A trypanosome oligopeptidase as a target for the trypanocidal agents pentamidine, diminazene and suramin

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Abstract African trypanosomes contain a cytosolic serine oligopeptidase, called OP-Tb, that is reversibly inhibited by the active principles of three of the five most commonly used trypanocidal drugs: pentamidine, diminazene and suramin. OP-Tb was inhibited by pentamidine in a competitive manner, and by suramin in a partial, non-competitive manner. The inhibition of OP-Tb by a variety of suramin analogues correlated with the trypanocidal efficacy of these analogues ($P = 0.03$; by paired Student's *t*-test). Since intracellular (therapeutic) concentrations of pentamidine and suramin are reported to reach approximately $206K_i$ and $15K_i$ respectively, we suggest that these drugs may exert part of their trypanocidal activity through the inhibition of OP-Tb.

Key words: Protease; Oligopeptidase; Suramin; Pentamidine; Diminazene; *Trypanosoma brucei*

1. Introduction

African trypanosomes are protozoan parasites that cause widespread disease in livestock (e.g. cattle) as well as in humans. Collectively, the diseases are referred to as African trypanosomiasis. A number of drugs are available for the chemotherapy of African trypanosomiasis, including sulfonated naphthylamines such as suramin [1]; aromatic diamidines, including pentamidine [2] and diminazene [3]; the trivalent arsenical, melarsoprol [4]; and α-DL-difluoromethylornithine (DFMO), a suicide inhibitor of ornithine decarboxylase [5]. With the exception of DFMO, the in vivo targets of the drugs have not been unequivocally identified. Consequently, the biochemical basis of their trypanocidal action is unclear [6]. During the course of our studies on the peptidases of African trypanosomes, we have identified and purified a cytosolic [7] oligopeptidase from the African trypanosome *Trypanosoma brucei brucei*, which we called OP-Tb [8,9]. The substrate specificity of OP-Tb, which preferentially cleaves peptides after pairs of basic amino acid residues [8,9], suggested that the aromatic diamidines (pentamidine and diminazene), would act as competitive inhibitors of OP-Tb. Similarly, previous reports of the inhibition of trypsin-like serine peptidases by suramin [10–12] raised the possibility that suramin might inhibit OP-Tb. Furthermore, OP-Tb activity is abolished in the presence of thiol-reactive compounds [9], and since melarsoprol forms adducts with thiol-containing proteins via disulfide bonds [13,14], it was also possible that melarsoprol could inactivate OP-Tb. Here we report that pentamidine, diminazene and suramin are indeed inhibitors of OP-Tb, and that, in the case of pentamidine and suramin, the inhibition of OP-Tb by these inhibitors is likely to be of in vivo significance. This represents the first report where peptidases have been identified as possible targets of trypanocidal drugs and we suggest that serine peptidase inhibitors may represent a class of lead compounds for the development of new chemotherapeutic agents that are active against OP-Tb. Recent reports of drug resistance by African trypanosomes [15–17] underscore the importance of developing new and improved therapeutic strategies.

2. Materials and methods

2.1. Materials

Melarsoprol and melarsen oxide were from Dr. Alan Fairlamb, Biomedical Sciences Institute, University of Dundee. DFMO was from Dr. Andrew Peregrine, International Livestock Research Institute, Nairobi, Kenya. Suramin was from Bayer (Leverkusen, Germany). Berenil was from Hoechst Veterinär (Munich, Germany). Pentamidine and diminazene aceturate (minimum 90% purity) were from Sigma (St. Louis, MO, USA). The structure of suramin (Fig. 1) illustrates that it is a symmetrical molecule and that each half, connected by a central urea group, can be sub-divided into three regions: an acidic naphthalene moiety (R_1), a methylbenzoyl moiety (R_2) and a benzoyl (R_3) moiety. Analogues were synthesized as described previously [18] with various substitutions at R_1, R_2 and R_3 (Tables 1 and 2). Analogue nomenclature is adapted from that described in [19].

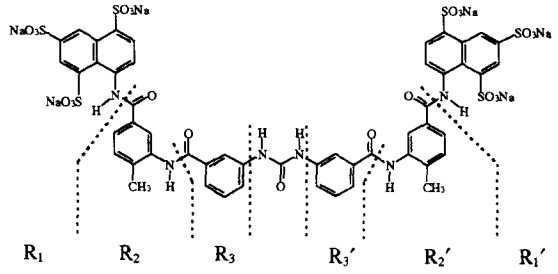

Fig. 1. Chemical formula of suramin and designated nomenclature for suramin analogues (modified from [18,19]).

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Abbreviations: AMC, 7-amino-4-methylcoumarin; Cbz, carbobenzoxy; DFMO, α-DL-difluoromethylornithine; PGK, phosphoglycerate kinase; Suc, succinyl

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2.2. Isolation of OP-Tb

OP-Tb was purified from *T. b. brucei* clone ILTat 1.1 [9] and active enzyme concentration determined using 4-methylumbelliferyl-*p*-guanidobenzoate as described [20].

2.3. Diagnosis of inhibition mechanism

Inhibition mechanisms were diagnosed from the effect of a drug on the K_m and V_{max}. OP-Tb (1.5 ng, 18.75 fmol active concentration) was pre-incubated in assay buffer (50 mM Tris-HCl, 10 mM dithiothreitol, pH 8) for 10 min at 37°C, with or without drug, followed by the addition of substrate. The initial steady-state velocity was determined by continuous assay for a range of substrate concentrations (45 nM–75 μM). While the kinetic data are represented graphically as Lineweaver-Burk plots (Fig. 2), due to the inherent error in the determination of these parameters graphically [21], actual values for K_m and V_{max} were determined by hyperbolic regression using the software package Hyper 1.01 (obtained from Dr. J.S. Easterby, University of Liverpool, UK). The k_{cat} was determined from $k_{cat} = V_{max}/[E]_0$, where $[E]_0$ represents the active enzyme concentration. Dithiothreitol was omitted from assays involving organo-arsenicals to prevent potential complex formation of arsenic with free thiols [22]. Organo-arsenicals were made up as stock solutions in dimethylformamide, and diluted with assay buffer, while the other inhibitors were made up as aqueous stock solutions. In the case of melarsoprol and melarsen oxide, the K_m and V_{max} values were determined in the presence of residual concentrations of dimethylformamide, in order to negate the effect of this solvent on these parameters. Non-competitive inhibition was distinguished from irreversible inhibition by diluting out the inhibitory effect of compounds exhibiting either mode of inhibition. This was achieved with a succession of four dilution/concentration cycles where enzyme-inhibitor samples were diluted with assay buffer and concentrated to the original volume in 3 ml polysulfone concentrators (7000×*g*, 10°C). Trypsin and chymotrypsin were assayed as described previously [12].

2.4. Calculation of inhibition constant

For competitive inhibitors, the apparent K_i ($K_{i(app)}$) was determined in the presence of Cbz-Arg-Arg-AMC as previously described [23], and corrected for the presence of substrate (K_m=245 nM) by dividing the $K_{i(app)}$ by the factor $1+[S]/K_m$ to give the true K_i [23]. For partial non-competitive inhibitors, the K_i was determined from the complex steady-state rate equation of Baici [24] as modified by Szedlacsek et al. [25],

$$\frac{v_i}{v_0} = \frac{v_0 - v_\infty}{2v_0}\sqrt{\left(\frac{1+\sigma}{\alpha+\sigma}\cdot\frac{\alpha K_i}{[E]_0} + \frac{[I]}{[E]_0} - 1\right)^2 + 4\frac{1+\sigma}{\alpha+\sigma}\cdot\frac{\alpha K_i}{[E]_0}} + \\ + \frac{v_0 + v_\infty}{v_0 - v_\infty} - \frac{1+\sigma}{\alpha+\sigma}\cdot\frac{\alpha K_i}{[E]_0} - \frac{[I]}{[E]_0} \quad (1)$$

where $\sigma = [S]/K_m$, $v_\infty = \beta\, k_{cat}\, [E]_0[S]/[S]+\alpha K_m$. The parameters α and β are dimensionless factors representing the change in the K_m and k_{cat}, respectively, in the presence of an inhibitor and v_∞ is the rate extrapolated at infinite inhibitor concentration, [I]. Once K_m, k_{cat}, α and β had been determined separately, the K_i was calculated by non-linear regression analysis, fitting (v_i, [I]) pairs to Eq. 1, in which all other parameters were fixed.

2.5. Cytotoxicity assays

Trypanosomes were cultured [26] and cytotoxicity assays performed as previously described [27]. Cultures were maintained in 24-well cell-culture plates (1 ml culture volume). Drugs were dissolved in dimethylsulfoxide, and added at various concentrations to trypanosome cultures. The final dimethylsulfoxide concentration was maintained at 1% (v/v). Control cultures (without drugs, but containing 1% (v/v) dimethylsulfoxide) were incubated under the same conditions. Cell numbers were determined (in triplicate) with a hemocytometer, and data analyzed graphically, plotting growth inhibition versus drug concentration, from which the effective concentration inhibiting the growth of trypanosome populations by 50% (EC_{50}) was calculated.

3. Results and discussion

The trivalent arsenicals, melarsoprol or melarsen oxide, and the ornithine analogue DFMO had no effect on the hydrolysis of Cbz-Arg-Arg-AMC by OP-Tb relative to uninhibited controls (data not shown). However, OP-Tb activity is enhanced in the presence of reducing agents and polyamines [8]. Since melarsoprol therapy depletes intracellular reducing agents (namely trypanothione) and since DFMO therapy depletes intracellular polyamine levels [28] either drug may down-regulate OP-Tb activity within trypanosomes through the depletion of intracellular OP-Tb activators.

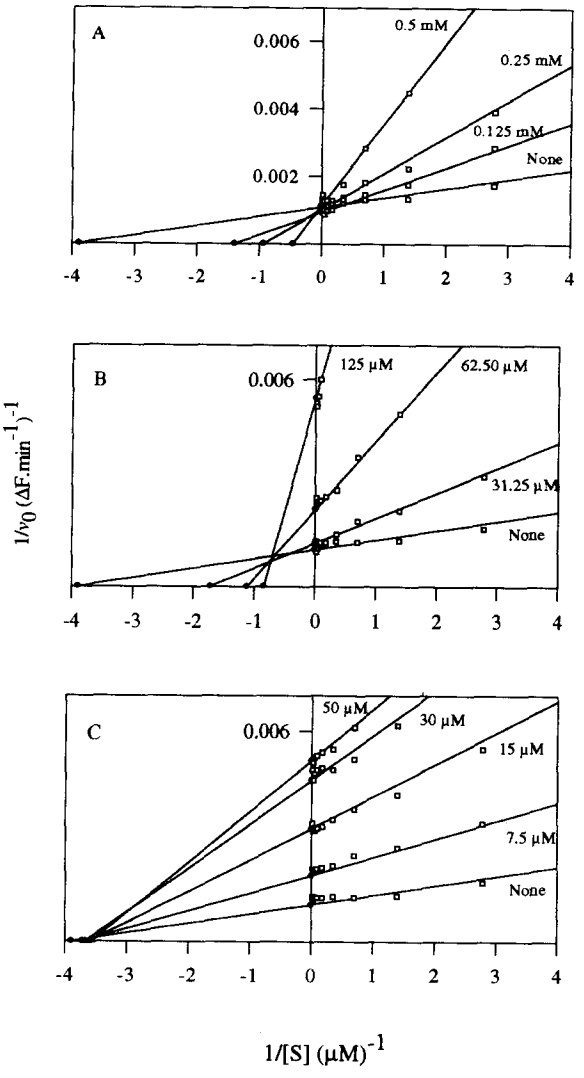


Fig. 2. Diagnosis of inhibition mechanisms by trypanocidal drugs. Lineweaver-Burk plots of kinetic data obtained for the inhibition of Cbz-Arg-Arg-AMC hydrolysis by OP-Tb over a range of inhibitor concentrations for (A) pentamidine, (B) Berenil and (C) suramin.

OP-Tb was competitively inhibited by pentamidine with a K_i of 3.4 μM (Fig. 2A), which compares well with the K_i observed for bovine β-trypsin (2.3 μM) [29], bovine mast-cell tryptase (1.2 μM) [30] and human plasmin (3.3 μM) [31]. Pentamidine isethionate is the active principle of the trypanocidal drug Pentacarinate. The mechanism of its trypanocidal activity is not understood, although it has previously been attributed to inhibition of trypanosome *S*-adenosyl-L-methionine decarboxylase [32] or to the intercalation with trypanosome DNA [33]. Structurally, the pentamidine molecule represents two benzamidine molecules joined together, resembling a pair of basic amino acid (arginine) residues in

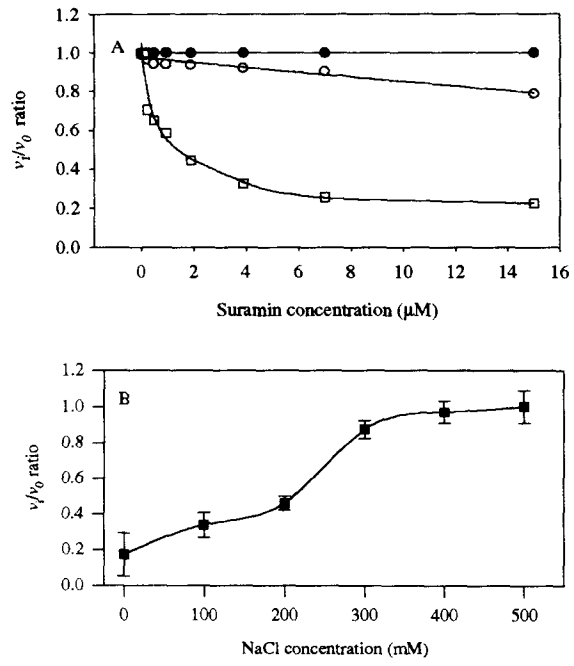


Fig. 3. Effect of suramin on substrate hydrolysis by serine proteases. A: Effect of increasing concentrations of suramin on the activity of OP-Tb (20 nM) against Cbz-Arg-Arg-AMC (□), chymotrypsin (25 nM) against Suc-Leu-Tyr-AMC (●) and trypsin (25 nM) against Cbz-Arg-AMC (○). Ordinates (v_i/v_0), initial rate in the presence of suramin/initial rate in the absence of suramin. B: Effect of NaCl on the inhibition of OP-Tb (20 nM) activity against Cbz-Arg-Arg-AMC by suramin (■). Error bars represent the mean $v_i/v_0 \pm$ S.D. ($n = 3$). All substrates at 5 μM.

a dipeptide. During therapeutic regimens, pentamidine typically reaches concentrations of up to 13 μM in the host bloodstream [34] where it has a plasma half-life of 47 h [35]. Furthermore, pentamidine is actively concentrated by trypanosomes which possess pentamidine transporters [36], reaching intracellular concentrations of between 700 μM and 1 mM in 3 h [37]. For an inhibitor to be considered physiologically relevant, its in vivo concentration must be $> 10K_i$ [38]. As the intracellular concentration of pentamidine within trypanosomes is likely to reach at least 700 μM ($= 206K_i$) during therapeutic regimes, it is possible that pentamidine may exert its trypanocidal activity through the inhibition of OP-Tb, which is a cytosolic enzyme.

Commercially available preparations of the trypanocidal drug Berenil contain 45% (m/m) diminazene aceturate, and 55% (m/m) phenyldimethyl pyrazolone [39]. Diminazene aceturate is the active principle of this drug and is known to inhibit *S*-adenosyl-L-methionine decarboxylase [32] and extranuclear DNA synthesis in trypanosomes [40]. However, the mechanism of the trypanocidal activity of diminazene has not been unequivocally determined. Diminazene is like pentamidine in that it resembles two benzamidine molecules joined together, albeit with a shorter spacer region. Thus, like pentamidine, it also mimics potential substrates for OP-Tb. This structural similarity to pentamidine suggests that diminazene molecules may behave similarly with respect to OP-Tb, within the parasites. Berenil exhibited mixed inhibition of OP-Tb (Fig. 2B). This raises the possibility that the diminazene aceturate competitively inhibits OP-Tb activity, while the phenyldimethyl pyrazolone contributes to the inhibition of OP-Tb by Berenil in a non-competitive manner. However, similar data were obtained using a 90% pure preparation of diminanizene aceturate (results not shown), suggesting that diminazene aceturate itself is entirely responsible for the mixed inhibition observed.

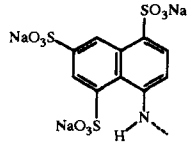
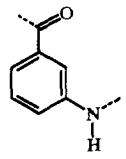
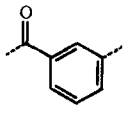
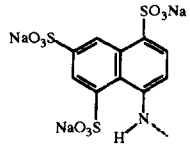
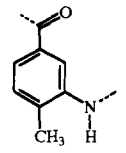
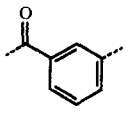
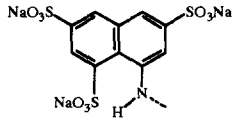
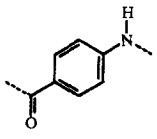
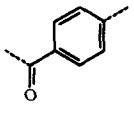
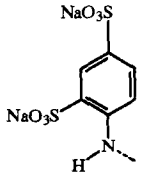
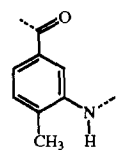
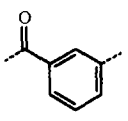
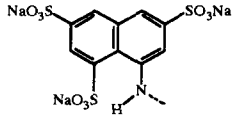
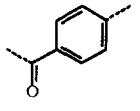
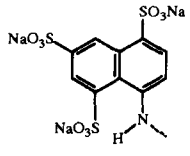
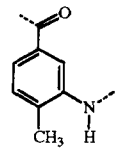
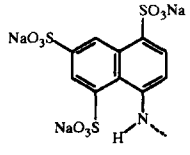
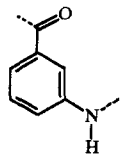
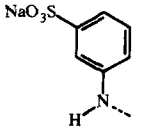
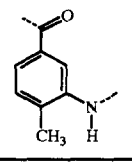
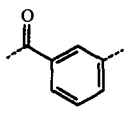
Suramin is known to inhibit a number of serine peptidases. These include kallikrein, thrombin and plasmin [10], C1 esterase [11] and trypsin [41]. However, the mechanism is unknown. Kinetic analysis of kallikrein inhibition by suramin revealed that it was not competitive. More detailed analysis of the suramin interaction with neutrophil elastase, cathepsin G and proteinase 3 indicated that suramin was a non-competitive inhibitor of these enzymes [12]. Here we show that the suramin inhibition of OP-Tb activity against Cbz-Arg-Arg-AMC resulted in a 4.5-fold reduction in V_{max} with no apparent effect on K_m (Fig. 2C). As it was possible to dilute out the effect of suramin with a series of four dilution/concentration cycles (results not shown), the inhibition by suramin was reversible, and therefore non-competitive. The clustering of the V_{max} values at high suramin concentrations (ordinate, Fig. 2C) suggested that a partial inhibition mechanism was operating [12,24,25], and this did indeed prove to be the case (Fig. 3A). The $[E]_0/K_i$ ratio for the inhibition of OP-Tb by suramin was < 0.01, indicating that there was no tight binding of inhibitor to enzyme [38]. A similar situation was observed for the inhibition of neutrophil proteinase 3 by suramin, which also exhibited partial, non-competitive inhibition by suramin with an $[E]_0/K_i$ ratio < 0.01 [12].

In general there is a good relationship between the basicity of an enzyme and its affinity for suramin [12], but this rule appears to break down with OP-Tb which has a p*I* of 5.1 [7]. However, despite its low p*I*, OP-Tb has 24 lysine residues and 59 arginine residues (unpublished data). We suspect that the sulfonic acid groups of suramin electrostatically interact with these basic residues, a conclusion supported by the observation that NaCl abolished the inhibition of OP-Tb by suramin (Fig. 3B). However, additional specific interactions appear to be involved, as polysulfonated glycosaminoglycans such as heparin do not inhibit OP-Tb (unpublished data), suggesting that the inhibition is not due to a non-specific electrostatic effect with polysulfonated compounds.

Both suramin and its demethylated analogue (NF037, Table 1) were potent inhibitors of OP-Tb activity, and were equally trypanocidal. These findings contrast with those of Forneau et al. [42], who reported that demethylation of suramin resulted in complete loss of trypanocidal activity. However, the EC_{50} values reported here were determined in vitro, whereas Forneau et al. [42] investigated the ability of suramin analogues to cure trypanosome infection in vivo, in mice. Taken together, these data suggest that, while the methyl groups are unimportant for OP-Tb-inhibitory activity and trypanocidal efficacy, they may be essential for the metabolism and/or transport of suramin in mice.

Both the number and spatial arrangement of the sulfonic acid groups on the aminonaphthyl (R_1) moiety were important for inhibitory and trypanocidal activity. Replacing each of the 1-naphthylamino-4,6,8-trisulfonic acid moieties of suramin with aniline-2,4-disulfonic acid (NF065; Table 1) resulted in a 12-fold increase in K_i, but only a 15% increase in EC_{50}. Removal of a second sulfonate group from each of the aniline moieties in NF065 (NF078; Table 1) elevated the K_i 25-fold when compared with suramin. This weak inhibitor

Table 1
Inhibition constants and EC_{50} values for symmetrical suramin analogues

Analogue	Structural substituents			K_i	EC50
	$R_1 = R_1'$	$R_2 = R_2'$	$R_3 = R_3'$	(μM)	(μM)
NF037				5.4	74.5
Suramin				6.7	88.2
NF031				62.2	96.7
NF065				77.3	104.3
NF013			absent	127.7	128.7
NF058			absent	159.2	167.4
NF023			absent	161.0	172.2
NF078				167.2	>220.0

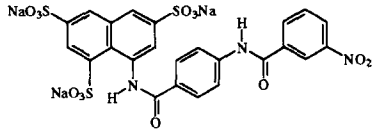
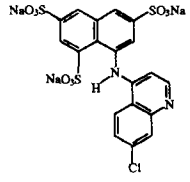
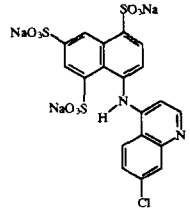
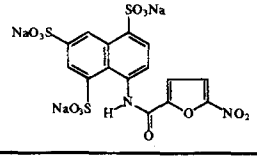
Analogue nomenclature is detailed in [19].

possessed no trypanocidal activity at the concentrations tested.

It is apparent from Tables 1 and 2 that the length of the molecule is also important for both inhibitory potency and trypanocidal activity. Removal of the benzoyl (R_3) group of suramin (NF058; Table 1) increased the K_i 24-fold and the EC_{50} two-fold. Demethylation of the methylbenzoyl (R_2) moiety of this shortened molecule (to generate NF023 from NF058; Table 1) had little effect on K_i or EC_{50} when compared with NF058. This is in agreement with our findings that these methyl groups are not important for inhibitory or trypanocidal activity.

Replacement of both the methylbenzoyl (R_2) and benzoyl (R_3) groups of suramin with 4-C-benzoyl groups (NF031; Table 1) elevated the K_i nine-fold, with a 10% increase in EC_{50}. Subsequent removal of two of these 4-C-benzoyl groups

Table 2
Inhibition constants and EC_{50} values for asymmetrical and half-suramin analogues

Analogue	Chemical structure	K_i (μM)	EC_{50} (μM)
NF033		308.8	>220.0
NF035		n.m.	>220.0
NF036		n.m.	>220.0
NF226		n.m.	>220.0

n.m. denotes that no inhibitory activity was observed at the concentrations tested. Analogue nomenclature is detailed in [19].

(NF013; Table 1) increased the K_i 19-fold and the EC_{50} by 31% when compared with suramin. This indicated that a reduction in the length of the molecule was accompanied by a reduction in both inhibitory and trypanocidal activity.

The importance of the length and symmetry of the molecule is also illustrated by the lack of inhibitory or trypanocidal activity of the 'half-suramin' analogue NF036 (Table 2). Neither the rearrangement of one of the sulfonate groups on the naphthylene ring (NF035; Table 2), nor the attachment of the 4,6,8-substituted aminonaphthylsulfonate to a 5-nitro-2-furyl group (NF226; Table 2) improved the inhibitory or trypanocidal activity.

In the absence of a three-dimensional structure of OP-Tb, it is difficult to predict how suramin may interact, in such a specific fashion, with OP-Tb. Hart et al. [43] examined the interactions of suramin and trypanosome phosphoglycerate kinase (PGK). Computer modelling of a putative PGK-suramin complex illustrated that electrostatic interactions between PGK and suramin would bridge the PGK active site and block the mechanism of action of the enzyme. However, it is premature to speculate whether or not suramin may somehow occlude the active-site 'pit' proposed to exist in oligopeptidases [44].

There was a significant correlation ($P = 0.03$; by paired Student's t-test; Table 1) between the inhibitory potency and trypanocidal efficacy of suramin analogues, which strongly suggests that OP-Tb represents an important intracellular target for suramin. During treatment regimens, suramin reaches concentrations of approximately 100 μM within the host bloodstream [45] and, although not actively concentrated in the parasite, it reaches intracellular concentrations of approximately 100 μM [6]. Since suramin has a K_i of 6.7 μM for OP-Tb, the inhibition of OP-Tb by suramin may be physiologically relevant and this may explain, at least in part, the trypanocidal action of suramin, which remains unelucidated [6].

The trypanocidal action of suramin has previously been attributed to the inhibition of trypanosome glycolytic enzymes, and while K_i values for the inhibition of these enzymes by suramin have not been determined, IC_{50} values of 10–100 μM have been reported [46]. Additionally, a K_i of 15 μM has been reported for the inhibition of trypanosome 6-phosphogluconate dehydrogenase by suramin [47]. However, Wang [6] pointed out that all nine glycolytic enzymes are protected from cytosolic suramin by compartmentalization in a glycosome, which is unlikely to take up suramin by diffusion or endocytosis. Suramin has been used for over 70 years [6] and appears still to be effective against the parasite as there are few reports of *T. b. brucei* resistance [48]. As trypanosomes do not readily develop resistance to suramin, it seems likely that the drug may act on multiple targets in the parasite. Given that the K_i for the inhibition of OP-Tb by suramin is lower than that reported for other enzymes, as well as the significant correlation between trypanocidal efficacy and OP-Tb inhibition, and the cytosolic location of OP-Tb, we propose that OP-Tb is one of these targets.

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References

[1] Kleine, P.K. and Fischer, W. (1923) Deutsch. Med. Wochenschr. 32, 1039–1041.
[2] Lourie, E.M. and Yorke, W. (1939) Ann. Trop. Med. Parasitol. 33, 289–304.
[3] Jensch, H. (1937) Angew. Chem. 50, 891–895.
[4] Friedham, E.A.H. (1949) Am. J. Trop. Med. Hyg. 29, 173–180.
[5] McCann, P.P., Bacchi, C.J., Hanson, W.L., Cain, G.D., Nathan, H.C., Hutner, S.H. and Sjoerdsma, A. (1981) Adv. Polyamine Res. 3, 97–110.
[6] Wang, C.C. (1995) Annu. Rev. Pharmacol. Toxicol. 35, 93–127.
[7] Kornblatt, J.D., Mpimbaza, G.W.N. and Lonsdale-Eccles, J.D. (1992) Arch. Biochem. Biophys. 293, 25–31.
[8] Troeberg, L., Pike, R.N., Morty, R., Berry, R.K., Coetzer, T.H.T. and Lonsdale-Eccles, J.D. (1996) Eur. J. Biochem. 238, 728–736.
[9] Lonsdale-Eccles, J.D. and Grab, D.J. (1987) Eur. J. Biochem. 169, 467–475.
[10] Eisen, V. and Loveday, C. (1973) Br. J. Pharmacol. 49, 678–687.
[11] Fong, J.S.C. and Good, R.A. (1972) Clin. Exp. Immunol. 10, 127–138.
[12] Cadène, M., Duranton, J., North, A., Si-Tahar, M., Chignard, M. and Bieth, J.G. (1997) J. Biol. Chem. 272, 9950–9955.
[13] Cunningham, M.L., Zvelebil, M.J. and Fairlamb, A.H. (1994) Eur. J. Biochem. 221, 285–295.
[14] Johnson, R.M. (1963) in: Metabolic Inhibitors (Hochster, R.M. and Quastel, J.H., Eds.), pp. 99–118, Academic Press, London.
[15] Pinder, M. and Authié, E. (1984) Acta Trop. 41, 247–252.
[16] Schönefeld, A., Röttcher, D. and Moloo, S.K. (1987) Tropen. Med. Parasitol. 38, 177–180.
[17] Sutherland, I.A. and Holmes, P.H. (1993) Acta Trop. 54, 271–278.
[18] Nickel, P., Haack, H.-J., Widjaja, H., Ardanuy, U., Gurgel, C., Düwel, D., Loewe, H. and Raether, W. (1986) Arzneim.-Forsch./Drug Res. 36, 1153–1157.
[19] Jentsch, K.D., Hunsman, G., Hartmann, H. and Nickel, P. (1987) J. Gen. Virol. 68, 2183–2192.
[20] Coleman, P.L., Latham, H.G. and Shaw, E. (1981) Methods Enzymol. 80, 12–25.
[21] Dixon, M. and Webb, E.C. (1979) Enzymes, Longman, London.
[22] Barron, E.S.G. (1951) Adv. Enzymol. 11, 201–253.
[23] Salvesen, G. and Nagase, H. (1989) in: Proteolytic Enzymes: A Practical Approach (Benyon, R.J. and Bond, J.S., Eds.), pp. 83–104, IRL Press, Oxford.
[24] Baici, A. (1987) Biochem. J. 244, 793–796.
[25] Szedlacsek, S.E., Ostafe, V., Serban, M. and Vlad, M.O. (1988) Biochem. J. 254, 311–312.
[26] Hesse, F., Selzer, P.M., Mühlstädt, K. and Duszenko, M. (1995) Mol. Biochem. Parasitol. 70, 157–166.
[27] Kaminsky, R. and Zweygarth, E. (1989) Antimicrob. Agents Chemother. 33, 881–885.
[28] Fairlamb, A.H., Henderson, G.B., Bacchi, C.J. and Cerami, A. (1987) Mol. Biochem. Parasitol. 24, 185–191.
[29] Caughey, G.H., Raymond, W.W., Bacci, E., Lombardy, R.J. and Tidwell, R.R. (1993) J. Pharmacol. Exp. Ther. 264, 676–682.
[30] Fiorucci, L., Erba, F., Bolognesi, M. and Ascoli, F. (1997) FEBS Lett. 408, 85–88.
[31] Geratz, J.D. (1973) Thromb. Diathes. Haemorrh. 29, 154–167.
[32] Bitonti, A.J., Dumont, J.A. and McCann, P.P. (1986) Biochem. J. 237, 685–689.
[33] Hajduck, S.L. (1978) Prog. Mol. Subcell. Biol. 6, 158–200.
[34] Sands, M., Kron, M.A. and Brown, R.B. (1985) Rev. Infect. Dis. 7, 625–634.
[35] Bronner, U., Doua, F., Ericsson, Ö., Gustaffson, L.L., Miézan, T.W., Rais, M. and Rombo, L. (1991) Trans. R. Soc. Trop. Med. Hyg. 85, 608–611.
[36] Damper, D. and Patton, C.L. (1976) Biochem. Pharmacol. 25, 271–276.
[37] Berger, B.J., Carter, N.S. and Fairlamb, A.H. (1995) Mol. Biochem. Parasitol. 69, 289–298.
[38] Beith, J.G. (1995) Methods Enzymol. 248, 59–84.
[39] Leach, T.M. and Roberts, C.J. (1981) Pharmacol. Ther. 13, 91–147.
[40] Newton, B.A. and Le Page, R.W.F. (1967) Biochem. J. 105, 50–51.
[41] Hawking, F. (1978) Adv. Pharmacol. Chemother. 15, 289–322.
[42] Forneau, E., Tréfouel, J., Tréfouel, M. and Vallée, J. (1924) Ann. Inst. Pasteur 38, 81–112.
[43] Hart, D., Langridge, A., Barlow, D. and Sutton, B. (1989) Parasitol. Today 5, 117–122.
[44] Barrett, A.J. and Rawlings, N.D. (1992) Biol. Chem. Hoppe-Seyler 373, 353–360.
[45] Fairlamb, A.H. and Bowman, I.B.R. (1980) Mol. Biochem. Parasitol. 1, 315–333.
[46] Wilson, M., Callens, M., Kunz, D.A., Pieré, J. and Opperdoes, F.R. (1993) Mol. Biochem. Parasitol. 59, 201–210.
[47] Hanau, S., Rippa, M., Bertelli, M., Dallocchio, F. and Barrett, M.P. (1996) Eur. J. Biochem. 240, 592–599.
[48] Scott, A.G., Tait, A. and Turner, M.R. (1996) Acta Trop. 60, 251–262.