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Specific peptide inhibitors of trypanothione reductase with backbone structures unrelated to that of substrate: potential rational drug design lead frameworks

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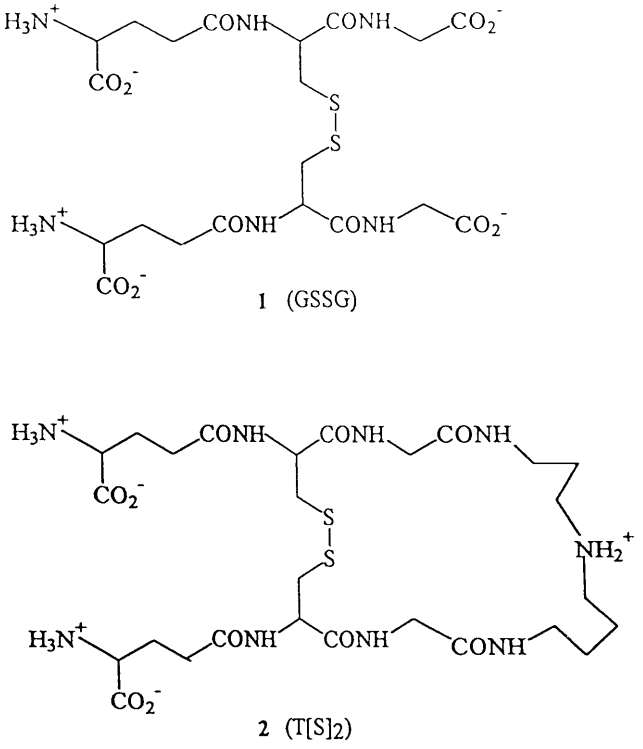
Summary. By introducing cationic charge sites novel peptide lead inhibitor structures for trypanothione reductase have been designed using molecular modelling methods. The inhibitors showed reversible, linear competitive inhibition and the strongest peptide inhibitor to date was found to be *N*-benzyloxycarbonyl-Ala-Arg-Arg-4-methoxy-β-naphthylamide with a K_i value of 2.4μM and a selectivity for parasitic enzyme (trypanothione reductase) over the host enzyme (human glutathione reductase) of over 3 orders of magnitude.

Keywords: Amino acids – Drug leads – Glutathione – Antiparasitics – Trypanosomiasis – Leishmaniasis

Abbreviations: Bz, benzoyl; GR, glutathione reductase; GSSG, glutathione disulphide; GSH, reduced glutathione; I_{50}, concentration of inhibitor giving 50% inhibition; $T[S]_2$, trypanothione disulphide; $T[SH]_2$, reduced trypanothione as dithiol; TR, trypanothione reductase; Z, benzyloxycarbonyl.

Introduction

The possibilities to design potential lead drug structures against trypanosomiasis and leishmaniasis, major fatal or debilitating diseases of the developing world, took a step forward when it was recognised that the redox defences of the causative parasites differed absolutely from those of their host organisms. The mammalian glutathione system is replaced by an analogously functioning, but molecularly distinct, trypanothione metabolism. The dithiol form of trypanothione serves a similar set of roles as the reduced GSH thiol group and in so doing is converted to trypanothione disulfide (Scheme 1). Just



Scheme 1. Structures of glutathione disulfide (GSSG) and trypanothione disulfide ($T[S]_2$)

as the energetically expensive framework of GSH is recycled by reduction under the catalysis of glutathione reductase, trypanothione disulfide is reduced back to its active form by an analogous enzyme, trypanothione reductase. Parasite TR differs from host GR in not processing GSSG. Conversely, host GR does not reduce $T[S]_2$ (Krauth-Siegel et al., 1987; Shames et al., 1986). Such mutual rejection of cognate substrates between host and parasite augured for the possibility of selective inhibitor design (Krauth-Siegel et al., 1987; Shames et al., 1986) and for TR as a target enzyme in rational drug design, a view underpinned by several subsequent suggestions (Benson et al., 1992; Hunter et al., 1992; Krauth-Siegel and Coombs, 1999; Schirmer et al., 1995). Inhibitors successfully designed by rational drug design approaches against TR have been reviewed (Austin et al., 1998). Efficient selective blockade of TR should compromise the redox defenses of the parasites, increasing their sensitivity to redox-damage based drugs, such as nifurtimox or benznidazole. A TR inhibitor might be a drug in its own right or (even synergistic) when co-administered with a redox-active drug such as nifurtimox (Chan et al., 1998). In spite of several studies to validate TR as a drug target on a biological basis (Dumas et al., 1997; Kelly et al., 1993; Tovar and Fairlamb, 1996) a null TR phenotype has not yet been produced (reviewed (Krauth-Siegel and Coombs, 1999)).

The X-ray structures of human GR (Karplus and Schulz, 1987) and of TRs from *Crithidia fasciculata* (Hunter et al., 1992; Kuriyan et al., 1991) and *Trypanosoma cruzi* have been solved (Lantwin et al., 1994; Zhang et al., 1996). The crystal structures of TR complexed with the alternative substrate glutathionyl spermidine (Bailey et al., 1993) and with the weak, but selective, inhibitor mepacrine, (Jacoby et al., 1996) have been reported. The differing substrate specificities of human GR and TR (Bailey et al., 1993; Hunter et al., 1992; Kuriyan et al., 1991) have been explained on the basis of the structure of the active site of native TR. Many inhibitors have now been reported for TR (reviewed (Austin et al., 1998)), but the first real advance in inhibitor design came from a rational design approach which identified tricyclic neuroleptic frameworks as selective TR lead-inhibitor structures (Benson et al., 1992). The initial design, using a homology model of the TR structure as the X-ray data were unavailable, targetted the hydrophobic wall formed by W21, M113 etc., which provides the major part of the substrate exclusively of TR for trypanothione (Shames et al., 1986). They proved strong, competitive inhibitors of TR (K_i values to low micromolar) with little detectable inhibition on human GR (even up to low millimolar levels) (Benson et al., 1992). Further analysis of the phenothiazine family, probing TR inhibition and activity against parasites in culture, showed considerable scope for substitution at the ring nitrogen position (Chan et al., 1995; Chan et al., 1998) and other ring structures (Garforth et al., 1997). Diphenyl sulfides (Baillet et al., 1996; Girault et al., 1997), the ring-opened variants, are also potent inhibitors of TR. The important advance provided by this family of compounds is that they have considerably lowered neuroleptic activity (Fernandez-Gomez et al., 1995).

For both the phenothiazine (Chan et al., 1998) and imipramine families (Garforth et al., 1997) kinetic evidence shows that the tricyclic framework's detailed binding orientation is highly influenced by its particular substitution pattern. There are clearly several distinct energetically equivalent binding orientations: the TR binding cavity is still far from optimally filled. To try address this from an angle different to the tricyclics we considered peptide structures. Peptides present enormous problems as potential drugs (e.g. metabolism, immune recognition, charge, oral bioavailability), but have the advantage of straightforward synthesis, especially amenable to combinatorial refinement, and they thus provide rapid lead information. Using our initial homology model of TR (Benson et al., 1992) we docked a number of possible peptide ligands with the strategy of using both glutamate side-chains in the TR active site to give spatial-directional components to the inhibitor binding as well as incorporating putative hydrophobic regions, such as the Z-site (Chan et al., 1998) and the hydrophobic wall formed by W21, M113. Thus, benzoyl-Leu-Arg-Arg-β-naphthylamide was predicted to bind specifically to TR, but not host GR, and indeed was found to be a linear competitive inhibitor of GR with $K_i = 13.8\mu M$ (Garforth et al., 1994). We now report results with other peptides, which do not use the peptide structure of the trypanothione substrate, but which nevertheless provide strong, inhibitors of TR, selective over GR.

Materials and methods

Trypanothione reductase from *T. cruzi* was isolated by means of overexpression of the gene in *E. coli* JM109 cells bearing the expression vection PBSTNAV (Meinnel et al., 1988) as previously described (Benson et al., 1992) was homogenous by the criterion of SDS PAGE and had a specific activity identical to wild-type TR (Krauth-Siegel et al., 1987). Enzyme activity was assayed at 25°C in 0.02 M HEPES buffer, pH 7.25, containing 0.15 M KCl, 1 mM EDTA, 0.12 mM TSST and 0.1 mM NADPH (Krauth-Siegel et al., 1987) at an enzyme concentration of approximately 0.3 μg.ml^{-1}. Recombinant human GR was isolated from plasmid-carrying *E. coli* SG5 cells as described (Nordhoff et al., 1993) and assayed following literature conditions (Worthington and Rosemeyer, 1974). The GR isolated had a K_m value for GSSG of 6.79×10^{-5} M and $k_{cat} = 1.50 \times 10^4 \text{min}^{-1}$ (cf. literature values of 6.5×10^{-5} M and $k_{cat} = 9.5 \times 10^3 \text{min}^{-1}$ for recombinant human GR (Nordhoff et al., 1993)). Sodium dodecylsulfate polyacrylamide gel electrophoresis showed the enzyme to be homogeneous with a subunit molecular weight of 51,800 daltons.

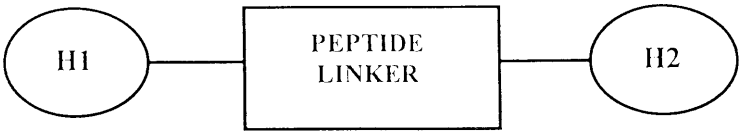
Initial inhibitor design used a molecular graphics homology model of *T. congolense* TR (McKie, 1993) constructed by modification of the published coordinates of human erythrocyte GR (Karplus and Schulz, 1987). Towards the end of the project, the published *C. fasciculata* (Bailey et al., 1993; Hunter et al., 1992; Kuriyan et al., 1991) and *T. cruzi* (Zhang et al., 1996) X-ray diffraction coordinates replaced the modelled coordinates. Potential inhibitors were designed to bind via specific interactions, hydrogen-bond charge-charge and hydrophobic. Structures were constructed using the CHEMNOTE function of QUANTA: energy minimisation was performed using CHARMM.

Inhibition type was assessed by analysing the patterns of three diagnostic classes of plot: $1/v_o$ *versus* $1/[S_o]$ for various [I]; $1/v_o$ *versus* [I] for various $[S_o]$; and $[S_o]/v_o$ *versus* [I] at various $[S_o]$. Values of K_i for competitive inhibition were determined by direct weighted ($1/v_o^2$ weighting) least squares nonlinear regression analysis of the raw data using the equation for linear competitive inhibition ($v = V_{max}[S_o]/([S_o] + K_m(1 + [1]/K_i))$). Values of V_{max} and K_m were obtained by least squares nonlinear regression analysis using the Grafit programme (distributed by Sigma Chemical Co.). Values of I_{50}, the concentration required to give 50% inhibition under the assay conditions described above, were determined by interpolation from a plot of assay velocity *versus* inhibitor concentration using nonlinear regression to fit to the equation for linear competitive inhibition suitably rearranged.

Gel filtration (PD-10) was used to investigate the reversibility of inhibition. All columns were pre-treated by loading an aliquot of BSA (1 ml of 1 mg/ml) to eliminate non-specific adsorption and washed using assay buffer (0.02 M Hepes, pH 7.25, containing 0.15 M KCl and 1 mM EDTA) before use. Samples (control TR and TR mixed with peptide **21** at 5 times its I_{50}) applied as aliquots (100 μl) to the PD-10 column and eluted with assay buffer. Enzymic activities and absorbancies at 280 nm of eluates were determined.

Results and discussion

A range of peptides found to inhibit trypanothione reductase is collected along with their I_{50} values in Table 1. For **18** and **13** a full kinetic analysis showed that the inhibition type was linear competitive based on the array of diagnostic plots. Plots for inhibition of TR by Z-Ala-Arg-Arg-4-methoxy-β-naphthylamide (**18**) are shown in Fig. 1A–C. Inhibition by **18** was shown to be 100 ± 0.5% reversible by gel filtration. The simplest explanation of such competitive inhibition is direct binding to the active site. The data of Table 1 can be discussed by dividing the molecule into three parts: two hydrophobes



Scheme 2. Structural arrangement of peptide inhibitors of trypanothione reductase

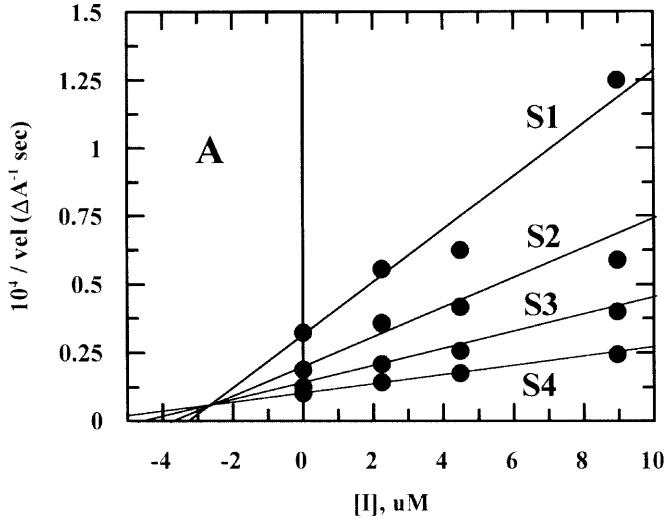
Table 1. Inhibition by peptides of recombinant trypanothione reductase from *T. cruzi* and human erythrocyte glutathione reductase. TR ($\sim$0.3 μg.ml^{-1}) was assayed at 25°C in 0.02 M HEPES buffer, pH 7.25, containing 0.15 M KCl, 1 mM EDTA, 0.12 mM TSST and 0.1 mM NADPH (Krauth-Siegel et al., 1987): assay conditions for recombinant human GR were as described (Nordhoff et al., 1993)

No.	Compound	I_{50} (K_i), μM
1	Bz-Arg-p-nitroanilide[a]	547 ± 113
2	Z-Arg-p-nitroanilide[a]	394 ± 52
3	H-Arg-β-naphthylamide[a]	1,520 ± 220
4	H-Trp-β-naphthylamide[e]	2,670 ± 560
5	Z-Lys-4-methoxy-β-naphthylamide[b]	816 ± 68
6	H-Trp-OH[d]	2,671 ± 556
7	Z-Arg-Arg-4-methoxy-β-naphthylamide[b]	38.5 ± 7.8
8	Z-Arg-Arg-p-nitroanilide[b]	59.9 ± 8.0
9	H-His-Trp-His-OH[f]	1,742 ± 61
10	H-His-Trp-Lys-OH[d]	3,911 ± 1,142
11	H-Phe-Arg-Trp-OH[d]	396 ± 70
12	H-Phe-Pro-Arg-4-methoxy-β-naphthylamide[a]	544 ± 32
13	Bz-Leu-Arg-Arg-β-naphthylamide[b]	11.8 ± 1.8 (13.8 μM)
14	Boc-Leu-Arg-Arg-7-amido-methylcoumarin[c]	356 ± 97
15	Boc-Leu-Lys-Arg-7-amido-methylcoumarin[a]	418 ± 37
16	Bz-Gly-Arg-Arg-Leu-β-naphthylamide[a]	510 ± 51
17	Z-Gly-Gly-Arg-7-amido-methylcoumarin[d]	296 ± 74
18	Z-Ala-Arg-Arg-4-methoxy-β-naphthylamide[d]	10.4 ± 0.6 (2.4 μM)
19	Z-Lys-Phe-Arg-p-nitroanilide[a]	242 ± 50
20	Bz-Phe-Val-Arg-7-amido-4-methylcoumarin[a]	247 ± 60
21	H-Phe-Met-Arg-Phe-NH_2[a]	2,740 ± 160

[a] No inhibition of human GR at 0.1 mM; [b] No inhibition of human GR at 0.5 mM; [c] No inhibition of human GR at 0.3 mM; [d] No inhibition of human GR at 1.0 mM; [e] I_{50} against human GR 1.4 mM; [f] I_{50} against human GR 2.4 mM.

(H1 and H2) joined by a peptide linker (Scheme 2). In the initial assessment of structures for potential inhibitors the hydrophobe H1 was modelled into the "Z-site" of TR (approximately near F396′) (Chan et al., 1998; El-Waer et al., 1991) and hydrophobe H2 into the hydrophobic cleft region near W21, M113 etc.

Comparison of 7 with **8** shows the 4-methoxy-β-naphthylamide and the p-nitroanilide to be of approximately equal potency with respect to I_{50} values, in spite of the 4-methoxy-β-naphthylamide being larger than the p-nitroanilide and predicted thus as a better complement to the hydrophobic cleft. 7-Amido-4-methylcoumarin possesses a lipophilic methyl group and a carbonyl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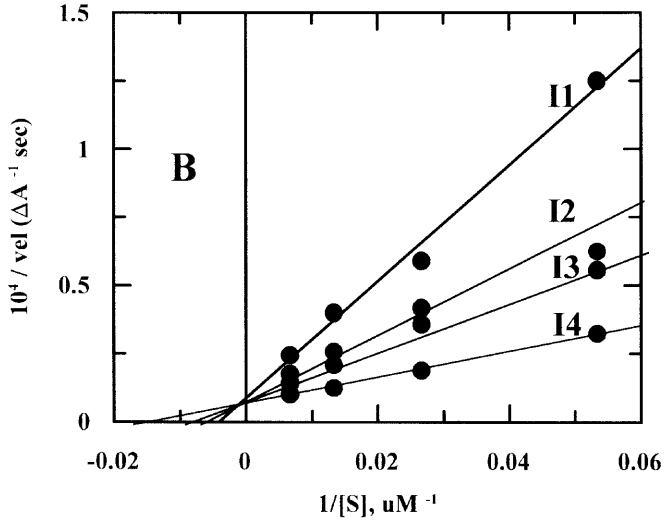
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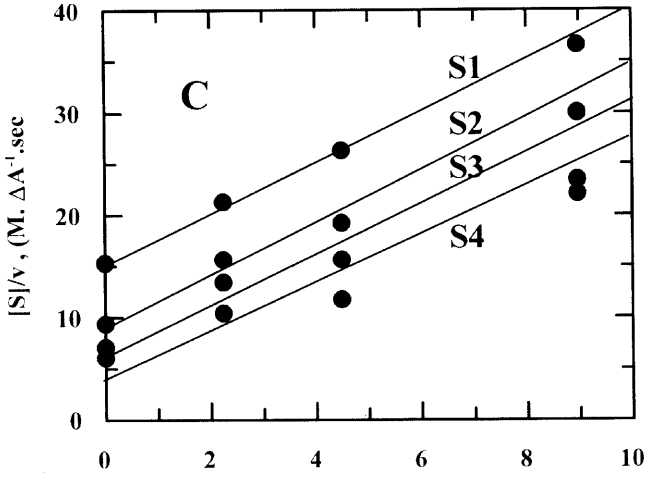
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function capable of hydrogen bonding, but these substituents do not seem to confer any marked advantages over unsubstituted ring systems.

A single arginine residue as the peptide linker (**1** and **2**) yields I_{50} values of 400–550μM. Two arginine residues (**7**, **8**) improves the I_{50} value by almost an order of magnitude relative to the single arginine (**1**, **2**). Introduction of a spacer residue (**18** and **13**) such as alanine or leucine improves binding even further to give Ki values around the 2.4–13.8μM. There is a 5-fold difference in K_i values for these two compounds. As there is no detectable inhibition of human GR by **18** at 1 mM, a reasonable estimate for the minimal value of K_i (should there be any weak, barely detectable inhibition of GR) would be >5 mM. This means that for **18** the selectivity for TR over GR is >2,500. There is as yet no X-ray derived structural data for a TR inhibitor except for the study of mepacrine (Jacoby et al., 1996). However in molecular modelling studies, the inhibitor benzoyl-Leu-Arg-Arg-β-naphthylamide has been docked into the TR active site (Garforth et al., 1994) such that the arginine residues are directed towards the complementary Glu466′ and Glu467′ site and Asp105, and the leucine residue is modelled towards the hydrophobic pocket formed by Val58 and Leu399′.

One of the difficulties with the large, and panoramically hydrophobic, tricyclics as TR inhibitors is the non-directional nature of the hydrophobic interactions which have to be included in the design process. This makes it difficult to predict unique binding orientations with any degree of certainty. Balanced against this the desirability of having a substantial hydrophobic component in a drug lead structure. By considering the peptide structures of the present study it is clear that the use of arginyl residues in a peptide inhibitor framework serves as an adequate starting basis for improved ligand design as low μM K_i values can be achieved, along with specificity for TR over GR.

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Fig. 1. Inhibition kinetic plots for *T. cruzi* trypanothione reductase inhibited by Z-Ala-Arg-Arg-4-methoxy-β-naphthylamide (**18**) assayed at 25°C in 0.02 M HEPES buffer, pH 7.25, containing 0.15 M KCl, 1 mM EDTA, 0.12 mM TSST and 0.1 mM NADPH. **A.** Dixon plot of 1/v versus [I] for the inhibition of *T. cruzi* trypanothione reductase by Z-Ala-Arg-Arg-4-methoxy-β-naphthylamide with trypanothione disulfide as variable substrate at the following concentrations: S1, 150μM; S2, 75μM; S3, 37.5μM; S4, 18.75μM. **B.** Lineweaver-Burk plot of 1/v versus 1/[S] for the inhibition of *T. cruzi* trypanothione reductase by Z-Ala-Arg-Arg-4-methoxy-β-naphthylamide with trypanothione disulfide as variable substrate at the following inhibitor concentrations: I1, 8.96μM; I2, 4.48μM; I3, 2.24μM; I4, 0μM. **C.** Cornish-Bowden plot of [S]/v versus [I] for the inhibition of *T. cruzi* trypanothione reductase by Z-Ala-Arg-Arg-4-methoxy-β-naphthylamide with trypanothione disulfide as variable substrate at the following concentrations: S1, 150μM; S2, 75μM; S3, 37.5μM; S4, 18.75μM

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