugs that contain both a group which specifically could target these drugs into the parasite via the P2-transporter, and a thionitrite-based or organic nitrate-based NO•-donor group that could exert the specific pharmacological effect by increasing the nitric oxide level. Furoxan **9** (Fig. 1), structurally-related to adenine, was also investigated.

Results

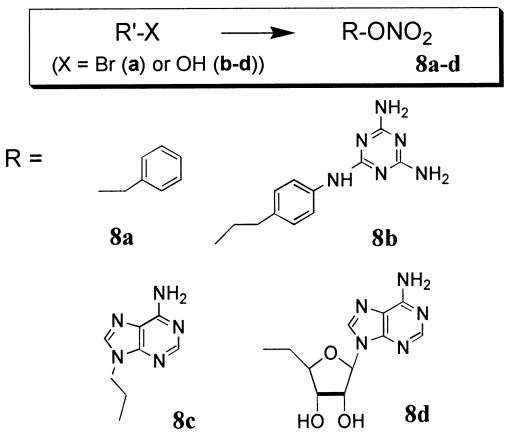
Synthesis and stability studies of thionitrites 7a–d (Scheme 1)

D,L-Penicillamine **4** was activated for coupling with amines by conversion into 3-acetamido-4,4-dimethyl-thietan-2-one **5**, which was prepared from the racemate of penicillamine by reaction with acetic anhydride in pyridine (40% yield).[12] The conversion of penicillamine into its corresponding thietanone for coupling with amino compounds was found to be a convenient and general method that can be run either in organic solvents or in biphasic conditions. Reaction of thietanone **5** with 2,4-diamino pyridine (commercially available), with 2-(4-aminophenylamino)-4,6-diamino-1,3,5-triazine,[13] and with 9-(2-amino-ethyl)-9*H*-purin-6-ylamine[14] gave the thiols **6a**, **6b** and **6c**, respectively. Compound **6d** was obtained by reaction of **5** with 5′-amino-2′,3′-(*O*-isopropylidene)-5′-deoxyadenosine[15] and subsequent deacetalization. Thiols **6a–d** were fully characterized by mass spectrometry, NMR and IR spectroscopies, and gave satisfactory elemental analysis.[16] For all these coupling reactions, no protection was required for amidine functions. The nitrosation reactions to obtain the thionitrites necessitated an investigation of various methods so that they precipitate once formed: only sodium nitrite as nitrosating agent in acidic methanolic conditions gave a satisfactory result in obtaining the thionitrite **7b**, and no diazotization/deamination reaction was observed. On the other hand, thionitrites **7a**, **7c** and **7d** were obtained by electrophilic nitrosation of the corresponding parent thiols with *tert*-butyl nitrite in chloroform (**7a**) or in acetone (**7c** and **7d**) with yields ranging from 60 to 86%. All thionitrites were characterized by $^{1}H/^{13}C$ NMR and IR spectroscopies.[17] They were stable at the solid stage and gave a green colour in solution in organic or aqueous media which indicates the presence of an *S*-nitroso group. Their decomposition studies were carried out by UV–visible spectroscopy noting the disappearance of the characteristic-S−N=O absorbance at 340 nm. Compound **7a–d** decomposed slowly in phosphate buffers (pH 7.5) with half-life times comprised between 2 and 3 h, comparable to the half-life of *S*-nitroso-*N*-acetylpenicillamine (SNAP; **7** where R = OH, Scheme 1) in the same conditions.[18] In the presence of transition metal ion chelators such as EDTA, release of nitric oxide from thionitrites **7a–d** decreased dramatically.[19]

Synthesis of organic nitrates 8a–d (Scheme 2) and furoxan 9. Organic nitrates **8b** and **8c** were readily prepared from the corresponding alcohol derivatives[20] by nitration using 60% nitric acid in the presence of acetic anhydride. They were fully characterized by mass spectrometry, NMR and IR spectroscopies, and gave satisfactory elemental analysis.[21] Synthesis of **8a** was achieved from benzyl bromide,[22] and **8d** was synthesized by nitration of 2′,3′-isopropylidene adenosine followed by an acidic deacetalization as previously published.[23] Nitration of 2,4,6-triaminopyrimidine followed by an oxidative intramolecular cyclization with iodosylbenzene diacetate gave furoxan **9**.[24]



Scheme 1. Synthesis of thionitrites.



Scheme 2. Synthesis of organic nitrates. For **8a**, **8b** and **8c**, R = R′. For **8d**, R′ is the *O*-isopropylidene derivative of R.

Biological evaluation[25]

All compounds were tested on *T. equiperdum* E1 for their ability to inhibit the uptake of [2-^{3}H]adenosine via transporter P2 in the presence of a saturating concentration of inosine to inhibit the P1 transporter. The values of K_i (Table 1) show that all compounds structurally-related to the melaminyl, adenine and adenosine derivatives efficiently inhibit adenosine transport in *T. equiperdum* in the presence of inosine, with affinity constant values (K_i) ranging from 0.2 to 12.3 μM, i.e., of the same order of magnitude of the K_m values of the natural substrate adenosine which enters through both adenosine transporters P1 ($K_m = 0.6$ μM) and P2 ($K_m = 0.7$ μM). In comparison, the melaminyl drug cymelarsen (compound **1** where R = R′ = -CH_2-CH_2-NH_2) uptake via P2 is less efficient ($K_i = 22.9$ μM). In the same conditions, thionitrite **7a**, which possesses a benzamidine function, and furoxan **9** exhibit a weak affinity for the transporter, whereas SNAP and **8c** which lack a P2-recognition moiety do not have any affinity for it with K_i values higher than 1 mM.

Discussion

The uncommon stability of SNAP[26] prompted us to choose penicillamine derivatives for the synthesis of thionitrites **7a**–**d**. In spite of the presence of amino groups, they exhibit significant stability to allow a structural characterization and a potential therapeutic use, and are capable of slowly generating NO• in physiological conditions with half-life times of several hours, i.e., a rate of decomposition comparable to those of SNAP. Beside *S*-nitrosothiols, organic nitrates constitute a class of drugs used as therapy for a large variety of cardiovascular diseases, but their use is often limited by the development of nitrate tolerance. Although a large body of evidence indicates that nitric oxide is formed from this class of NO•-donors, the mechanisms by which this occurs that, it is thought, involve both flavins and thiols, are not fully understood.[27] In the case of furoxans, an analogous mechanism involving thiols as cofactors has been assumed to occur resulting in the NO• generation. Stability studies of compounds **8a**–**d** revealed that they are stable in physiological conditions. All compounds which possess a melaminyl-, adenine- or adenosine-based P2 recognition motif strongly inhibit the uptake of adenosine by the transporter P2, that is, these compounds, like the melaminophenyl arsenical cymelarsen, have a specific interaction with this transporter. In the same experimental conditions, **7a** (benzamidine) and **9** (furoxan) are poor substrates of P2, whereas **8a** and SNAP do not compete with adenosine for transport, suggesting that recognition is strongly dependent on the nature of the structural features carried by the NO•-releasing moiety. Production of nitric oxide from NO• donors, including SNAP, has been shown to inhibit the growth of *Trypanosoma cruzi* and *Leishmania major*.[28] The mechanism of this toxicity has been attributed, at least in part, to the inhibition of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, or to its conversion into more reactive species like peroxynitrite. Conversely, all NO• donors of this study show a weak in vitro antiparasitic activity against *T. equiperdum* at relevant concentrations, except for compound **8c** which shows a cytotoxic ($LD_{100} = 60$ μM) and a cytostatic activity (8 μM). A better enzymatic defence system in the case of *T. equiperdum* could account for this discrepancy. In the case of thionitrites, these poor activities may be also related to their relatively rapid homolytic clivage in physiological conditions, and thus to a reduced NO• release when the molecules reach the parasite.

Table 1. Inhibition of P2 adenosine transporter by cymelarsen and NO•-donors in *T. equiperdum*

Compounds	K_i (μM)	Compounds	K_i (μM)
Adenosine	0.70[a]±0.01	**8a**	>1000
Cymelarsen	22.9±0.2	**8b**	0.20±0.02
7a	404±54	**8c**	1.9±0.3
7b	0.58±0.05	**8d**	12.3±0.2
7c	0.62±0.05	**9**	117±20
7d	3.47±0.35	SNAP	>1000

[a]K_m = value of adenosine.

Conclusion

The presence of purine transporters constitutes a weighty difference between parasites and their hosts that could be exploited for the development of drugs with trypanocidal activities. The molecules that bear a melaminyl, adenine or adenosine moiety were found to have a high affinity for the P2-transporter in *T. equiperdum* suggesting that these recognition moieties could be considered as a mode of transport for such drugs. By analogy with the detrimental effect of nitric oxide in the growth of *T. cruzi* or *Leishmania*, we expected that these NO•-carriers could by themselves provoke a nitrosative stress on *T. equiperdum*, leading to the death of the parasite. The poor in vitro trypanotoxic activity of these compounds could be attributed either to their rapid metabolization or to an efficient antioxidant defence system. In a complementary approach to the present

work, further studies are being carried out to investigate the inhibition of parasitic iron superoxide dismutase for a synergistically production of nitric oxide and superoxide.

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References and Notes

1. Wink, D. A.; Mitchell, J. B. *Free Radic. Biol. Med.* **1998**, *25*, 434 and references therein.
2. James, S. L. *Microbiol. Rev.* **1995**, *59*, 533.
3. Stamler, J. S.; Simon, D. I.; Osborne, J. A.; Mullins, M. E.; Jaraki, O.; Michel, T.; Singel, D. J.; Loscalzo, J. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 444.
4. Koppenol, W. H. *Free Radic. Biol. Med.* **1998**, *25*, 385.
5. Rubbo, H.; Denicola, A.; Radi, R. *Arch. Biochem. Biophys.* **1994**, *308*, 96.
6. Fairlamb, A. H.; Blackburn, P.; Ulrich, P.; Chait, B. T.; Cerami, A. *Science* **1985**, *227*, 1485.
7. Carter, N. S.; Fairlamb, A. H. *Nature* **1993**, *361*, 173.
8. Carter, N. S.; Berger, B. J.; Fairlamb, A. H. *J. Biol. Chem.* **1995**, *270*, 28153.
9. Barrett, M. P.; Fairlamb, A. H.; Rousseau, B.; Perié, J.; Chauvière, G. *Biochem. Pharmacol.* (in press).
10. Mäser, P.; Sütterlin, C.; Kralli, A.; Kaminsky, R. *Science* **1999**, *285*, 242.
11. Tye, C.-K.; Kasinathan, G.; Barrett, M. P.; Brun, R.; Doyle, V. E.; Fairlamb, A. H.; Weaver, R.; Gilbert, I. H. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 811.
12. Al-Zaidi, S. M. R.; Crilley, M. M. L.; Stoodley, R. J. *J. Chem. Soc. Perkin Trans. 1* **1983**, 2259.
13. Synthesized from 2-chloro-4,6-diamino-1,3,5-triazine and 4-aminoacetanilide in refluxing aq NaOH (1 eq.) and subsequent deacetalization with aq HCl (yield: 95%).
14. Synthesized from adenine and 2-bromo-1-[(*tert*-butyloxycarbonyl)amino]ethane in dry *N*,*N*′-dimethylformamide in presence of tetrabutylammonium iodide and potassium carbonate, and subsequent Boc-deprotection with trifluoroacetic acid (yield: 20%).
15. Kolb, M.; Danzin, C.; Barth, J.; Claverie, N. *J. Med. Chem.* **1982**, *25*, 550.
16. **6a**: Anal. calcd for $C_{12}H_{18}N_4O_2S$: C, 51.04; H, 6.43; N, 19.84. Found C, 50.99; H, 6.41; N, 19.81. **6b**: Anal. calcd for $C_{16}H_{22}N_8O_2S$, 1.5 H_2CO_3: C, 43.47; H, 5.21; N, 23.18. Found C, 42.71; H, 5.67; N 22.98. **6c**: Anal. calcd for $C_{14}H_{21}N_7O_2S$, 1.5 H_2CO_3: C, 41.89; H, 5.44; N, 22.06. Found C, 41.01; H, 5.40; N, 22.06. *O*-isopropylidene derivative of **6d**: Anal. calcd for $C_{20}H_{29}N_7O_5S$, 1.5 H_2CO_3: C, 46.57; H, 5.77; N, 18.10. Found C, 46.06; H, 5.84; N, 18.08.
17. **7a**: IR (KBr) 1657, 1531, 667 cm^{-1}. ^1H NMR (DMSO-d_6, 250 MHz): δ 1.89 (s, 3H), 1.98 (s, 3H), 2.03 (s, 3H), 5.40 (d, $J=9.5$ Hz, 1H), 7.06 (d, $J=9.5$ Hz, 1H), 7.96 (d, $J=9$ Hz, 1H), 8.41 (s, 1H), 8.69 (d, $J=9$ Hz, 1H), 10.94 (s, 1H). ^{13}C NMR (DMSO-d_6, 60 MHz): 22.2, 24.7, 26.4, 59.1, 59.5, 114.1, 124.9, 125.0, 137.6, 151.3, 167.6, 169.5. **7b**: IR (KBr) 1654, 1510, 668 cm^{-1}. ^1H NMR (C_5D_5N, 300 MHz): δ 2.12 (s, 3H), 2.17 (s, 3H), 2.24 (s, 3H), 6.16 (d, $J=9.5$ Hz, 1H), 6.57 (br. s, 6H), 7.89 (d, $J=9$ Hz, 2H), 7.97 (d, $J=9$ Hz, 2H), 9.53 (d, $J=9.5$ Hz, 1H), 10.74 (s, 1H); 11.73 (s, 1H). ^{13}C NMR (C_5D_5N, 75 MHz): δ 22.9, 25.6, 27.0, 60.0, 61.1, 121.2, 122.2, 134.6, 136.7, 164.4, 168.5, 170.4. **7c**: IR (KBr) 1700, 1655, 1512, 668 cm^{-1}. ^1H NMR (DMSO-d_6, 200 MHz): δ 1.82 (s, 6H), 1.85 (s, 3H), 3.59.62 (m, 2H), 4.33 (m, 2H), 5.05 (d, $J=9.5$ Hz, 1H), 8.35 (d, $J=9.5$ Hz, 1H), 8.43 (s, 1H), 8.45 (s, 1H), 8.59 (m, 1H), 8.77 (br. s, 1H), 9.55 (br. s, 1H). ^{13}C NMR (DMSO-d_6, 50 MHz): δ 22.2, 24.6, 26.7, 38.2, 43.3, 58.8, 59.1, 117.9, 144.1, 144.6, 148.7, 148.8, 168.6, 169.2. **7d**: IR (KBr) 1686, 1654, 1510, 669 cm^{-1} ^1H NMR (DMSO-d_6, 250 MHz): δ 1.84, 1.90 (2 s, 3H), 1.92, 1.96 (2 s, 3H), 1.95 (s, 3H), 3.37.50 (m, 2H), 3.99.10 (m, 2H), 4.60.65 (m, 1H), 5.22, 5.24 (d, $J=9.5$ Hz, 1H), 5.92.94 (m, 1H), 8.40.53 (m, 2H), 8.67.73 (m, 2H). ^{13}C NMR (DMSO-d_6, 60 MHz): δ 22.4, 24.5, 24.7, 26.6, 40.0, 40.4, 58.8, 58.9, 59.6, 71.2, 73.2, 83.2, 83.5, 87.5, 87.6, 118.8, 119.0, 142.3, 146.6, 148.4, 148.5, 151.0, 151.2, 168.5, 169.3.
18. Singh, R. J.; Hogg, N.; Joseph, J.; Kalyanaraman, B. *J. Biol. Chem.* **1996**, *271*, 18596.
19. Askew, S. C.; Barnett, D. J.; McAninly, J.; Williams, D. L. H. *J. Chem. Soc. Perkin Trans. 2* **1995**, 741.
20. Precursor of **8b** was synthesized from 2-chloro-4,6-diamino-1,3,5-triazine and 4-aminophenethyl alcohol in refluxing aq NaOH (yield: 96%). Precursor of **8c** was synthesized from adenine and ethylene carbonate in refluxing DMF with a catalytic amount of NaOH (yield: 46%).
21. **8b**: IR (KBr) 3411, 3323, 1632, 1277 cm^{-1}. ^1H NMR (DMSO-d_6, 250 MHz): δ 2.92 (t, $J=5$ Hz, 2H), 4.70 (t, $J=5$ Hz, 2H), 6.30 (s, 4H), 7.12 (d, $J=8.5$ Hz, 2H), 7.71 (d, $J=8.5$ Hz, 2H), 8.81 (s, 1H). ^{13}C NMR (DMSO-d_6, 60 MHz): δ 31.6, 73.0, 119.7, 128.57, 129.1, 139.19, 164.7, 166.0. Mass (DCI/NH_3) m/z 292 $[M+H]^+$. Anal. calcd for $C_{11}H_{13}N_7O_3$: C, 45.36; H, 4.50; N, 33.66. Found C, 45.14; H, 4.50; N, 33.77. **8c**: IR (KBr) 3301, 3157, 1689, 1632, 1281 cm^{-1} ^1H NMR (DMSO-d_6, 250 MHz): δ 4.55 (t, $J=5$ Hz, 2H), 4.93 (t, $J=5$ Hz, 2H), 7.27 (s, 2H), 8.15 (s, 1H), 8.18 (s, 1H). ^{13}C NMR (DMSO-d_6, 60 MHz): δ 40.4, 71.4, 118.4, 140.7, 149.6, 152.5, 155.9. Mass (DCI/NH_3) m/z 225 $[M+H]^+$, 242 $[M+NH_4]^+$. Anal. calcd for $C_7H_8N_6O_3$: C, 37.50; H, 3.60; N, 37.49. Found C, 37.68; H, 3.60; N, 37.12.
22. **8a** was prepared from benzyl bromide and silver nitrate in acetonitrile at room temperature (yield: 97%).
23. Lichtenthaler, F. W.; Müller, H. J. *Synthesis* **1974**, 199.
24. Sako, M.; Oda, S.; Ohara, S.; Hirota, K.; Maki, Y. *J. Org. Chem.* **1998**, *63*, 6947.
25. For the experimental determination of K_i, see: Barrett, M. P.; Zhang, Z. Q.; Denise, H.; Giroud, C.; Baltz, T. *Mol. Biochem. Parasitol.* **1995**, *73*, 223.
26. Field, L.; Dilts, R. V.; Ravichandran, R.; Lenhert, P. G.; Carnahan, G. E. *J. Chem. Soc. Chem. Commun.* **1978**, 249
27. Thatcher, G. R. J.; Weldon, H. *Chem. Soc. Rev.* **1998**, *27*, 331.
28. Bourguignon, S. C.; Alves, C. R.; Giovanni De Simone, S. *Acta Trop.* **1997**, *66*, 109.