Spectrophotometric monitoring of the photoreactivity of fluphenazine and its use as a photoaffinity label for trypanothione reductase

Application Note

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Introduction

Trypanosomal and leishmanial parasites, in contrast to their mammalian hosts, do not use a glutathione-based redox defence system, but rather one involving trypanothione. Thus, in trypanosomes the role equivalent to that of glutathione reductase (GR) in mammals (reducing glutathione disulphide, Figure 1, back to its reduced form) is played by trypanothione reductase (TR, which reduces trypanothione disulphide, Figure 2). TR and GR have mutually exclusive substrate specificities, neither processing the other's substrate[1]. This selective recognition of parasite and host redox-disulphide structures has led to the rational design of TR-specific inhibitors as potential drug leads against African sleeping sickness and other diseases caused by these parasite groups. Thus, the phenothiazine inhibitors of TR[2] were designed on the basis that the major difference in the active-sites of TR and GR is the hydrophobic pocket in TR containing Trp-21, Met-113 and Ala-343[3]. To obtain insight into the precise binding details of this set of inhibitors we have made use of the known photochemistry of fluphenazine (Figure 3), which forms reactive radical-ions under the influence of light[4] to photolabel recombinant TR from *T. cruzi*, giving a new structural probe of the binding site(s) for tricyclics. Substituted phenothiazines have been used to photolabel viruses[5], nucleic acids[6] and membrane proteins[7] and the dopamine receptor has been specifically tagged[8].

The Cary 1E Spectrophotometer was used to follow the photolysis in various media of fluphenazine and to measure the change in UV-visible spectrum of trypanothione reductase after photoinhibition in the presence of fluphenazine.

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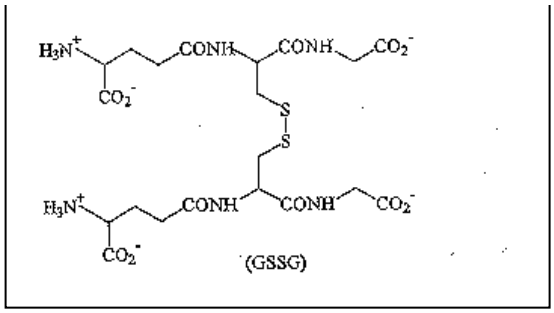

Figure 1. Structure of Glutathione Disulphide (GSSG)

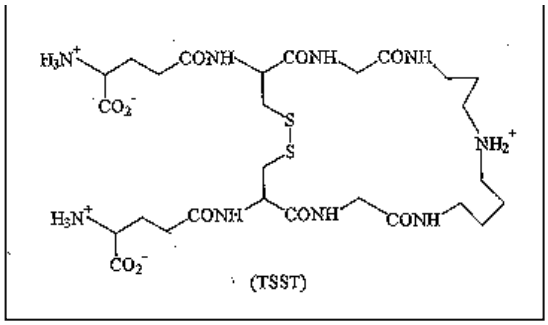

Figure 2. Structure of Trypanothione Disulphide (TSST)

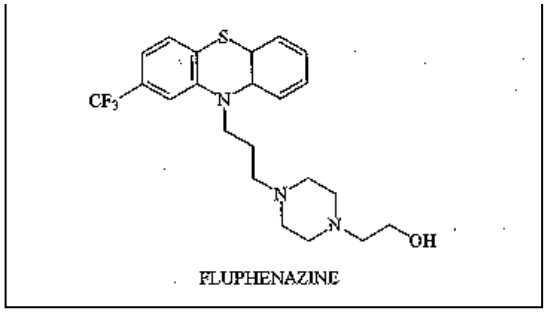

Figure 3. Structure of Fluphenazine

Experimental

Equipment

- Cary1E spectrophotometer equipped with a Cary temperature controller
- HP Deskjet-510 printer.
- Quartz cuvettes for readings in the UV range; otherwise disposable plastic cuvettes
- Applied Photophysics Model 2007 Quantum Yield Photoreactor

Reagents

- Freshly prepared fluphenazine solutions (0.03 mM indimethyl sulphoxide and 0.02 mM in Hepes buffer)
- Dry chloroform
- $SnCl_2$ (0.13 M in 36% HCl, w/v)

Method

To determine optimal absorption wavelengths and photostabilities under the conditions available, the following experiments were carried out. Freshly prepared fluphenazine solutions (0.03 mM in dimethyl sulphoxide and 0.02 mM in Hepes buffer) were irradiated at 20 °C by a source of incident light of wavelength range above 300 nm. The solution was removed at fixed times and its UV spectrum recorded until no further spectral changes occurred.

Photoirradiation was carried out by means of an Applied Photophysics Model 2007 Quantum Yield Photoreactor using a filtered, high pressure 250 W mercury lamp. To isolate light of wavelengths greater than 300 nm, the quartz filter compartments contained $SnCl_2$ (0.13 M in 36% HCl, w/v) pre-irradiated for 1 hour. Prior to irradiation all surfaces were cleaned with dry chloroform. Samples were irradiated at 15 cm from the light source in quartz cuvettes in a cuvette holder mounted on an optical bench and which was immersed in a medium of appropriate temperature in a Dewar flask with a quartz window (the aperture to which was regulated by a shutter).

Extreme care must be used with such lamps because of the high UV emission. UV-proof glasses should be worn and warning signs displayed to exclude people from the laboratory area at times when the lamp is exposed.

Results

Photolysis of fluphenazine

UV spectra of fluphenazine in DMSO and in buffer after irradiation under the above conditions for the times indicated are shown in Figures 4 and 5.

The UV spectrum of this compound changes as the irradiation time increased, showing the drug's photosensitivity and the formation of photolysis products. Plots of the absorbances at 258 and 271 nm taken from the data in Figure 4 are shown in Figure 6. The data are fitted to the equation for first order kinetics and the rate constants are 3.60 (±0.20) x 10^{-3} s^{-1} at 258 nm and 2.50 (±0.20) x 10^{-3} s^{-1} at 271 nm. This difference in rate constants measured for the same data but at different wavelengths indicates that the photolytic process being monitored spectrophotometrically is complex. Close inspection of the isosbestic regions of Figure 4 shows that the isosbestic points alter with time, in agreement with a multistep process.

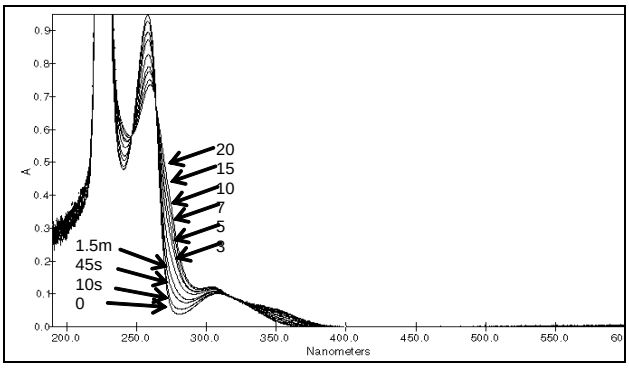

Figure 4. UV-visible spectral changes on irradiation of 3.00 x 10^{-5} M fluphenazine at 25 °C in dimethyl sulphoxide with light of wavelengths greater than 300 nm. Spectra are annotated with irradiation times in minutes

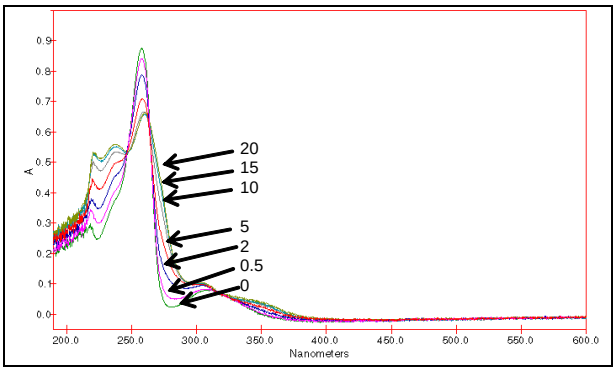

Figure 5. UV-visible spectral changes on irradiation of 2.00 x 10^{-5} M fluphenazine with light of wavelengths greater than 300 nm at 25 °C in 0.02 M Hepes buffer, pH 7.25, containing 0.15 M KCl and 1 mM EDTA. Spectra are annotated with irradiation times in minutes

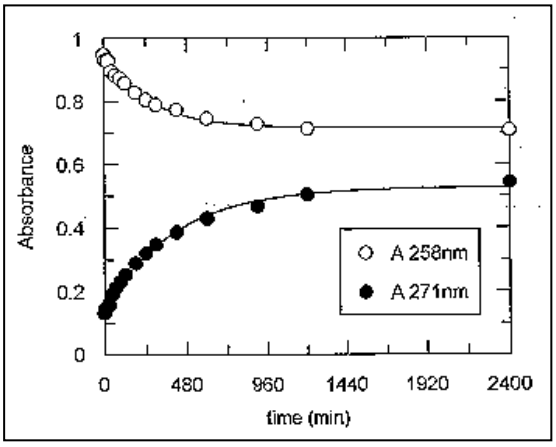

Figure 6. Plots of absorbance against photoexposure time for fluphenazine at 258 nm and 271 nm using the data in the spectral overlays of Figure 4. Points are experimental; lines are theoretical assuming first order kinetics

Spectral analysis of TR modified by fluphenazine-phototreatment

TR was modified by photolyzing fluphenazine (2.5 mM) in its presence and the UV-Visible spectrum of the modified protein was measured after its reisolation and separation from excess fluphenazine and lower molecular weight photo-products by gel filtration over a PD-10 column. The spectra are shown in Figure 7.

To allow for slight differences in absolute concentrations of native and photo-inhibited TR samples as a result of gel-filtration, the Cary software can be used to normalize both spectra to 280 nm, an arbitrary wavelength but one which is dominated by the aromatic amino-acid content of the protein.

It is clear from spectra (a) and (b) that the enzyme has been altered chromophorically by photolyzing fluphenazine, a difference brought out by using the Cary software to produce a difference spectrum (c), by subtracting spectrum (b) from spectrum (a).

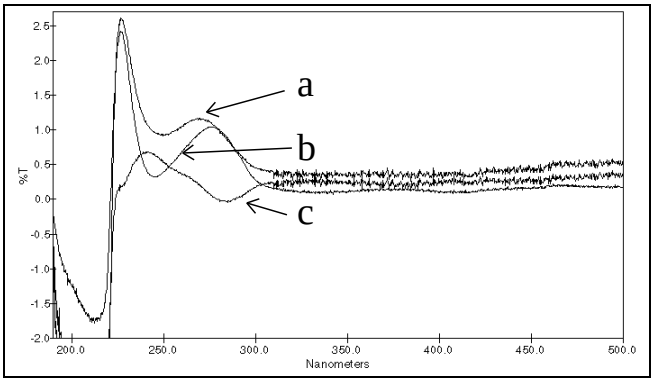

Figure 7. UV-Visible spectra of (a) native recombinant trypanothione reductase and (b) trypanothione reductase photo-inhibited by photolysis in the presence of fluphenazine and reisolated by gel flitration. Both spectra are normalized to A = 1.00 at 280 nm and spectrum (c) is the subtracted spectrum [(a) - (b)]

Discussion

The gel filtration studies and the resulting difference spectrum of Figure 7(c) provide good evidence that photo-inhibition of TR by fluphenazine leads to covalent incorporation of the phenothiazine nucleus into the protein. In more detailed analysis of this photo-inhibition of TR we have shown that fluphenazine is a competitive inhibitor of trypanothione reductase (K_i 19.1 μM) in the dark. We also found that photolysis of fluphenazine in the presence of trypanothione reductase led to irreversible, time-dependent inactivation, which was not dependent on the presence of molecular oxygen in the medium and could be protected against by the presence of trypanothione substrate. MALDI and electrospray mass spectrometric analysis showed that 2 to 5 mole equivalents of the phenothiazine were incorporated per enzyme subunit [9]. This mass spectrometric analysis provides powerful confirmation of the spectrophotometric results.

Acknowledgements

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