Ellman's-reagent-mediated regeneration of trypanothione *in situ*: substrate-economical microplate and time-dependent inhibition assays for trypanothione reductase

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Trypanothione reductase (TryR) is a key enzyme involved in the oxidative stress management of the *Trypanosoma* and *Leishmania* parasites, which helps to maintain an intracellular reducing environment by reduction of the small-molecular-mass disulphide trypanothione ($T[S]_2$) to its di-thiol derivative dihydrotrypanothione ($T[SH]_2$). TryR inhibition studies are currently impaired by the prohibitive costs of the native enzyme substrate $T[S]_2$. Such costs are particularly notable in time-dependent and high-throughput inhibition assays. In the present study we report a protocol that greatly decreases the substrate quantities needed for such assays. This is achieved by coupling the assay with the chemical oxidant 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), which can rapidly re-oxidize the $T[SH]_2$ product back into the disulphide substrate $T[S]_2$, thereby maintaining constant substrate concentrations and avoiding deviations from rate linearity due to substrate depletion. This has enabled the development of a continuous microplate assay for both classical and time-dependent TryR inhibition in which linear reaction rates can be maintained for 60 min or more using minimal substrate concentrations ($< 1\ \mu$M, compared with a substrate K_m value of $30\ \mu$M) that would normally be completely consumed within seconds. In this manner, substrate requirements are decreased by orders of magnitude. The characterization of a novel time-dependent inhibitor, *cis*-3-oxo-8,9b-bis-(N^1-acrylamidospermidyl)-1,2,3,4,4a,9b-hexahydrobenzofuran (PK43), is also described using these procedures.

Key words: disulphide recycling, 5,5′-dithio-bis-(2-nitrobenzoic acid), high-throughput screening.

INTRODUCTION

The parasitic protozoa *Trypanosoma* and *Leishmania* cause a number of human diseases, including Chagas' disease, Leishmaniasis and African sleeping sickness. Current treatments for these diseases are ineffective, impractical or severely toxic, hence there is a pressing need to find new molecules that can selectively inhibit the function of enzymes that are unique to these organisms. One such target is trypanothione reductase (TryR), an NADPH-dependent oxidoreductase that helps to protect the parasite from oxidative stress by maintaining an intracellular reducing environment. TryR achieves this by reduction of the N^1,N^8-bisglutathionyl spermidine conjugate trypanothione ($T[S]_2$) to dihydrotrypanothione ($T[SH]_2$). $T[SH]_2$ plays a key role in the inactivation of potentially damaging radicals and reactive oxygen species, and is re-oxidized to $T[S]_2$ in the process. Disabling the function of TryR in *Leishmania* [1–3] and *T. brucei* [4] markedly increases the sensitivity of these parasites to oxidative stress, a consequence that makes TryR an attractive therapeutic target.

A major limitation in the screening of large synthetic or natural product libraries against TryR is the commercial expense of the natural substrate, $T[S]_2$ (Figure 1). In the search for more economical assay procedures, several alternative substrates have been developed. These include both simplified analogues of $T[S]_2$ [5–8] and non-peptidic substrates such as bis-polyamine derivatives of Ellman's reagent, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) [9]. A colorimetric microtitre plate assay based upon the derivatization of unconsumed $T[S]_2$ has also been developed [10]. This is a stopped assay that requires additional chemical manipulation and, despite the increase in assay sensitivity, unreasonable quantities of $T[S]_2$ are still required for the screening of large compound libraries. Use of the physiological substrate

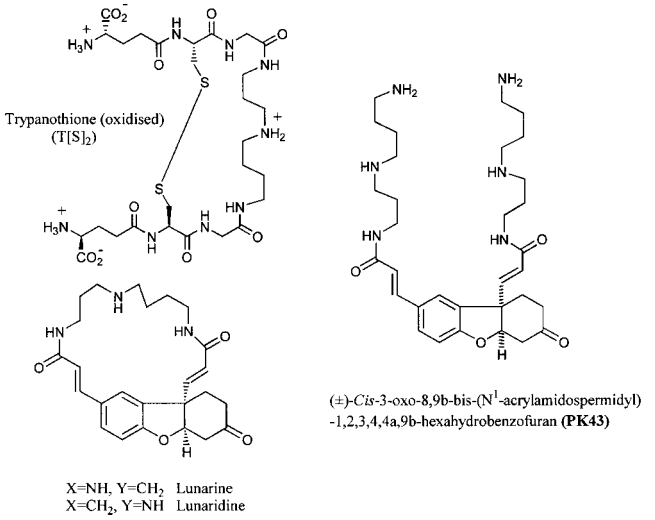

Figure 1 Structure of the substrate ($T[S]_2$) and of time-dependent inhibitors of TryR

Abbreviations used: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); PK43, *cis*-3-oxo-8,9b-bis-(N^1-acrylamidospermidyl)-1,2,3,4,4a,9b-hexahydrobenzofuran; TNB, 2-nitro-5-mercaptobenzoic acid; TryR, trypanothione reductase; $T[S]_2$, trypanothione; $T[SH]_2$, dihydrotrypanothione.

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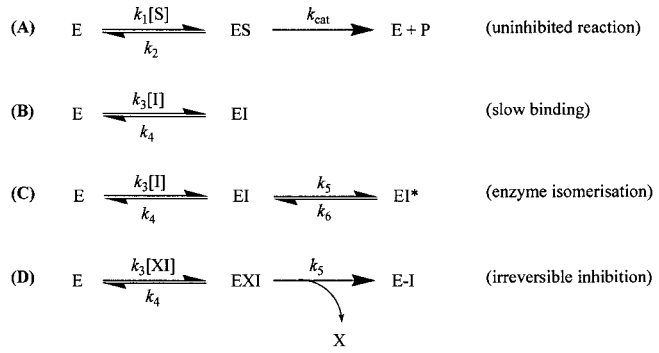


Scheme 1 Basic mechanisms of time-dependent inhibition

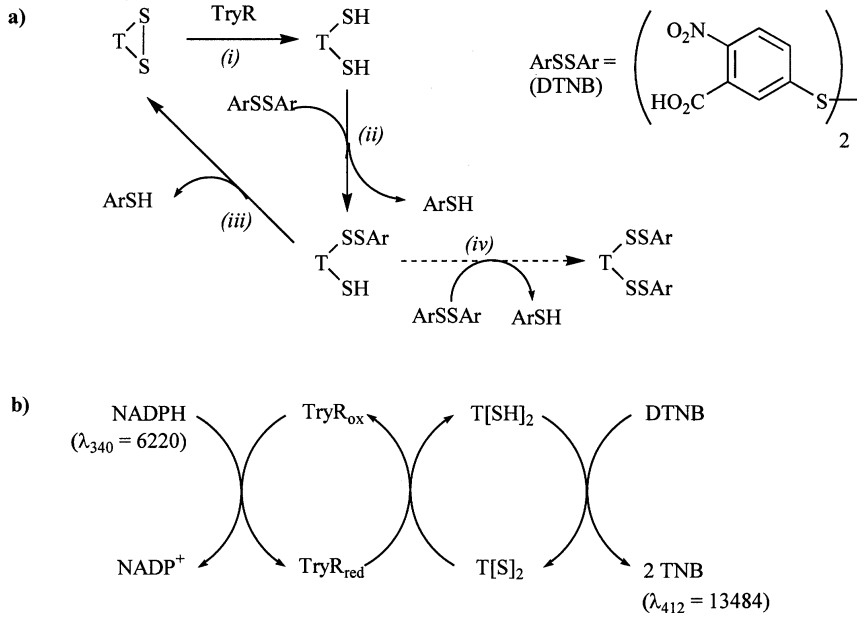
See the text for details.

has the advantage that inhibition assays are based on the native mechanism of substrate recognition.

Following the discovery of the macrocyclic polyamine alkaloid lunarine (Figure 1) as a time-dependent inhibitor of TryR [11], we were interested in developing efficient screening methods, using the physiological substrate, to evaluate libraries of synthetic lunarine analogues for both classical and time-dependent inhibition of TryR. Time-dependent (or slow-binding) inhibition can result from three basic mechanisms (Scheme 1). In classical inhibition mechanisms, the steady-state equilibrium between enzyme and inhibitor is established rapidly (usually within milliseconds). Simple slow binding (Scheme 1B) occurs when this steady state takes several seconds or even minutes to become established. With an enzyme isomerization mechanism (Scheme 1C), a rapid inhibitor binding step is followed by a slower enzyme/inhibitor conformational change. Irreversible inhibition proceeds by the same rapid binding step followed by a rate-limiting covalent modification of the enzyme (Scheme 1D).

A diagnostic feature of time-dependent inhibition is an observable decrease in enzyme activity over a time scale of seconds or minutes. One way of identifying time-dependent inhibition is to pre-incubate the enzyme with inhibitor prior to substrate addition and then assay for residual enzyme activity as a function of pre-incubation time [12]. These methods are not appropriate for high-throughput assays, as several experiments must be carried out (over a range of pre-incubation times) in order to observe any decrease in enzyme activity over time. Alternatively, extended continuous assays can be performed in the presence of inhibitor to reveal any time-dependent decrease in enzyme activity. For such experiments to be valid, reactions must not be allowed to run beyond the time over which the reaction rate is linear in the absence of inhibitor (i.e. deviation from linearity due to substrate depletion must be avoided). Using a large excess of substrate will prevent such deviations from linearity, but this is undesirable for TryR screens due to the expensive substrate. Continuous assays for competitive time-dependent inhibition in the presence of large substrate excesses also retards the rate of time-dependent inactivation. Consequently, prolonged reaction times may be required in order to observe the slow onset of enzyme inhibition, and thus in high-throughput screens potential slow-binding inhibitors could go unnoticed. Lower enzyme concentrations will minimize substrate turnover, but this will be at the expense of assay sensitivity.

To avoid substrate depletion, we chose to mediate the *in situ* chemical regeneration of $T[S]_2$ from $T[SH]_2$ using Ellman's reagent (DTNB). While there have been reports on the use of DTNB to titrate the product formed by the reduction of glutathione in a glutathione reductase endpoint assay, no mention was made of the role of DTNB in the *in situ* recycling of GSH to



Scheme 2 DTNB-coupled TryR assay

(**a**) Mechanism of DTNB-mediated re-oxidation of $T[SH]_2$: (i) TryR-catalysed reduction of $T[S]_2$; (ii) mixed-disulphide formation by reaction with DTNB *in situ*; (iii) a rapid intramolecular disulphide exchange reaction regenerates $T[S]_2$; (iv) possible reaction of the mixed-disulphide intermediate with DTNB (when in too large an excess) to give the bis-disulphide. (**b**) Schematic of the DTNB-coupled assay.

GSSG, and it is a procedure that has since received scant attention [13–16].

We postulated that DTNB could be used to chemically regenerate $T[S]_2$ from $T[SH]_2$, *in situ*, by first forming the mixed disulphide ArS-T-SH and a 2-nitro-5-mercaptobenzoic acid (TNB, ArSH) molecule (step ii Scheme 2a), followed by the rapid intramolecular displacement of a second TNB molecule to regenerate $T[S]_2$ (step iii in Scheme 2a) [15,16]. Reaction progress could be monitored by measuring the absorbance of the TNB chromophore at 412 nm, using a molar absorption coefficient of approx. 13600 $M^{-1} \cdot cm^{-1}$ [17]. In standard TryR assay protocols, enzyme activity is usually monitored by the decrease in absorbance at 340 nm due to NADPH oxidation, with a molar absorption coefficient of 6200 $M^{-1} \cdot cm^{-1}$. While only one equivalent of NADPH is oxidized per molecule of $T[S]_2$ that is reduced, two equivalents of TNB will be generated from the same catalytic cycle when DTNB is used to re-oxidize the $T[SH]_2$ product (Scheme 2b). Considering the differences in stoichiometry and chromophore molar absorption coefficients, it is evident that monitoring TNB production in an assay coupled to DTNB will give a 4-fold increase in assay sensitivity.

It also seemed reasonable that, provided that substrate regeneration was much faster than enzyme turnover, it should be possible to maintain constant substrate levels (and linear reaction rates) for extended periods of time while using concentrations of $T[S]_2$ that are only a fraction of its K_m value (i.e. 30 μM). Reported herein is the development of such a procedure that is appropriate for both time-dependent and high-throughput TryR inhibition assays. The characterization of a novel time-dependent inhibitor, *cis*-3-oxo-8,9b-bis-(N^1-acrylamidospermidyl)-1,2,3,4,4a,9b-hexahydrobenzofuran (PK43) (Figure 1), is also described using these procedures.

MATERIALS AND METHODS

Materials

Recombinant *T. cruzi* TryR was purified from *Escherichia coli* as described previously [18]. The enzyme was stored as a suspension in 70 %-satd ammonium sulphate solution at 4 °C, and dialysed extensively against the assay buffer before use. $T[S]_2$ was purchased from Bachem, and DTNB was from Sigma. The concentrations of stock solutions of $T[S]_2$ were calibrated by titration with an excess of TryR and monitoring the change in NADPH absorption at 340 nm following total substrate depletion. Spectrophotometric assays of TryR were carried out in 1 ml cuvettes on a temperature-controlled Shimadzu UV–visible recording spectrophotometer. Microtitre plate assays were carried out in 96-well plates on a Molecular Devices Thermomax microplate reader. One unit of enzyme activity is defined as the amount of TryR required to reduce 1 μmol of $T[S]_2$ in the presence of 150 μM NADPH under saturating substrate conditions at 27 °C and pH 7.5 in the absence of DTNB. Kinetic data analysis was performed using GraFit version 5 (Erithacus Software Ltd). Details of the synthesis of PK43 will be reported elsewhere, but can be obtained from A.H.F. on request.

Enzyme assays

For assay of TryR, the standard cuvette assay mixture (1 ml) contained TryR (1 m-unit), 40 mM Hepes (pH 7.5), 1 mM EDTA, 0.15 mM NADPH, 1 μM $T[S]_2$, 25 μM DTNB and various concentrations of inhibitor. Inhibitor stock solutions were made up using co-solvent mixtures of assay buffer and DMSO, such that final assay mixtures contained no more than 1 % (v/v) DMSO. Enzyme mixtures were pre-incubated with NADPH for 5 min at 27 °C prior to initiating the reaction by the addition of substrate. Enzyme activity was monitored by the increase in absorbance at 412 nm due to the formation of TNB. To determine time-dependent inhibition constants, assays were carried out at five different inhibitor concentrations ($0–2 \times K_i$) and one substrate concentration (1 μM). K_i values and rate constants were determined using the appropriate integrated rate equations as described below.

For the microplate assays, the final assay mixtures (0.25 ml) contained TryR (1 m-unit) in the presence of 40 mM Hepes (pH 7.5), 1 mM EDTA, 0.15 mM NADPH, 25 μM DTNB, 1 μM $T[S]_2$ and inhibitor (100 μM). Enzyme mixtures were pre-incubated for 5 min with NADPH at 27 °C prior to initiating the enzyme reaction by the addition of substrate, followed by inhibitor. Following inhibitor addition, enzyme activity was monitored by the increase in absorbance at 410 nm due to the formation of TNB. The microplate assays were monitored at 410 nm, as this was the UV filter that showed the closest match to 412 nm on the microplate reader.

RESULTS

Effects of DTNB concentration on linearity

DTNB was made up as a 10 mM stock solution in 40 mM Hepes buffer (pH 7.5) containing 10 % (v/v) DMSO. DMSO was necessary to solubilize DTNB at high concentrations, but the proportion of DMSO in the final assay mixtures never exceeded 1 % (v/v), so as not to interfere significantly with enzyme activity

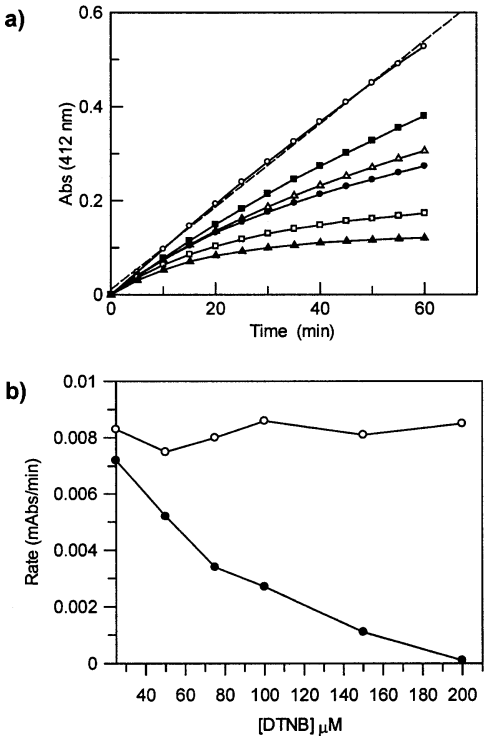


Figure 2 Effects of DTNB on TryR activity during prolonged incubations

(**a**) TryR (1 m-unit), NADPH (0.15 mM) and $T[S]_2$ (1 μM) were incubated at 27 °C, pH 7.5, in the presence of various concentrations of DTNB: 25 (○), 50 (■), 75 (△), 100 (●), 150 (□) and 200 (▲) μM. $R = 0.9994$ for a linear regression fit to the data at 25 μM DTNB (broken line). (**b**) Effect of increasing DTNB concentrations on the rate of reaction at 0 (○) and 60 (●) min. Abs, absorbance.

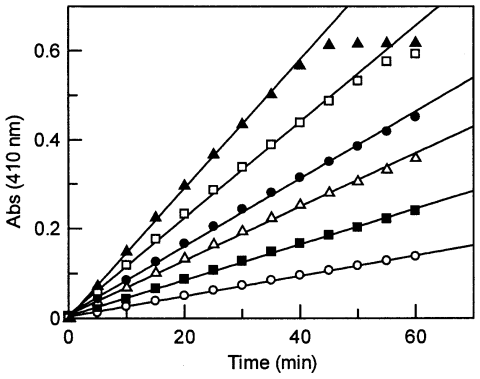


Figure 3 Effect of substrate concentration on linearity

TryR (1 m-unit), NADPH (0.15 mM) and DTNB (25 μM) were incubated at 27 °C, pH 7.5, for 60 min in the presence of various concentrations of $T[S]_2$ substrate: 0.25 (○), 0.5 (■), 0.75 (△), 1.0 (●), 1.5 (□) and 2.0 (▲) μM. At 0.25 and 0.5 μM DTNB, linear fits were calculated using data points up to 40 min, since beyond this time deviations from linearity were evident due to DTNB exhaustion. Abs, absorbance.

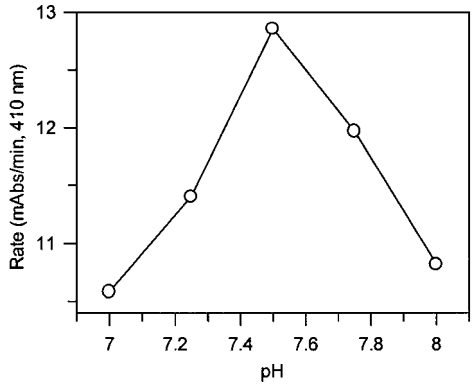


Figure 4 pH profile for the DTNB-coupled TryR assay

TryR (1 m-unit), NADPH (0.15 mM), DTNB (25 μM) and $T[S]_2$ (1 μM) were incubated at 27 °C at various pH values.

(discussed below). Initial experiments in which DTNB was added via a pipette to the reaction mixture were hampered by the slow precipitation of DTNB interfering with the absorbance measurements. This problem (encountered at DTNB concentrations ranging from 50 to 500 μM) was overcome by ensuring complete dilution of DTNB in the assay mixture by rapid mixing prior to the addition of other reaction components. At high concentrations, DTNB appeared to behave as a time-dependent inhibitor of TryR, and a consistently linear reaction rate was only attainable when DTNB concentrations were lowered to 25 μM (Figure 2a). Higher concentrations of DTNB increased the rate of enzyme inactivation, but had no effect on the initial rate of the reaction (Figure 2b). At 25 μM DTNB, the reaction rate was effectively linear and the time-dependent decrease in enzyme activity over 1 h was minimal; a best fit to this progress curve (Figure 2a), by linear regression, gives an R value of 0.9994. Increasing the NADPH concentration did not alter the rate of enzyme inactivation. Due to the complex nature of the DTNB-mediated inhibition of TryR (discussed below), and because no significant inhibition was evident at DTNB concentrations that were appropriate for these assays (25 μM), inhibition constants for DTNB were not obtained. It has been reported that DTNB is an unsuitable reagent for the *in situ* titration of $T[S]_2$, as it is slowly reduced in a TryR-catalysed process [10]. DTNB concentrations and turnover rates in these previous experiments were not reported, but in our hands there was no evidence of TNB formation when 150 μM DTNB was incubated with TryR (3 m-units) for 60 min (results not shown).

Effects of substrate concentration on linearity

The choice of a 1 μM substrate concentration was arbitrary, and satisfactory results were obtained over a range of $T[S]_2$ concentrations (Figure 3). The lowest substrate concentration tested was 0.25 μM, but in theory lower concentrations could be used. The lower limit of substrate concentration is likely to be governed by the increased quantities of TryR required to achieve an appropriate reaction rate.

Optimizing the pH

When $T[S]_2$ is the substrate, TryR has a broad pH optimum in the pH range 7.5–8.0 [19]. Experiments were carried out over the pH range 7.0–8.0 to see if pH affected the DTNB-mediated regeneration of $T[SH]_2$. A plot of reaction rate against pH showed an optimum pH value of 7.5 (Figure 4). These data are

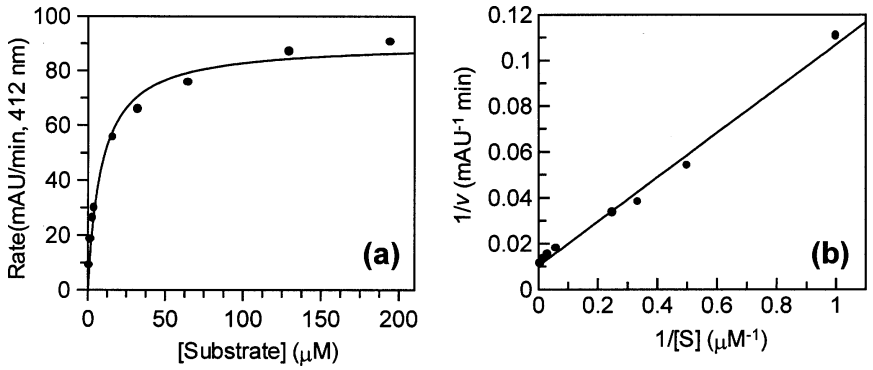


Figure 5 K_m determination for $T[S]_2$ in the presence of DTNB

(**a**) Various concentrations of $T[S]_2$ were incubated with TryR, NADPH (0.15 mM) and DTNB (25 μM) at pH 7.5 and 27 °C. (**b**) Double-reciprocal plot. AU, absorbance units.

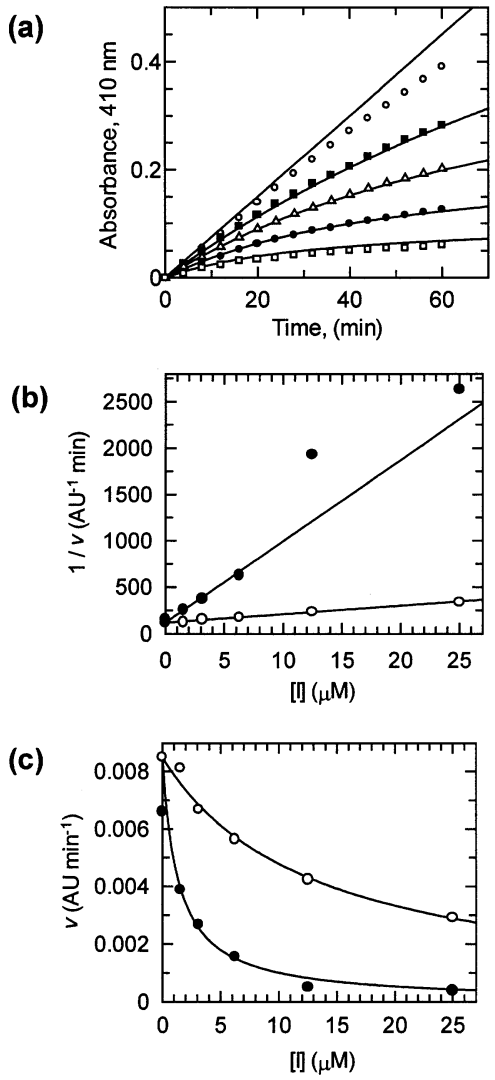


Figure 6 Time-dependent inhibition of TryR by melarsen oxide

TryR (1 m-unit), NADPH (0.15 mM), DTNB (25 μM) and $T[S]_2$ (1 μM) were assayed at 27 °C, pH 7.5, in the presence of various concentrations of melarsen oxide for 60 min. (**a**) Decrease in enzyme activity over time as a function of inhibitor concentration: 0 μM (○), 3.125 μM (■), 6.25 μM (△), 12.5 μM (●), and 25 μM (□). (**b**) Variation in the reciprocal of v_i (○) and v_s (●) as a function of inhibitor concentration. (**c**) Re-plot of the variation in v_i (○) and v_s (●) with respect to inhibitor concentration. AU, absorbance units.

in agreement with the pH profile of TryR that is obtained in the absence of DTNB [19,20].

Rate is proportional to TryR concentration

The velocity of the reaction was directly proportional to the concentration of enzyme used (results not shown). The rate constant for the initial thiol–disulphide exchange reaction between DTNB and $T[SH]_2$ ($3.5 \times 10^4\ M^{-1} \cdot s^{-1}$) [15] (step ii, Scheme 2a) is several orders of magnitude greater than the k_{cat} for TryR from *T. cruzi* (14200 min^{-1}) [20], and the intramolecular displacement of TNB that regenerates $T[S]_2$ is even faster (step iii, Scheme 2a) [15,16]. The DTNB concentration is in vast excess compared with TryR in these experiments. Consequently, the enzyme-catalysed turnover of $T[S]_2$ remains rate-limiting, and the linearity of the reaction is therefore not affected due to gradual substrate depletion at higher enzyme concentrations.

Effects of assay conditions on substrate K_m

Using standard TryR assay conditions (i.e. enzyme, NADPH and $T[S]_2$ substrate), the K_m value for $T[S]_2$ was in the 30 μM range for experiments carried out at pH 7.5 in 40 mM Hepes buffer solution. When substrate assays were carried out under these conditions, but with the addition of 25 μM DTNB, the determined K_m value for $T[S]_2$ was significantly decreased to just 9.1 ± 1.0 μM (Figure 5). We have previously noted similar variations in substrate K_m values for disulphide reductase assays that are carried out at the same pH but in the presence of different buffer salt compositions (C. J. Hamilton, A. Saravanamuthu and A. H. Fairlamb, unpublished work). When using this system to assay competitive time-dependent inhibitors, it is the determined K_m value of 9 μM that is used in the appropriate rate equations to extrapolate the inhibition constants.

Effects of DMSO on enzyme activity

When enzyme inhibitors show poor solubility in the aqueous assay buffers being used, concentrated stock solutions are usually made up in DMSO, and the required inhibitor concentrations are then obtained by serial dilution into assay buffer. The upper limits of DMSO concentration that would be tolerated by TryR under DTNB-coupled assay conditions were therefore established. As expected, enzyme activity was adversely affected by the presence of increasing DMSO concentrations (results not shown). In order to minimize enzyme inactivation, where DMSO must be used it should not exceed 1 % (v/v) of the final assay mixture. To minimize experimental error, the same percentage of DMSO should be maintained in all assays carried out (in the presence or absence of inhibitor) for each compound being assayed.

Optimum assay conditions

Following these preliminary experiments, the optimum assay conditions for subsequent enzyme inhibition assays were defined as follows: pH 7.5, 1 μM $T[S]_2$, 27 °C, 25 μM DTNB, 1 m-unit of TryR, 0.15 mM NADPH, maximum DMSO content 1 % (v/v). DTNB is always the first component to be dissolved in the assay mixture, with rapid mixing to ensure complete dissolution, thereby avoiding subsequent precipitation.

Time-dependent inhibition assay for melarsen oxide

To confirm the experimental validity of the DTNB-coupled continuous assay, the time-dependent inhibition of TryR by the tervalent arsenical melarsen oxide was studied, and the results were compared with those determined previously by other methods [21]. Using the pre-incubation methods outlined above, melarsen oxide has been characterized previously as a reversible time-dependent inhibitor of TryR that operates by the two-stage inhibition mechanism (Scheme 1C). The time-dependent inhibition of TryR by melarsen oxide was monitored under the optimized DTNB assay conditions, and the change in the rate of TNB production at 412 nm was monitored continuously for 60 min. These and all subsequent detailed inhibition experiments were carried out as 1 ml solutions in UV cuvettes.

For the purpose of curve-fitting, approx. 15–30 evenly spaced data points were taken from each progress curve. The concentration-dependent values for the initial rate (v_i), the steady-state rate (v_s), the apparent rate constant for establishing the steady-state equilibrium between EI and EI* (k') (see Scheme 1) and the displacement of the curve from the vertical ordinate (d)

Table 1 Comparison of some time-dependent inhibitors of TryR

*Results determined using the DTNB-coupled assay procedures; †results determined previously using pre-incubation methods [21]; ‡data taken from [25]. Error values are standard errors calculated by the matrix inversion method.

Compound	K_i (μM)	K_i^* (μM)	k_5/k_6	k_5 (min^{-1})	k_6 (min^{-1})
Melarsen oxide*	18 ($\pm$2)	1.4 ($\pm$0.4)	10.7	0.0859 ($\pm$0.0257)	0.0054 ($\pm$0.0064)
Melarsen oxide†	17.2			0.0858	
Lunarine‡	304 ($\pm$2)	114 ($\pm$2)	1.7	0.0128 ($\pm$0.0008)	0.0077 ($\pm$0.0024)
PK43	59 ($\pm$3)	3.5 ($\pm$0.9)	10.8	0.2320 ($\pm$0.0153)	0.0215 ($\pm$0.0015)

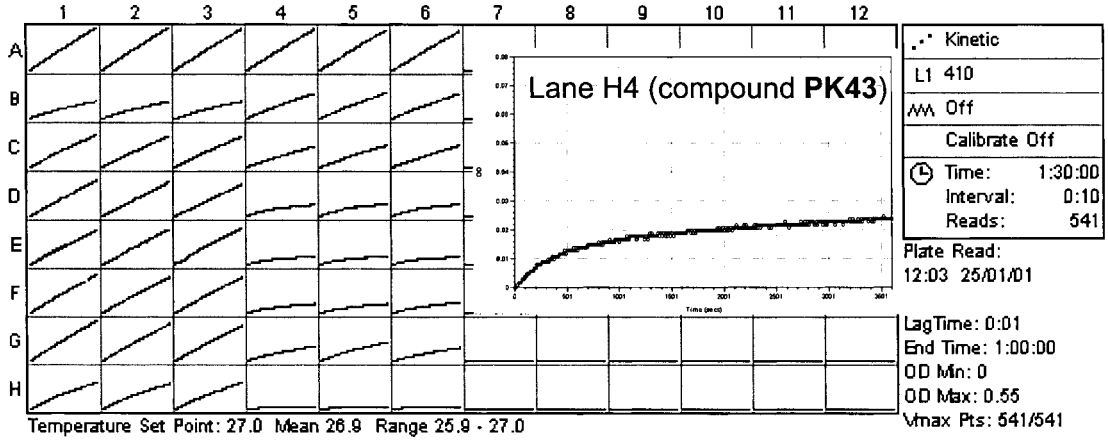


Figure 7 Microplate assay for TryR inhibition

A total of 14 synthetic lunarine analogues were screened as potential inhibitors of TryR. TryR (1 m-unit), NADPH (0.15 mM), DTNB (25 μM) and $T[S]_2$ (1 μM) were assayed at 27 °C, pH 7.5, in the presence of 100 μM inhibitor. Each inhibitor assay was carried out in triplicate (lanes B1–B3 to H1–H3 and B4–B6 to H4–H6), and control experiments were carried out in the absence of inhibitor (lanes A1–A6). The inset shows an expansion of lane H4 demonstrating reversible time-dependent inactivation of TryR by compound PK43.

were determined by non-linear fitting of the above data to eqn (1) [22]:

$$P = v_s t + (v_i - v_s)(1 - e^{-k't})/k' + d \quad (1)$$

where P is amount of product formed at time t. Fitting of these data, by non-linear regression, using equations (2) and (3) gives values for the initial K_i and the overall steady-state dissociation constant (K_i^*) respectively:

$$v_i = v_{i=0}/[1 + I/K_i(1 + S/K_m)] \quad (2)$$

$$v_s = v_{i=0}/[1 + I/K_i^*(1 + S/K_m)] \quad (3)$$

where I is the inhibitor concentration, S is the substrate concentration and K_m is the apparent Michaelis–Menten constant for TryR (determined in a separate experiment to be 9.1 ± 1.0 μM). The ratio between the forward and reverse isomerization steps (k_5/k_6) for the interconversion between EI and EI* was calculated using eqn (4):

$$k_5/k_6 = K_i/K_i^* - 1 \quad (4)$$

For each inhibitor concentration, the value for k_6 can be determined from the relationship in eqn (5):

$$k_6 = v_s k'/v_i \quad (5)$$

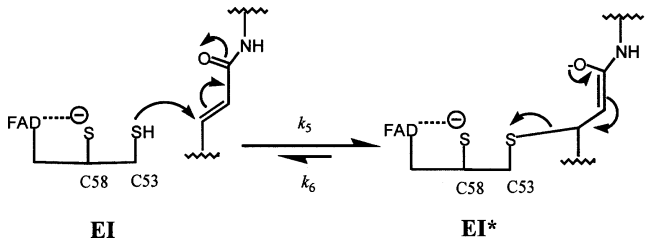
Finally, the mean value for k_6 is then substituted into eqn (4) to calculate k_5. For the inhibition mechanism shown in Scheme 1(C), the relationship between k' and inhibitor concentration can also be expressed by eqn (6):

$$k' = k_6 + k_5(I/K_i)/(1 + S/K_m + I/K_i) \quad (6)$$

By substituting eqn (6) into eqn (1), an integrated rate equation is obtained that can be used to make an overall fit of several progress curves with different inhibitor concentrations [22]. In this manner K_i, k_5 and k_6 can be obtained directly, and K_i^* can then be determined using eqn (4). The data from these experiments are shown in Figure 6, and the derived inhibition constants are compared with those that have been determined previously for melarsen oxide using pre-incubation methods in Table 1 [21]. Earlier reports on melarsen oxide [21] only give values for K_i and k_5 (or k_{inact} as it is sometimes called), but here we were able to also determine values for K_i^* and k_6. The kinetic constants derived from these DTNB-mediated assays are in close agreement with those published previously [21], but the error margins for k_6 are greater than 100 %. When k_6 is determined using eqn (5), a comparable value of 0.0060 ± 0.0022 is obtained, but with more acceptable error margins.

Microplate assay

An assay procedure was developed for the microplate reader to enable the high-throughput screening of compound libraries for both classical and time-dependent inhibitors of TryR. For these experiments, the $T[S]_2$ concentration was set at 1 μM and inhibitor concentrations of 100 μM were used in all assays. These inhibitor concentrations were chosen to reflect the K_i values expected in the first generation of compound libraries we wanted to screen. The results of initial screening experiments for inhibition of TryR with a range of synthetic analogues of the alkaloid lunarine are shown in Figure 7. By using low enzyme



Scheme 3 Proposed mechanism for the reversible time-dependent inhibition of TryR by the *Lunaria* alkaloids

See the text for details.

and DTNB concentrations, it was possible to run continuous experiments in which the absorbance at 410 nm did not exceed 1.0 for at least 60 min in the absence of inhibitor. In this manner, it is possible to identify both classical inhibitors (by a decrease in the linear gradient) and time-dependent inhibitors (by a gradual deviation from linearity over time). Compound PK43 (Figure 1), which demonstrates notable time-dependent inhibition of TryR (Figure 7, lanes H4–H6), is discussed in more detail below.

Detailed evaluation of PK43, a novel time-dependent inhibitor of TryR

The spermidine-based macrocyclic alkaloids lunarine and lunaridine have been identified previously as competitive, time-dependent inhibitors of TryR [11]. Pre-incubation of TryR with these alkaloids has shown that time-dependent inhibition only occurs when TryR is in the reduced state when the redox-active disulphide pair, Cys-53 and Cys-58, are in the di-thiol form. A two step mechanism for this time-dependent inactivation of reduced TryR has been proposed (Scheme 1C) [11], whereby the EI* complex is formed as a result of reversible conjugate addition of an active-site thiol to one of the α,β-unsaturated amide moieties of the lunarine macrocycle (Scheme 3). During studies into the mechanism of TryR inhibition by the lunaria alkaloids and the design of more potent analogues, we have prepared an acyclic bis-spermidine-N^1-amide derivative of lunarine, called PK43. This compound was prepared to help establish whether the macrocyclic spermidine moiety of lunarine is an essential feature for time-dependent and/or competitive inhibition. Lunarine was originally selected for inhibition studies with TryR [11] based on the presence of the bridging spermidine-N^1,N^8-bis-amide moiety, which is also found in the native substrate $T[S]_2$, where it is an essential component for substrate binding [23]. Whereas lunarine can be likened to $T[S]_2$, comparisons can also be drawn between compound PK43 and N^1-glutathionyl-spermidine disulphide, an alternative, naturally occurring substrate for TryR in which the bridging spermidine unit of $T[S]_2$ is replaced by two linear spermidine chains [2,24]. The inhibition data obtained for PK43 using the DTNB-coupled assay procedures are shown in Figure 8 and Table 1. As can be seen, PK43 displayed time-dependent inhibition when incubated with TryR under the standard assay conditions for 60 min. The inhibition constants for this compound were calculated from these data as described above. The initial rate (v_i) varied with inhibitor concentration, which clearly indicates that mechanism (C) in Scheme 1 is operating, with a Michaelis-type EI complex forming as an intermediate.

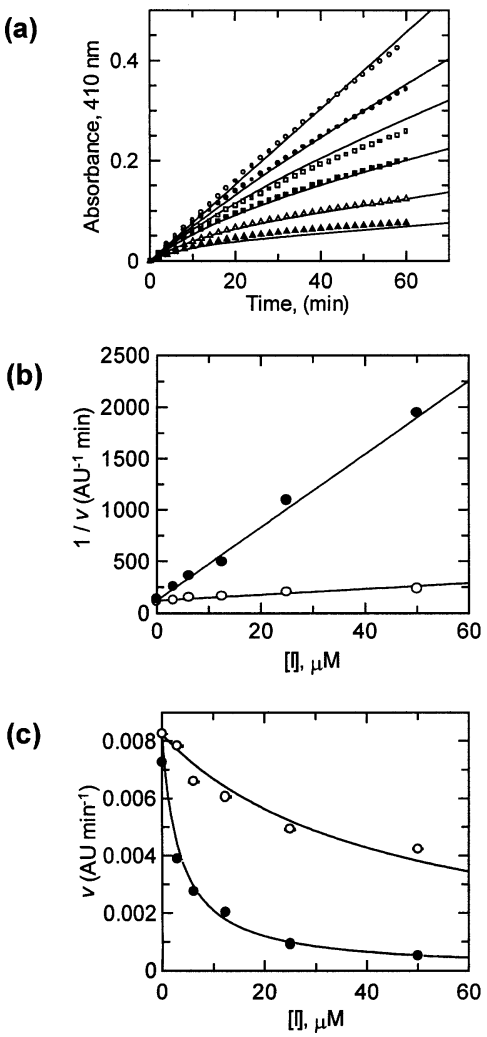


Figure 8 Time-dependent inhibition of TryR by PK43

TryR (1 m-unit), NADPH (0.15 mM), DTNB (25 μM) and $T[S]_2$ (1 μM) were assayed at 27 °C, pH 7.5, in the presence of various concentrations of PK43 for 60 min. (**a**) Decrease in enzyme activity over time as a function of inhibitor concentration: 0 μM (○), 3.125 μM (●), 6.25 μM (□), 12.5 μM (■), 25 μM (△) and 50 μM (▲). (**b**) Variation in the reciprocal of v_i (○) and v_s (●) as a function of the concentration of PK43. (**c**) Re-plot of the variation in v_i (○) and v_s (●) with respect to the concentration of PK43. AU, absorbance units.

DISCUSSION

In these experiments we have demonstrated how DTNB can be used to improve economy in the use of native substrate in TryR assays by chemically recycling the enzyme product $T[SH]_2$ back into the disulphide substrate $T[S]_2$. As substrate regeneration is much faster than enzyme turnover, it is possible to maintain constant substrate concentrations (and linear reaction rates) for at least 60 min using substrate concentrations that would be consumed within seconds under standard assay conditions (Figure 2a). For inhibition studies, particularly with time-dependent inhibitors, it was important to define a set of assay conditions for which progress curves would not deviate significantly from linearity in the absence of inhibitor. The choice of DTNB concentration appears to be critical in this respect. High concentrations of DTNB appear to inactivate TryR in a time-

dependent manner, but do not have any effect on the initial rate of the reaction (Figure 2b). One possible explanation is that, rather than inactivating TryR, high concentrations of DTNB may actually prevent substrate regeneration. The DTNB-mediated regeneration of $T[S]_2$ operates through reaction with $T[SH]_2$ to give a mixed-disulphide intermediate, ArS-T-SH (step ii, Scheme 2a), which is rapidly converted into $T[S]_2$ via intramolecular disulphide exchange (step iii, Scheme 2a). In this process, a possible side reaction is the reaction of the mixed disulphide ArS-T-SH with another DTNB molecule to give the bis-mixed disulphide ArSS-T-SSAr, resulting in effective substrate depletion (step iv, Scheme 2a). As $T[S]_2$ regeneration is a rapid intramolecular process, this competitive side reaction would only become significant at elevated DTNB concentrations, where the apparent time-dependent decrease in enzyme activity could actually be due to gradual substrate depletion. For the purpose of the present studies, DTNB concentrations of 25 μM or less did not appear to significantly affect the linear reaction rate over a 60 min incubation period, with a linear best fit for the reaction progress curve giving an R value of 0.9994, so further studies of DTNB effects at higher concentrations were not pursued.

Under these assay conditions, complete consumption of DTNB gave a maximum absorbance value of 0.68 in a cuvette with a 1 cm path-length, and deviations from linearity became apparent at an absorbance value of approx. 0.55 (Figure 3) due to severely depleted DTNB concentrations resulting in less efficient substrate recycling. It is interesting to note that the NADPH concentration appears to have little effect on reaction linearity during prolonged incubations. When TryR was subjected to prolonged incubation with NADPH (0.15 mM) in the absence of substrate, as in pre-incubation time-dependent inhibitor assays, gradual NADPH-induced inactivation of TryR has been reported (30–40% inactivation over 60 min) [19,20]. This is believed to result from over-reduction of the enzyme (to EH_4). In our continuous DTNB-coupled experiments using a comparable NADPH concentration (0.15 mM), lower levels of inactivation were observed (only 10–15% over 60 min), and this might be attributed to DNTB-mediated substrate depletion, as described above (Figure 2). The constant substrate turnover in these assays appears to prevent over-reduction of the enzyme, as once it is in the reduced state (EH_2) it is re-oxidized back to FAD (through substrate turnover) much more rapidly that the apparent rate of over-reduction by a second molecule of NADPH.

Due to poor compound solubility, TryR inhibition assay systems will often contain traces of DMSO when stock concentrations of inhibitor must be made up using this solvent. We have shown that, in order to minimize enzyme inactivation, assay concentrations of DMSO should never exceed 1% (v/v), at which level TryR inactivation is limited to 15%. Based on these observations, standard assay conditions can now be defined as 25 μM DTNB, 1 μM $T[S]_2$, 0.15 mM NADPH and 1 m-unit of TryR at pH 7.5 in 40 mM Hepes, with a maximum DMSO content of 1% (v/v). These conditions are appropriate for experiments carried out over a 60 min time period, although enzyme quantities can obviously be varied according to assay duration and other experimental criteria.

The potential application of these procedures to economical microtitre plate high-throughput screening assays for classical competitive and time-dependent inhibitors of TryR has been demonstrated (Figure 7). At a 1 μM substrate concentration, the DTNB-coupled procedure lowers substrate requirements by at least two orders of magnitude compared with standard TryR inhibition assays that use a concentration of approx. 100 μM. The possibility of extended assays using the DTNB method enables compound libraries to be screened simultaneously for both classical and time-dependent inhibition. The use of low substrate concentrations means that less potent, but potentially useful, competitive time-dependent inhibitors of TryR may be revealed that may otherwise have gone undetected using procedures requiring much greater substrate concentrations.

The validity of the DTNB method for conducting detailed kinetic experiments using time-dependent inhibitors was established in assays with melarsen oxide (Figure 7). The data obtained from these experiments were in close agreement with previously reported values that were obtained using pre-incubation procedures (Table 1). The increases in both substrate and labour efficiency using the DTNB-coupled procedures are significant. The pre-incubation assay for melarsen oxide is an endpoint assay, which required at least six pre-incubation experiments of various time periods to be carried out at each of six inhibitor concentrations using 50 μM $T[S]_2$ [21]. The DTNB-coupled assay required just one continuous experiment to be carried out at each of six inhibitor concentrations using 1 μM $T[S]_2$. While the pre-incubation experiments require careful planning and involve at least 36 separate enzyme activity experiments, with a six-channel spectrophotometer it is possible to set up all of the DTNB-coupled experiments to run simultaneously. In theory, substrate economy could be improved further by running these assays as 200 μl solutions in a microplate format. In our hands such experiments did not provide data sets that were appropriate for curve fitting due to occasional irregularities in the reaction progress curves (a consequence of measuring absorbance through the top of the solution).

Classical TryR assays are monitored by the decrease in absorbance at 340 nm due to NADPH consumption, with a molar absorption coefficient of 6200 $M^{-1} \cdot cm^{-1}$. Assays at this wavelength have proven previously to be problematic with compounds such as lunarine that also absorb at this wavelength. To avoid this background interference, it is usually necessary to monitor NADPH consumption at 366 nm. At 366 nm there is no overlapping absorption by lunarine, but the molar absorption coefficient of NADPH is lowered to 3300 $M^{-1} \cdot cm^{-1}$, which means that there is a 2-fold decrease in assay sensitivity. DTNB assays are monitored at 412 nm, a wavelength at which the *Lunaria* alkaloids do not absorb. These assay procedures are now being used to support our ongoing studies of *Lunaria* alkaloids as time-dependent inhibitors of TryR, and have helped to identify and characterize a more potent synthetic lunarine analogue. PK43 is a bis-spermidine functionalized open-chain analogue of the macrocyclic alkaloid lunarine that displays time-dependent inhibition of TryR (Figure 8) via the two-step mechanism (Scheme 1C). Compared with lunarine [25] the apparent K_i and K_i* values for PK43 are 7-fold and 32-fold lower respectively (Table 1). The lower K_i for PK43 could be due to the greater flexibility of its open-chain structure (compared with the more conformationally constrained lunarine macrocycle), enabling it to mould more readily into the relatively rigid active site of TryR [24]. In this class of compounds we believe that the conversion from EI into EI* involves an alteration in the enzyme-bound inhibitor conformation that positions one of the α,β-unsaturated amide moieties for reversible conjugate addition with an active-site cysteine (Scheme 3). The 18-fold enhanced k_5 value for PK43 may again reflect its increased flexibility, enabling it to adopt more readily an appropriate orientation from which conjugate addition may occur. The difference between the off rates (k_6) for PK43 and lunarine are much less significant. This is to be expected if k_6 relates to a retro-conjugate addition process, since k_6 will be governed primarily by the leaving-group ability of the β-carbon substituent, which would be the same Cys-53 thiol side chain of TryR in both cases.

While the efficacy of PK43 as a time-dependent inhibitor is a notable improvement on lunarine, the isomerization constant k_5/k_6 is still relatively low. A high k_5/k_6 value is desirable, as this would reflect a higher ratio of the rates of forward to reverse isomerization (or conjugate addition) between the EI and EI* complexes. If these lunarine alkaloids do interact with TryR via a reversible conjugate addition process, then attempts to further decrease k_6 for subsequent analogues could prove difficult if this reaction step is governed predominantly by protein rather than inhibitor structure. The reversibility of conjugate addition to these α,β-unsaturated amides could be prevented if it were possible for what is presumably the enol amide (EI*) (Scheme 3) to tautomerize to the more stable saturated β-amide. To achieve this requires more detailed mechanistic information about time-dependent inhibition by this class of compounds. Further studies are currently in progress and will be reported in due course.

We note that these DTNB methods will not be appropriate for the analysis of certain classes of TryR inhibitors where DTNB and/or TNB react with the inhibitor compounds to give erroneous results. Such problems were encountered when we tried using these procedures to analyse TryR inhibition by nitric oxide donor drugs (results not shown). For the assays detailed herein, control experiments were carried out to confirm that no background reaction was taking place when 100 μM concentrations of melarsen oxide or α,β-unsaturated amido alkaloids such as lunarine and PK43 were incubated with 100 μM DTNB or TNB for 60 min (results not shown).

In summary, an efficient procedure has been developed for high-throughput screening and detailed time-dependent inhibition assays with TryR. These procedures require only minimal quantities of the expensive substrate $T[S]_2$, a factor that will be of significant benefit in the screening of large compound libraries. These methods may also have broader applications in DTNB-coupled assays for other disulphide reductase systems that utilize small-molecule thiols, such as glutathione, mycothione [26] and CoA disulphide [27].

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