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Rational design of peptide-based inhibitors of trypanothione reductase as potential antitrypanosomal drugs

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Summary. The rational design of ligands for the substrate-binding site of a homology-modelled trypanothione reductase (TR) was performed. Peptides were designed to be selective for TR over human glutathione reductase (GR). The design process capitalized on the proposed differences between the active-sites of TR and human GR, subsequently confirmed by the TR crystal structure. Enzyme kinetics confirmed that for *T. cruzi* TR benzoyl-Leu-Arg-Arg-ß-naphthylamide was an inhibitor (K_i 13.8 μM) linearly competitive with the native substrate, trypanothione disulphide, and did not inhibit glutathione reductase.

Keywords: Amino acids – Trypanothione reductase – Glutathione reductase – Trypanosomiasis – Leishmaniasis – Homology model – Drug design

Introduction

Parasitic protozoa are the aetological agents of a number of major diseases including malaria, leishmaniasis, toxoplasmosis and trypanosomiasis, whose chemotherapy needs considerable further development. One target for rational antileishmanial and antitrypanosomal drug design is trypanothione reductase (TR) (Walsh, et al., 1991), a disulphide-oxidoreductase found in both trypanosomes and leishmanias but not in their mammalian hosts. TR reduces the disulphide of trypanothione (**1**). A comparison of amino-acid sequences of glutathione reductase (GR) (the equivalent host enzyme which reduces glutathione disulphide (**2**)) and TR indicates that the enzymes are quite similar and may have arisen from a common ancestral gene (Sullivan et al., 1989).

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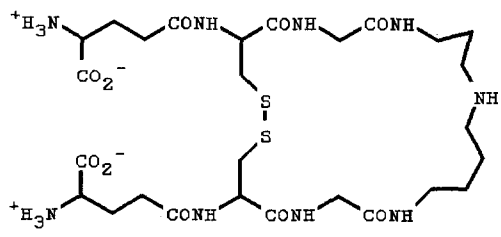

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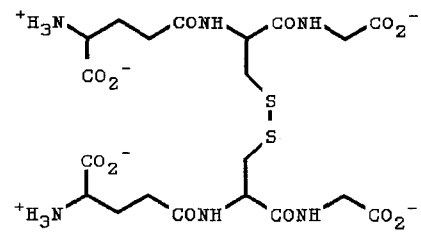

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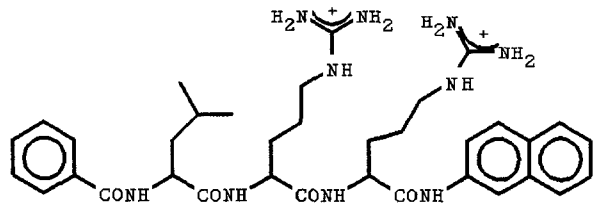

As an approach to designing inhibitors of TR, which would not block the action of mammalian GR, we undertook computer-graphically directed ligand design using the three dimensional structures of TR and GR comparatively. With the close sequence homology ($\approx$40% similarity) of TR and human erythrocyte GR, for which a high resolution X-ray structure was available (Karplus and Schulz, 1987), we were able to build a homology model of TR and use this as a basis for selective inhibitor design, discovering that tricyclic neuroleptics were such selective TR ligands by this means (Benson et al., 1992). This approach was later put on a firmer basis with the availability of X-ray diffraction data for TR, (Hunter et al., 1992). In this study we present the design and study of a peptide, unrelated in structure to trypanothione or glutathione, which we predicted would be a selective inhibitor of TR over GR. This peptide is the lead structure of a new class of TR-selective ligands.

Materials and methods

Inhibitor design

Our initial homology model of *T. congolense* TR was based on the high resolution crystal structure of human erythrocyte GR (Karplus and Schulz, 1987). The subsequent availability to us of a TR crystal structure, (Hunter et al., 1992); Kuriyan et al., 1991), allowed us to

compare the model and check the fit of putative ligands in the active-site of the crystal structure.

The hydrophobic residues in TR (L17, W21, Y110, M113 and F114) define a hydrophobic cleft which distinguishes the substrate-binding site of TR from the corresponding positively charged environment in GR. Ligands for TR were designed to incorporate a complementary hydrophobic surface to this region to promote binding to TR and ablate possible binding to GR. A second hydrophobic site, which we had previously defined as the 'Z-site', (El-Waer et al., 1991), incorporates the side-chains of F396', P398', and L399', in the vicinity of the gamma-glutamate binding-site of the native substrates. The glutamate side-chains (E466', E467') which bind the gamma-glutamate amino groups of trypanothione to TR also provide appropriate target groups for ligand binding. To simultaneously occlude the hydrophobic Z-site and the hydrophobic-cleft (L17, W21, etc.) imposes severe constraints on ligand design, a minimum of one intermediary residue being necessary to link hydrophobic side-chains of designed ligand peptides at the two sites.

Enzymology

The gene for trypanothione reductase of *T. cruzi* was isolated, amplified by PCR, and TR protein expressed and purified as described (Benson et al., 1992). Enzyme activity was measured spectrophotometrically at 340nm, at 25 °C in 0.02M Hepes buffer, pH 7.25, containing 0.15M KCl, 1mM EDTA and 0.1mM NADPH with an enzyme concentration of 0.3 $\mu g \mu l^{-1}$. Human GR was purified from blood as described (Krohne-Ehrich et al., 1977), with an additional gel filtration column chromatography step using Superose-12 (purchased from Pharmacia). The enzyme was assayed (Worthington and Rosemeyer, 1974) using 1mM GSSG and 0.1mM NADPH in 0.1M potassium phosphate buffer containing 0.2M KCl and 1mM EDTA at pH 7.0 at 25 °C. Diagnosis of inhibition type required enzyme kinetic data to be plotted in three ways: 1/V against $1/[S_0]$ for various [I]; $[S_0]/V$ against [I] at various $[S_0]$; and 1/V against [I] for various $[S_0]$. The K_i values were estimated by weighted least squares non-linear regression analysis using the Dixon Plot (1/V vs [I]). Benzoyl-Leu-Arg-Arg-ß-Naphthylamide was purchased from Nova-Biochem.

Results and discussion

By means of molecular graphics modelling, Benzoyl-Leu-Arg-Arg-ß-Naphthylamide (**3**) was predicted to be an inhibitor of TR but not of human GR. In agreement with this it was found not to inhibit human GR up to 0.5mM. However, it did inhibit TR from *T. cruzi*. Diagnostic plots to determine inhibition type against *T. cruzi* TR for Benzoyl-Leu-Arg-Arg-ß-Naphthylamide indicated that the peptide is a linear competitive inhibitor of TR with trypanothione as variable substrate. This result is in keeping with the prediction. The K_i value was determined to be 13.8 + 1.5μM (see Fig. 1) at pH 7.25, 25 °C. In Fig. 2, the docked structure of the inhibitor in the active-site is shown with some of the postulated interactions.

The structure of this novel peptide inhibitor of TR is interesting in that it is not similar to the native (peptidic) substrate of TR, trypanothione. Further it can distinguish GR and TR. Although unlikely to serve as a drug (because of its high charge, likely metabolic cleavage *in vivo*, etc.) such a lead peptide inhibitor will be useful in mapping out those residues of TR important for ligand-binding and hence assist in rational drug design using TR as target. It also introduces a second class of TR-specific (over GR) enzyme inhibitor, in addition to the tricyclic neuroleptics which we recently described (Benson et al., 1992). Binding

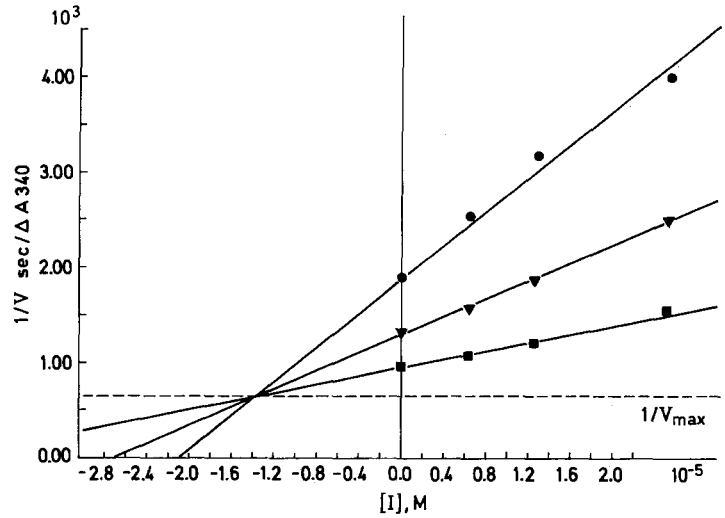

Fig. 1. Dixon Plot of 1/V (initial velocity) *versus* inhibitor concentration for the inhibition of *T. cruzi* trypanothione reductase at pH 7.25, 25 °C in 0.02M Hepes buffer containing 0.15M KCl, 1mM EDTA and 0.1mM NADPH. Points are experimental; lines are theoretical for linear competitive inhibition with $K_i = 13.8\ \mu M$, $V_{max} = 1.56 \times 10^{-3} \Delta A/sec$. The trypanothione concentrations used were 114 μM (–■–), 57μM (–▼–) and 28.5 μM (–●–)

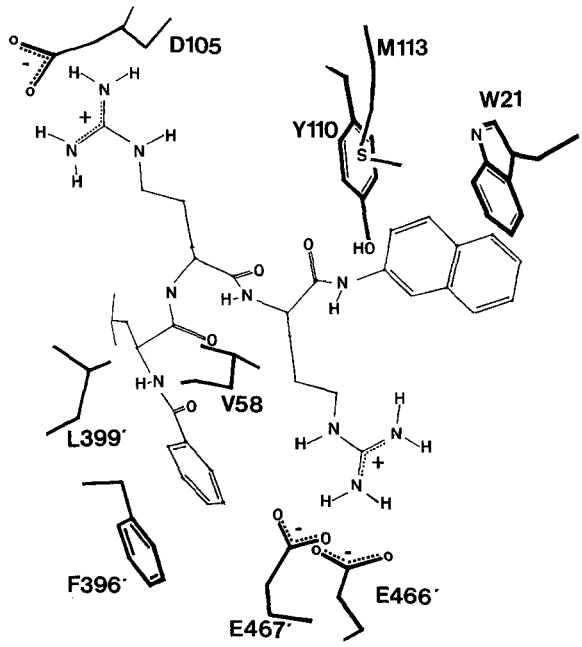

Fig. 2. Docked structure of Benzoyl-Leu-Arg-Arg-ß-Naphthylamide in the active-site of *T. cruzi* trypanothione reductase. The ß-Naphthylamide region contacts the hydrophobic wall formed by W21, M113, etc., whilst the N-terminal arginine of the ligand is directed towards D105 and the next arginine makes a bifurcated salt-link to the side-chains of Glu-466' and Glu-467'. The leucine residue of the ligand is docked against a hydrophobic surface formed by L399, P462 and V58, whilst the N-terminal blocking benzoyl group stacks against Phe-396' of the enzyme. The ligand is shown feint and key side-chains of TR are shown in bold; only polar hydrogens are represented

of this inhibitor is reasonably tight (at $K_i \approx 13\mu M$) for the first-generation ligand in this family and is comparable to the binding strengths achieved for the tricyclic inhibitors ($K_i \approx 6\mu M$). Whilst K_m is not necessarily a measure of binding, except in some exceptional circumstances, it is worth noting that the K_m for trypanothione with TR from *T. cruzi* is $55\mu M$ (El-Waer et al., 1991). Most important, however, is the lack of detectable inhibition of GR by Benzoyl-Leu-Arg-Arg-ß-Naphthylamide even at 0.5mM. Assuming conservatively that an inhibition of 5% had been detectable at this concentration, the I_{50} value (a rough guide to K_i for the presently used assay conditions) would be predicted to be ≥ 5mM. This makes the minimum selectivity for TR over GR (K_i(GR) by extrapolation K_i(TR)) ≥ 385 (5/0.013). Further studies to improve binding strength for TR, whilst retaining selectivity, are in progress.

Acknowledgements

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