Fluphenazine photoaffinity labelling of binding sites for phenothiazine inhibitors of trypanothione reductase

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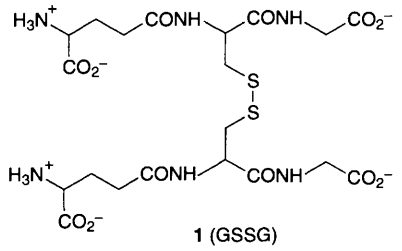
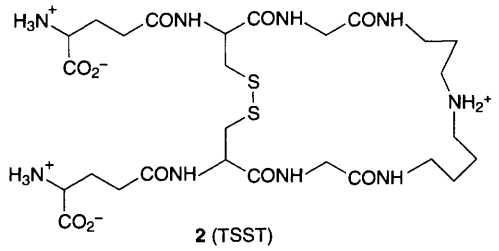
Photolysis of fluphenazine, a competitive inhibitor of trypanothione reductase in the presence of trypanothione reductase leads to irreversible, time-dependent inactivation, which is not dependent on the presence of molecular oxygen in the medium and can be protected against by the presence of trypanothione substrate; MALDI and electrospray mass spectrometric analyses shows that 2–5 equiv. of the phenothiazine are incorporated per enzyme subunit.

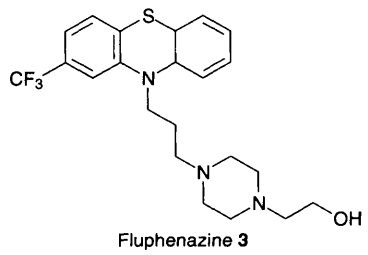
Trypanosomal and leishmanial parasites, in contrast to their mammalian hosts, do not use a glutathione-based redox defence system, but rather one involving trypanothione.[1] Thus, in trypanosomes the role equivalent to that of glutathione reductase (GR) in mammals (reducing glutathione disulfide **1** back to its reduced form) is played by trypanothione reductase (TR, which reduces trypanothione disulfide, TSST, **2**) TR and GR have mutually exclusive substrate specificities, neither processing the other's substrate.[2,3] This selective recognition of parasite and host redox-disulfide structures has led to the rational design of TR-specific inhibitors,[4] which are potential drug leads against African sleeping sickness and other diseases caused by these parasite groups. Selective TR inhibitors have been designed based on peptide backbones unrelated to trypanothione,[5] and on heterocyclic frameworks.[6] The tricyclic inhibitors of TR were designed[6] on the basis that the major difference in the active-sites of TR and GR is the hydrophobic pocket in TR containing W21, M113 and A343.[7–9] However, there is only circumstantial evidence that they indeed bind in this region, although a recent report has appeared of X-ray crystallographic data for TR with quinacrine bound.[10] To obtain complementary insight into the binding details of this set of inhibitors we have used fluphenazine **3**, which forms reactive radical-ions under the influence of light,[11] to photolabel recombinant TR from *T. cruzi*, giving a new structural probe of the binding site(s) for tricyclics.



1 (GSSG)



2 (TSST)



Fluphenazine **3**

Recombinant TR from *T. cruzi*, over-expressed in *E. coli*,[6] was assayed at 25 °C in 0.02 mol dm^{-3} Hepes buffer pH 7.25, containing 0.15 mol dm^{-3} KCl, 1 mmol dm^{-3} EDTA, 0.12 mmol dm^{-3} trypanothione and 0.1 mmol dm^{-3} NADPH. Photoirradiation was carried out (Applied Photophysics Model 2007 Quantum Yield Photoreactor, filtered, high-pressure 250 W mercury lamp) with light of wavelengths greater than 300 nm.[12] A fresh solution of **3** was incubated in assay buffer containing TR with the light shutter closed, the TR activity of an aliquot determined, irradiation initiated and at timed intervals aliquots removed for assay. Control samples of TR in the absence of **3** were also studied. Test and control samples were studied in the presence and absence of oxygen by sparging with N_2 before initiating reaction. In studies of protection by TSST, TR (4.1 nmol dm^{-3}) was incubated at 25 °C for 5 min in degassed assay buffer with TSST (0.12 mmol dm^{-3}) and fluphenazine (1.6 μmol dm^{-3}, 0.2 I_{50}) before photoactivation. Aliquots were removed to assay buffer and assay initiated by adding NADPH. A control was carried out in the absence of TSST.

To prepare TR modified by **3** for mass spectrometric analysis, freshly prepared fluphenazine solution (50 μl of 6.4 × 10^{-2} mol dm^{-3} stock protected by Al foil) was mixed with TR (20 μl of 4.1 × 10^{-5} mol dm^{-3} stock TR plus 930 μl of assay buffer, freshly prepared and degassed by N_2 bubbling for 20 min). The mixture was further sparged with N_2 for 5 min prior to photolysis and TR activity of an aliquot measured. After 30 s irradiation, an aliquot (5 μl) was removed to check complete TR inactivation. Control TR was treated identically but in the absence of **3**. Unbound **3** was separated from TR by gel filtration. HPLC of the protein-containing fraction thus obtained was performed using a Vydac 2.1 × 150 mm C_8 column (300 Å pore size) (Vydac, Hesperia, CA, USA) eluted at 0.5 ml min^{-1} with binary gradient elution from 80% mobile phase A to 20% A over 60 min; solvent A was 0.1% trifluoroacetic acid (TFA) in water and solvent B, 0.075% TFA in acetonitrile with UV absorbance detection at 280 nm. Electrospray ionization (ESI) mass spectrometry was performed on a VG Quattro triple quadrupole instrument, upgraded to Quattro II

specifications, and equipped with an electrospray (ESI) source (VG Organic, Manchester, UK). The resolution was set to give a peak width of 0.5 Da at half-height for a singly charged ion, and calibrated using equine myoglobin. Molecular masses were determined from maximum entropy processing (MaxEnt, VG Organic) which yields resolution-enhanced spectra showing signal intensity as a function of mass, rather than mass-to-charge. Matrix-assisted laser desorption ionisation (MALDI) mass spectrometry was performed on a VG TofSpec time-of-flight mass spectrometer operated in linear mode and equipped with a nitrogen laser, with a matrix of saturated sinapinic acid in water–acetonitrile (1 : 1 with 0.1% TFA).

Fluphenazine inhibited TR from *T. cruzi* in the dark, linearly competitive with TSST with K_i 1.91 ± 0.22 × 10^{-5} mol dm^{-3}. Reisolation of TR incubated in the dark with **3** by PD-10 gel filtration gave 99.0 ± 0.8% activity, relative to control TR. At 6 × 10^{-8} mol dm^{-3} TR there was no loss of activity over 120 s exposure at 25 °C to light $\lambda \geq 300$ nm. Photoexposure of TR in the presence of **3** led to time-dependent loss of TR activity, but with no detectable difference in inactivation rates in the presence and absence of oxygen. Irreversibility of TR inhibition after photo-exposure in the presence of **3** was confirmed by gel filtration and dialysis. The time-dependence of the photo-induced inactivation of TR by **3** obeyed first-order kinetics with k_{inact} (the pseudo first order rate constant for inactivation of TR) = 3.26 (±0.14) × 10^{-2} s^{-1} and a final loss of 94.6 (±1.6)% of the initial activity. In the presence of 1.2 mmol dm^{-3} TSST, the values of k_{inact} were 1.53 (±0.33) × 10^{-2} s^{-1} and 1.83 (±0.34) × 10^{-2} s^{-1} (duplicate runs), but complete inhibition was no longer achievable under these conditions, 31.1 ± 1.9% activity remaining.

The mass-to-charge spectra obtained (ESI MS) of TR irradiated in the absence of **3** and of TR incubated with **3**, but not irradiated, were essentially identical to that of native TR, with determined molecular masses within 48 Da of that for native TR, indicating no significant covalent modification of TR in the absence of either irradiation or **3**. Initial mass spectrometric analysis of fluphenazine-modified TR by MALDI-TOF MS showed an $(M + H)^+$ ion at approximately *m/z* 55 236, with a lower relative abundance $(M + 2H)^{2+}$ ion at *m/z* 27 702, substantiating the estimate of molecular mass of the modified protein. ESI MS was then used to assess the heterogeneity of the modified protein and estimate molecular masses precisely. The resulting mass-to-charge spectrum was substantially more complex than that from native TR due to the presence of several components whose distributions of multiply protonated states overlapped. MaxEnt processing (with a specified molecular mass range of 53 400–56 500) revealed at least 4 components (apparent molecular masses 54 705.9, 55 106.4, 55 501.0 and 55 893.9). The average difference between these masses is 396.0 ± 3.3 Da. This value, 41.3 Da less than the molecular mass of **3**, suggests more complex chemistry than simple addition in the interaction between **3** and TR on irradiation. Based on a mass increase of approximately 396 Da per fluphenazine-derived modification to TR, the components detected correspond to fluphenazine/TR subunit molar ratios of 2–5.

The simplest model consistent with reversible, linear competitive inhibition of TR by **3** in the dark is binding in the region of the active site as originally proposed.[6] Protection by TSST shows that some of the radicals formed react specifically at the fluphenazine-binding site which is the origin of the dark inhibition of TR, most likely the active-site. The time-dependence of the light-induced inhibition indicates chemical modification of TR, but photosensitised oxidation of Trp21 in the active site is unlikely to cause inactivation from the lack of an O_2 requirement. It has been reported recently that TR and GR are inactivated by photosensitised oxidation by porphyrins and phthalocyanines.[13] Strong evidence of covalent modification of TR by **3** under photoactivation comes from the mass spectrometric observation that 2–5 equiv. of **3** become covalently attached to TR on photoactivation, indicating either that there are additional *exo*-sites for tricyclics and/or that more than one tricyclic nucleus can attach to the active-site at the same time. Phenothiazine-derived radical(s) provide the most likely inactivating species, the lack of an oxygen requirement arguing against hydroxyl radical or singlet oxygen attack on TR, as does the molecular mass increase determined by mass spectrometry. Phenothiazine drugs stack in an aqueous medium[14] and their photoproducts include dimers and higher oligomers.[15] Longer-lived (than radicals) photoproducts of phenothiazines have been reported to inhibit dehydrogenases in addition to the photogenerated free radicals.[16] Thus, it is possible that photoproduced oligomers of **3** form more rapidly than they react with TR and move as a unit to covalently label TR. Further studies are required to elucidate the details of the modification.

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