Received: 24 March 2020 | Revised: 2 August 2020 | Accepted: 20 August 2020

DOI: 10.1111/bph.15250

RESEARCH PAPER

Pseudoirreversible slow-binding inhibition of trypanothione reductase by a protein–protein interaction disruptor

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Funding information
Consejería de Educación e Investigación. Comunidad de Madrid, Grant/Award Number: S-2018/BAA-4370; Secretaría de Estado de Investigación, Desarrollo e Innovación. Ministerio de Economía y Competitividad, Grant/Award Number: SAF2015-64629-C2; Consejo Superior de Investigaciones Científicas, Grant/Award Number: 201980E028

Background and Purpose: Peptide P4 was described as a dimerization disruptor of trypanothione reductase (TryR), a homodimeric enzyme essential for survival of trypanosomatids. Determination of the true inhibitory constant (K_i) for P4 was not achieved because reaction rates continuously decreased with time, even when substrate concentration was kept constant. The aim of this study was to find a suitable kinetic model that could allow characterization of the complex pattern of TryR inhibition caused by P4.

Experimental Approach: After showing the slow-binding and pseudoirreversible activity of P4 against *Leishmania infantum* trypanothione reductase (Li-TryR), analysis of the curvatures of the reaction progress curves at different inhibitor concentrations allowed us to define the apparent inhibitory constants (K_i^{app}) at five different substrate concentrations. Analysis of the changes in K_i^{app} values allowed precise definition of the type of inhibition.

Key Results: *Li*-TryR inhibition by P4 requires two sequential steps that involve rapid generation of a reversible enzyme–inhibitor complex followed by a pseudoirreversible slow inactivation of the enzyme. Recovery of enzyme activity after inhibitor dissociation is barely detectable. P4 is a non-competitive pseudoirreversible inhibitor of *Li*- TryR that displays an overall inhibition constant (K_i^*) smaller than 0.02 μM.

Conclusion and Implications: *Li*-TryRdimer disruption by peptide P4 is a pseudoirreversible time-dependent process which is non-competitive with respect to the oxidized trypanothione (TS_2) substrate. Therefore, unlike reversible *Li*-TryR competitive inhibitors, enzyme inhibition by P4 is not affected by the TS_2 accumulation observed during oxidant processes such as the oxidative burst in host macrophages.

KEYWORDS

dimerization disruptor, *Leishmania infantum*, protein–protein interactions, pseudoirreversible inhibition, slow-binding inhibitor, time-dependent inhibition, trypanothione reductase

Abbreviations: DTNB, Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid); EI, enzyme/inhibitor; ESI, enzyme/substrate/inhibitor; ES, enzyme/substrate; *Li*-Try R, *Leishmania infantum* trypanothione reductase; TS_2, oxidized trypanothione.

1 | INTRODUCTION

Protozoan parasites from the *Leishmania* genus are the causative agents of the disease known as leishmaniasis, a zoonosis transmitted by dipterous insects from the Psychodidae family. Leishmaniasis shows a wide clinical spectrum ranging from cutaneous manifestations that can heal spontaneously to visceral infections that may lead to death. The different clinical outcomes depend on both the immune status of the host and the species involved. The Trypanosomatidae family to which *Leishmania* belongs also includes other parasitic organisms such as *Trypanosoma cruzi* and *Trypanosoma brucei*, causative agents of Chagas disease and sleeping sickness, respectively (Akhoundi et al., 2017, 2016; Burza, Croft, & Boelaert, 2018).

According to World Health Organization (WHO), leishmaniasis is one of the main neglected diseases, mainly affecting low-income populations in underdeveloped or developing countries. Although several alternatives are available for the treatment of leishmaniasis, none of the drugs currently in use can be considered ideal due to high toxicity, subsequent side effects and long duration of treatments, which lead to frequent therapeutic noncompliance. In addition, in most cases, these treatments do not completely eliminate the parasite (Bekhit et al., 2018; Hendrickx, Caljon, & Maes, 2019).

Trypanothione or N^1,N^8-bis(glutathionyl)spermidine is a unique low MW molecule characteristic of trypanosomatids that replaces **glutathione (GSH)** as the main electron donor in the cell. It is made up of two molecules of GSH linked by a **spermidine** bridge (Battista, Colotti, Ilari, & Fiorillo, 2020). Trypanothione is necessary for many highly relevant cellular processes such as (i) protection of DNA from the damage generated by radicals (Awad, Henderson, Cerami, & Held, 1992); (ii) elimination of hydrogen peroxide, being much more efficient than other thiols present in trypanosomatids such as GSH, mono-glutathionylspermidine and ovothiol A (Ariyanayagam & Fairlamb, 2001); (iii) detoxification of peroxynitrite (Thomson, Denicola, & Radi, 2003); (iv) reduction of protein and non-protein disulfides (Ariyanayagam & Fairlamb, 2001); (v) synthesis of deoxyribonucleotides by ribonucleotide reductase (Dormeyer, Reckenfelderbäumer, Ludemann, & Krauth-Siegel, 2001); (vi) resistance to chemotherapeutic agents (Baiocco, Colotti, Franceschini, & Ilari, 2009) and (vii) detoxification of methylglyoxal which, in almost all organisms, is detoxified by a system of GSH-dependent glyoxalases I and II (Ariza et al., 2006; Irsch & Krauth-Siegel, 2004).

Trypanothione, like glutathione, may exist in a reduced ($T[SH]_2$) or an oxidized (TS_2) form and it is maintained as $T[SH]_2$ by trypanothione reductase, an enzyme that fulfils the same function that **glutathione reductase (now known as glutathione-disulfide reductase)** does in the systems based on GSH (Battista et al., 2020; Krauth-Siegel & Comini, 2008). Trypanothione reductase (EC1.8.1.12) is a flavoenzyme that is responsible for reducing oxidized trypanothione and oxidized mono-glutathionylspermidine using NADPH as an electron donor. This enzyme is present only in trypanosomatids and is homologous to the ubiquitous glutathione reductase that reduces oxidized GSH in many organisms.

What is already known

- Peptide P4 is a dimer disruptor of *Leishmania infantum* trypanothione reductase.

What does this study add

- Two-step inhibition of *Leishmania infantum trypanothione* reductase by P4 matches the expected kinetics of a dimer disruptor.
- P4 is a non-competitive, pseudoirreversible, slow-binding inhibitor of *Leishmania infantum trypanothione* reductase.

What is the clinical significance

- P4 novel properties make it an excellent lead molecule for development of promising antileishmanial derivatives.

Trypanothione reductase is a validated therapeutic target in these parasitic protozoa since, due to the lack of glutathione reductase and thioredoxin reductase, it is the only link that connects the NADPH metabolism with that of thiols (Fairlamb & Cerami, 1992; Krauth-Siegel & Comini, 2008). For all the trypanosomatids studied so far, this enzyme is essential, for example, *L. donovani* parasites lacking the two endogenous copies of the trypanothione reductase (*TPR*) gene could only be obtained after episomal expression of the *TPR* coding sequence (Tovar, Wilkinson, Mottram, & Fairlamb, 1998). On the other hand, experiments with an inducible copy of *TPR* demonstrate that bloodstream-form *T. brucei* parasites are still able to grow after a 90% reduction in the usual intracellular concentration of this enzyme (Krieger et al., 2002). Bearing in mind that, upon oxidative stress, oxidized trypanothione may reach concentrations 1 or 2 orders of magnitude above its K_m, trypanothione reductase competitive inhibitors with K_i values in the micromolar range do not seem to be appropriate for the treatment of trypanosomiasis and leishmaniasis (Fairlamb, 2003; Krauth-Siegel & Comini, 2008; Krieger et al., 2002). Consequently, the search for drugs whose therapeutic target is trypanothione reductase should be concentrated on molecules that cause a non-competitive inhibition in the case of reversible inhibitors or, directly, on irreversible inhibitors (Krieger et al., 2002).

In this regard, because trypanothione reductase is only active in its dimeric form, our research group is focused on the development of non-competitive inhibitors of *Leishmania infantum*-trypanothione reductase that mimic the α2 helix of the *Li*-trypanothione reductase dimerization interface thereby causing dimer disruption. The 13-mer peptide P3 of sequence $PEIIQSVGISN_LKN_L$ and peptide P4, in which the E residue at position 2 was replaced by a K ($PKIIQSVGISN_LKN_L$), emerged as potent *Leishmania infantum*-trypanothione reductase dimerization inhibitors in the low micromolar range (Toro et al., 2013).

P4 also displays potent inhibitory activity in the oxidoreductase assays, but because of the non-linear behaviour of the reaction progress curves determination of its mode of inhibition has not been feasible up to now.

The objective of the current study was to find a suitable kinetic model that could describe the non-linear process of inhibition observed for our P4 prototype. For that purpose, we have analysed the reaction progress curves at different substrate and inhibitor concentrations. This has allowed us to define the apparent inhibitory constant K_i^{app} at five different substrate concentrations without any initial assumption of the specific mode of inhibition. Analysis of the changes in these constants as the substrate concentration increases demonstrates that P4 is a non-competitive, pseudoirreversible, slow-binding inhibitor of *Leishmania infantum*-trypanothione reductase.

2 | METHODS

2.1 | Materials and reagents

All reagents for the *Leishmania infantum*-trypanothione reductase activity assay were obtained from Sigma-Aldrich (Saint Louis, MO, USA) except for oxidized trypanothione, which was purchased from Bachem (Bubendorf, Switzerland). P4 was prepared following the previously reported solid-phase peptide synthesis protocols (Toro et al., 2013).

2.2 | *Leishmania infantum*-trypanothione reductase purification

Recombinant *Leishmania infantum*-trypanothione reductase HIS- and FLAG-tagged was purified from *Escherichia coli* as previously described (Toro et al., 2013). Briefly, *pRSETA-HIS-Li-TPR* and *pET24a-FLAG-Li-TPR* constructs were transformed into BL21 (DE3) Rosetta *E. coli* strain. An overnight *E. coli* culture grown at 37°C in LB medium with suitable antibiotics and vigorous shaking was diluted (1:100) in the same medium and allowed to grow in the same conditions until the OD_{600} was 0.4–0.6. Then *Li*-trypanothione reductase gene *(Li-TPR)* expression was induced by addition of 1-mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) during 16 h at 26°C. The cells were centrifuged for 5 min at 9,000× *g* and 4°C and the wet pellet was resuspended in a lysis buffer containing 50-mM Tris pH 7, 300-mM NaCl, 25-mM imidazole, protease inhibitor cocktail and 1 $mg{\cdot}ml^{-1}$ of lysozyme. Following a 30-min incubation on ice, the cell lysate was sonicated on ice (50% pulses, potency 7) for 30 min using a Sonifier Cell Disruptor B15 (Branson, Danbury, CT, USA) and centrifuged during 1 h at 50,000× *g* and 4°C. Supernatant was sonicated again as previously described for 10 min and loaded on a HisTrap column (GE Healthcare, Chicago, IL, USA) during 16 h at 4°C using a peristaltic pump P-1 (GE Healthcare). Once loaded, the HisTrap column was connected to an ÄKTApurifier UPC 10 (GE Healthcare) and extensively washed using a 10% gradient between buffers A (50-mM Tris pH 7 and 300-mM NaCl) and B (50-mM Tris pH 7, 300-mM NaCl and 500-mM imidazole). *Li*-trypanothione reductase was eluted using a 40% gradient between buffers A and B. Fractions containing recombinant *Leishmania infantum*-trypanothione reductase were pooled and loaded into a HiPrep™ 26/10 Desalting Column (GE Healthcare). Finally, *Leishmania infantum*-trypanothione reductase was concentrated to 2 $mg{\cdot}ml^{-1}$ using an Amicon® Ultra-15 50K (Merck Millipore, Burlington, MA, USA) and an equal volume of glycerol was added before storing at −20°C.

2.3 | *Leishmania infantum*-trypanothione reductase oxidoreductase activity

Oxidoreductase activity was determined spectrophotometrically using a modified version of the DTNB-coupled assay described by Hamilton, Saravanamuthu, Eggleston Fairlamb (2003). Briefly, reactions (250 µl) were carried out at 26°C in HEPES buffer (pH 7.5, 40 mM) containing EDTA (1 mM), NADPH (150 µM), $NADP^+$ (30 µM), DTNB (25 µM), oxidized trypanothione (1 µM), glycerol (0.02%), DMSO (1.75%) and recombinant *Leishmania infantum*-trypanothione reductase (7 nM). At the concentration used in this assay, DTNB does not have any relevant effect on the kinetics of the enzymatic reaction. Enzyme activity was monitored at 26°C by the increase in absorbance at 412 nm in an EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). TNB (2-nitro-5-metabercaptobenzoic acid) concentration (µM) was obtained by multiplying the absorbance values by 100 (50-µM TNB generates 0.5 arbitrary units of absorbance at 412 nm). One molecule of reduced trypanothione reduces one molecule of DTNB to generate two TNB molecules. All the assays were conducted in three independent experiments.

2.4 | Preincubation time dependence of *Leishmania infantum*-trypanothione reductase inhibition by P4

Leishmania infantum-trypanothione reductase (8.8 nM) was incubated at 26°C with increasing concentrations of P4 or the vehicle (DMSO) during different time periods in activity buffer (200 µl) containing HEPES (pH 7.5, 40 mM), EDTA (1.2 mM), NADPH (375 µM), $NADP^+$ (75 µM) and DTNB (31.25 µM). After incubation, all reactions were initiated at the same time by addition of 50 µl of HEPES buffer (pH 7.5, 40 mM) containing oxidized trypanothione (5 µM). The final assay mixtures (250 µl) contained HEPES (pH 7.5, 40 mM), EDTA (1 mM), NADPH (300 µM), $NADP^+$ (60 µM), DTNB (25 µM), oxidized trypanothione (1 µM), glycerol (0.02%), DMSO (1.75%) and recombinant *Leishmania infantum*-trypanothione reductase (7 nM). Enzyme activity was monitored at 26°C by the increase in absorbance at 412 nm in an EnSpire Multimode Plate Reader (PerkinElmer). All the assays were conducted in three independent experiments.

2.5 | Reversibility of *Leishmania infantum*-trypanothione reductase inhibition by P4

The reversibility of *Leishmania infantum*-trypanothione reductase inactivation by P4 was assessed by measuring the oxidoreductase activity of extensively diluted samples of the enzyme preincubated with or without inhibitor. *Leishmania infantum*-trypanothione reductase (400 nM) was incubated with 25 μM of either P4 or mepacrine in a buffer containing 50-mM Tris pH 7 and 300-mM NaCl during 16 h at 37°C with agitation and in a humid atmosphere. After incubation, samples were centrifuged for 15 min at 18,000× *g* and 4°C and supernatants were recovered. Finally, oxidoreductase activity was determined in a solution 2,500-fold diluted (0.16-nM *Leishmania infantum*-trypanothione reductase with or without 10 nM of either P4 or mepacrine) in activity buffer:- HEPES (pH 7.5, 40 mM), EDTA (1 mM), NADPH (300 μM), $NADP^+$ (60 μM), DTNB (150 μM) and oxidized trypanothione (50 μM). Enzyme activity was monitored at 26°C by the increase in absorbance at 412 nm in an EnSpire Multimode Plate Reader (PerkinElmer). All the assays were conducted in three independent experiments.

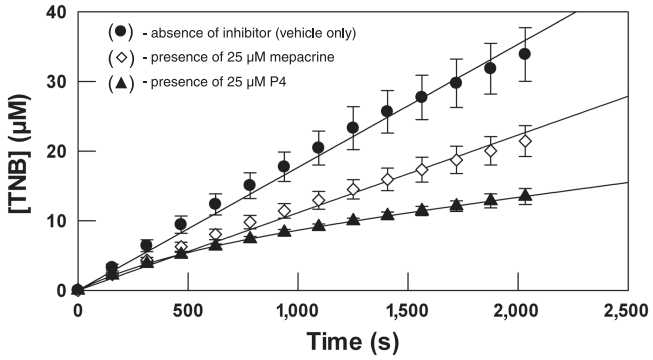


FIGURE 1 Time-dependent inhibition of *Leishmania infantum*-trypanothione reductase by P4. Reaction progress curves of *Li*-TryR in the absence of inhibitor or in the presence of 25-μM mepacrine or 25-μM P4. TNB production was monitored by the increase in absorbance at 412 nm. Data represent the mean ± SD of three independent experiments. Reaction progress curves of *Li*-TryR in the absence or in the presence of 25-μM mepacrine were fitted to a linear trend line whereas reaction progress curve of *Li*-Try R in the presence of 25-μM P4 was fitted to Equation 1

2.6 | Determination of P4 inhibitory constant

The inhibitory constant (K_i) for P4 was calculated following a slightly modified version of the standard DTNB-coupled assay (Hamilton et al., 2003). oxidized trypanothione was serially diluted (5-point dilution from 833 to 52 μM) in a buffer containing 40-mM HEPES (pH 7.5) and 1-mM EDTA. The different oxidized trypanothione solutions were dispensed (15 μl) into a 96-well microplate. P4 was serially diluted in DMSO (6-point dilution from 1.25 mM to 39 μM). The different aliquots of P4 and the equivalent amount of the vehicle (DMSO) were added to a pre-assay mixture yielding different mixtures containing 40-mM HEPES (pH 7.5), 1-mM EDTA, 416.67-μM NADPH, 208.33-μM DTNB, 1.45% DMSO and different P4 concentrations ranging from 0.54 to 34.72 μM. These mixtures (180 μl) were subsequently added to the appropriate wells previously filled with different oxidized trypanothione solutions. The assay was initiated by addition of 55 μl of a buffer containing 40-mM HEPES (pH 7.5), 1-mM EDTA, 272.73-μM $NADP^+$, 0.01% glycerol and 3.5-nM recombinant *Leishmania infantum*-trypanothione reductase using an automated dispensing system (PerkinElmer). The order of addition was essential to avoid enzyme preincubation with the inhibitor or the substrates. The final 250-μl assay contained 40-mM HEPES buffer (pH 7.5), 1-mM EDTA, 300-μM NADPH, 60-μM $NADP^+$, 150-μM DTNB, 3.12–50 μM of oxidized trypanothione, 1.04% DMSO, 0.002% glycerol and 0.8-nM recombinant *Leishmania infantum*-trypanothione reductase. Enzyme activity was monitored at 26°C by the increase in absorbance at 412 nm in an EnSpire Multimode Plate Reader (PerkinElmer). Coupling

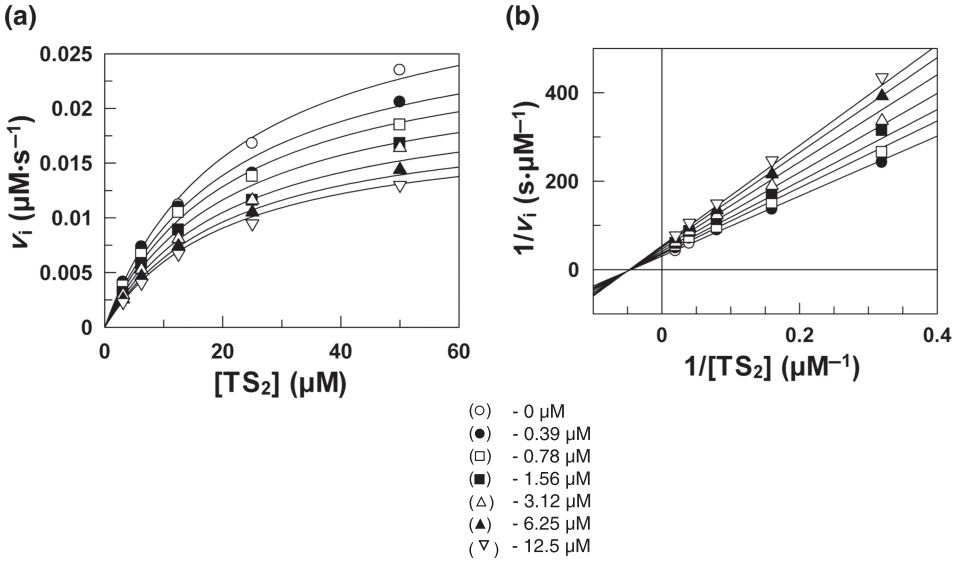


FIGURE 2 Non-competitive hyperbolic inhibition of *Leishmania infantum*-trypanothione reductase by P4. (a) Plot of the initial velocities (v_i) as a function of oxidized trypanothione (TS_2) concentration at seven different P4 concentrations: 0, 0.39, 0.78, 1.56, 3.12, 6.25 and 12.5 μM. 2-nitro-5-metabercaptobenzoic acid (TNB) production was monitored by the increase in absorbance at 412 nm. Curve was fitted using Equation 2 and results of the fit are shown in Table 1. (b) Double-reciprocal plot. Data are the results obtained in a representative assay from three independent experiments

an automated dispenser module to the spectrophotometer enabled real-time detection of the enzymatic reaction, which was crucial for high-quality non-linear fits of the experimental data and, especially, to estimate the initial velocity of the progress curves in the presence of P4. All the assays were conducted in three independent experiments.

2.7 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). No outliers were excluded in the data analysis nor in data presentation.

GraFit 6.0 software (Erithacus, Horley, SRY, UK) was used to perform linear and non-linear regressions. All experiments were undertaken in triplicate to ensure the reliability of single values.

For the experiments conducted in the absence of any preincubation period, k_{obs} values were estimated for every P4 concentration at all oxidized trypanothione concentrations by fitting the values of TNB concentration produced during each enzymatic reaction to Equation 1 (Copeland, 2013):

$$[P] = v_s t + \frac{v_i - v_s}{k_{obs}}[1 - \exp(-k_{obs} t)] + d \quad (1)$$

where v_i and v_s are the initial and steady state velocities, respectively, k_{obs} is the apparent first-order rate constant for the conversion of v_i into the steady state velocity (v_s) and d is a parameter that indicates the displacement of the curve on the vertical ordinate and takes into account any nonzero value of the measured signal at time zero caused by some of the reactants (i.e. small amounts of the reduced forms of Ellman's reagent or trypanothione) (Baici, 2015; Hamilton et al., 2003; Morrison & Cleland, 1983).

Dependency of v_i on [P4] and [oxidized trypanothione] was performed using Equation 2 (Leskovac, 2003):

$$v = \frac{V_{max}[S]}{K_m\left(\frac{1+\frac{[I]}{K_i}}{1+\frac{\beta[I]}{\alpha K_i}}\right) + [S]\left(\frac{1+\frac{[I]}{\alpha K_i}}{1+\frac{\beta[I]}{\alpha K_i}}\right)} \quad (2)$$

The time dependence of *Leishmania infantum*-trypanothione reductase inhibition by preincubation with P4 was analysed by fitting the experimental data to Equation 3 (Copeland, 2013):

$$\frac{v_i}{v_0} = \exp(-k_{obs} t) \quad (3)$$

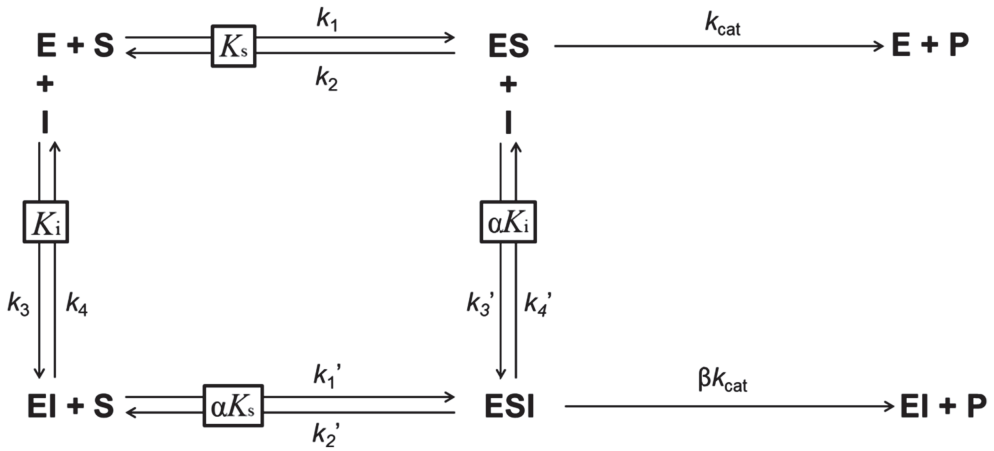
where v_i is the initial velocity at each inhibitor concentration and preincubation period and v_0 is the initial velocity without preincubation and in the absence of inhibitor.

The apparent inhibition constants (K_i^{app}) for each oxidized trypanothione concentration were determined by fitting the k_{obs} values obtained at different P4 concentrations to Equation 4:

TABLE 1 Estimation of α, β and the inhibition constant in the non-competitive hyperbolic inhibition mechanism

α	K_i (μM)	β
0.95 ± 0.18	1.34 ± 0.30	0.52 ± 0.05

Note: Estimated values ± standard error of α, β and K_i were obtained by fitting to Equation 2 the v_i values for every P4 concentration at the different fixed oxidized trypanothione concentrations assayed.



SCHEME 1 Mechanism of the hyperbolic inhibition of *Leishmania infantum*-trypanothione reductase by P4. Modified version of the classical scheme for linear simple inhibition that accounts for the possibility of a productive enzyme/substrate/inhibitor (ESI) complex with a catalytic rate constant (k_{cat}) value modified by the β factor. K_s is the equilibrium constant for the association of free enzyme (E) and substrate (S) to form the enzyme/substrate (ES) complex. k_1 and k_2 are the first-order rates of association and dissociation, respectively. K_i is the equilibrium constant for the association of enzyme (E) and inhibitor (I) to form the EI complex. k_3 and k_4 are the first-order rates of association and dissociation, respectively. α is the factor that modifies the equilibrium constants K_s and K_i when the substrate or the inhibitor binds to the EI or the ES complexes, respectively

$$k_{obs} = k_6 + \frac{k_5[I]}{K_i^{app} + [I]} \quad (4)$$

where K_i^{app} is the apparent value of the K_i for the initial encounter complex (k_4/k_3). k_5 and k_6 are the first-order rate constants that define the isomerization equilibrium between enzyme/inhibitor (EI) and E*I (Copeland, 2000, 2013).

The overall inhibition constant (K_i^*) for a slow-binding inhibitor that conforms to a two-step mechanism is defined by Equation 5:

$$K_i^* = \frac{K_i k_6}{k_5 + k_6} \quad (5)$$

The inhibition modality of P4 was assessed graphically by fitting the K_i^{app} values obtained at different oxidized trypanothione concentrations (Equation 6). The value obtained for α defines the mode of inhibition (competitive: $\alpha \rightarrow$ infinite; uncompetitive: $\alpha \rightarrow 0$; pure non-competitive $\alpha = 1$; mixed: $1 < \alpha < 10$; Copeland, 2000).

$$K_i^{app} = \frac{[S] + K_m}{\frac{K_m}{K_i} + \frac{[S]}{\alpha K_i}} \quad (6)$$

The inhibition modality of P4 was also assessed graphically by fitting the k_{obs} values obtained at different oxidized trypanothione concentrations at a fixed P4 concentration to derivations of the equations formulated by Tian and Tsou (1982; Copeland, 2000, 2013). These equations describe the relationship between k_{obs} and the substrate concentration for competitive (Equation 7A), uncompetitive (Equation 7B) and pure non-competitive (Equation 7C) slow-binding inhibitors:

$$k_{obs} = \frac{k}{1 + [S]/K_m} \quad (7A)$$

$$k_{obs} = \frac{k'}{1 + K_m/[S]} \quad (7B)$$

$$k_{obs} = k \quad (7C)$$

where the Michaelis constant K_m is the substrate concentration at which the reaction rate is half of the maximal velocity, [S] is the substrate concentration, k is the rate constant between the inhibitor and the enzyme (y intercept of the plot of k_{obs} as a function of substrate concentration) and k' is the rate constant between the inhibitor and the enzyme/substrate (ES) complex. Comparison of these three equations with those that describe the relationship between the true K_i and the K_i^{app} observed at different substrate concentrations

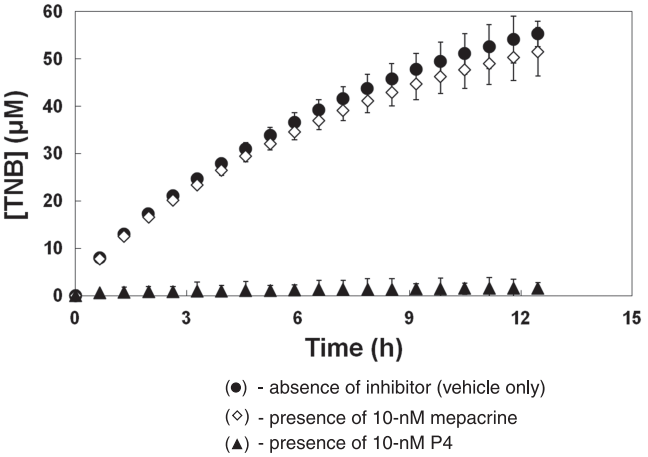


FIGURE 4 Pseudoirreversible inhibition of *Leishmania infantum*-trypanothione reductase by P4. *Li*-TryR (400 nM) was incubated during 16 h in the absence of inhibitor or in the presence of 25 μM of mepacrine or P4. Samples were diluted (2,500-fold) and *Li*-TryR activity was evaluated in enzymatic reaction mixtures containing trypanothione reductase (TryR; 0.16 nM) in the absence of inhibitor or in the presence of 10 nM of mepacrine or 10 nM of P4. 2-nitro-5-metabercaptobenzoic acid (TNB) production was monitored by the increase in absorbance at 412 nm. Data represent the mean ± SD of three independent experiments

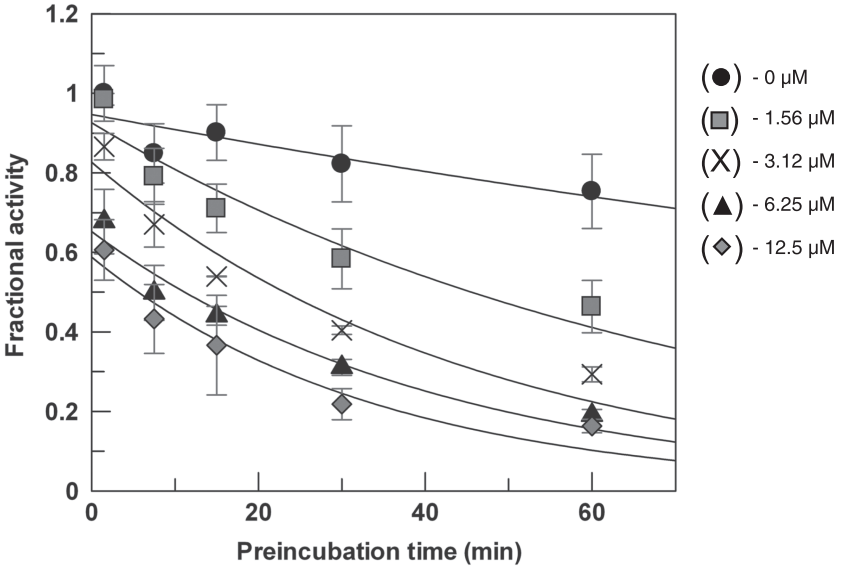


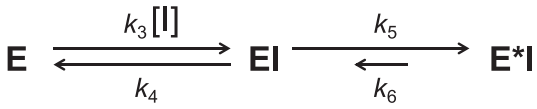
FIGURE 3 Effect of preincubation time with P4 on the fractional activity of *Leishmania infantum*-trypanothione reductase (*Li*-TryR). Fractional velocity is the quotient between the initial velocity (v_i) at each inhibitor concentration and the initial velocity in the absence of inhibitor and without preincubation (v_0). Preincubation time dependence of the fractional velocity was measured at different P4 concentrations: 0, 1.56, 3.12, 6.25 and 12.5 μM. Data represent the mean ± SD of three independent experiments. All curves were fitted to Equation 3

(Copeland, 2000) renders a general expression (Equation 8), which includes the α constant that defines each inhibitory mechanism.

$$k_{obs} = \frac{k(K_m + [S]/\alpha)}{K_m + [S]} \quad (8)$$

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).



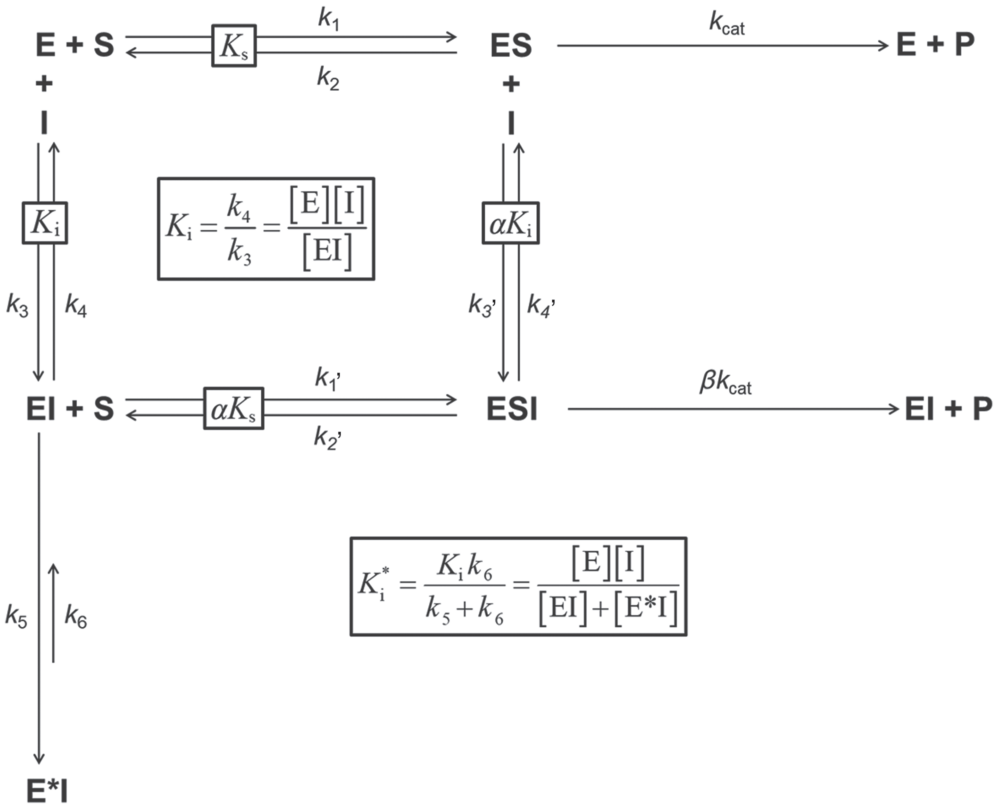
SCHEME 2 Two-step mechanism of time-dependent inhibition of *Leishmania infantum*-trypanothione reductase (*Li*-TryR) by P4. The first step is a rapid equilibrium that generates the enzyme/inhibitor (EI) complex. This process is governed by the association rate (k_3) and the dissociation rate (k_4). The second step consists of a slow and reversible inactivation of the enzyme that is governed by the forward isomerization rate (k_5) and the much smaller reverse rate constant (k_6)

3 | RESULTS

3.1 | TRL 35 is a slow-binding inhibitor

Data obtained from *Leishmania infantum*-trypanothione reductase activity in the presence of P4 indicate a progressive loss of activity of the enzyme that is not observed with other inhibitors. Observation of the reaction progress curves in the presence of mepacrine and P4 (Figure 1) reveals that, in the case of mepacrine, the reaction rate is almost constant until DTNB (that allows substrate regeneration) is consumed. This behaviour is characteristic of classical inhibitors that display a rapid and reversible inhibition. In contrast, in the case of P4, the velocity for the reaction progressively decreases with time until it reaches a value very close to zero long before all the DTNB is consumed. In fact, even after more than 24 h of incubation, analysis of the colour intensity in the wells showed that DTNB was not completely exhausted. The progress curve of this reaction is typical of a slow-binding inhibitor (Copeland, 2000, 2013) and further suggested that P4 could be an irreversible or pseudoirreversible inhibitor of *Leishmania infantum*-trypanothione reductase.

Despite the loss of activity observed during the reaction, dependency of the initial velocities (v_i) on P4 and oxidized trypanothione concentrations could be fitted to a model in which a rapid equilibrium is reached between free enzyme and inhibitor (E + I) and the enzyme/inhibitor (EI) complex (Figure 2; Scheme 1). Our data reveal a non-linear non-competitive hyperbolic inhibition mechanism in which the enzyme/substrate/inhibitor (ESI) complex is still able to yield product although less readily than does the enzyme/substrate



SCHEME 3 Global mechanism for trypanothione reductase activity in the presence of substrate (S) and inhibitor (I). Productive enzyme/substrate (ES) and enzyme/substrate/inhibitor (ESI) complexes generated through different pathways are able to generate product (P) with catalytic rate constants k_{cat} and $\beta^* k_{cat}$, respectively. E*I is an inactive form of E produced through a slow process governed by the forward isomerization rate (k_5) and the very small reverse rate constant (k_6)

(ES) complex. Kinetic data are indicated in Table 1. For this rapid equilibrium process, a K_i value of 1.34 ± 0.3 μM is obtained. An α value of 1 is indicative of a non-competitive mechanism and a β value of 0.5 reveals that the enzyme/substrate/inhibitor complex is still able to generate product at half velocity than enzyme/substrate complex.

3.2 | Preincubation with P4 enhances *Leishmania infantum*-trypanothione reductase inactivation

Different concentrations of P4 were incubated with *Leishmania infantum*-trypanothione reductase for different time lengths before initiating the reaction by addition of oxidized trypanothione. The

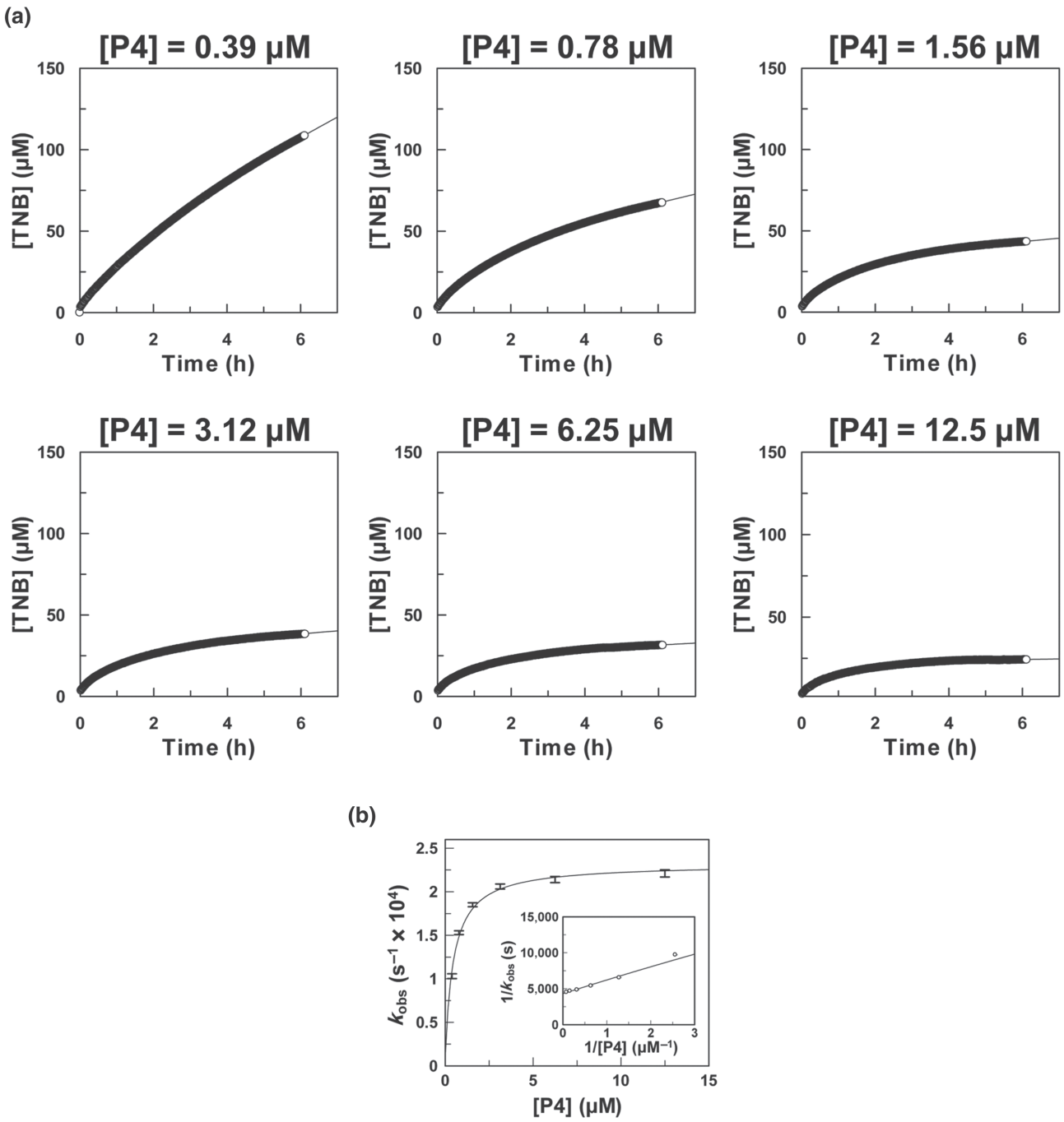


FIGURE 5 P4 concentration dependence of the observed rate constant for *Leishmania infantum* trypanothione reductase inactivation at 6.25-μM oxidized trypanothione (TS_2). (a) Progress curves for *Li*-TryR enzymatic reactions in the presence of increasing concentrations of P4 were fitted to Equation 1. Data are the results obtained in a representative assay from three independent experiments. The estimated k_{obs} values are shown in Table 2. (b) Plot of the k_{obs} values (± standard errors) as a function of P4 concentration. A double-reciprocal plot is shown in the inset. Curve was fitted using Equation 4 and results of the fit are shown in Table 3 (6.25-μM TS_2)

residual activities of the enzyme (relative to a [P4] = 0) as a function of the preincubation time are shown in Figure 3. As already described for slow-binding inhibitors (Copeland, 2000, 2013), the residual activity exponentially decays as the preincubation time increases. This effect becomes more pronounced with increasing inhibitor concentrations. It must be pointed out that the *y* intercepts of the exponential fits shown in the figure are significantly different from 1, which is considered an indication of a two-step inhibitory mechanism (Copeland, 2000, 2013). This reduction in the initial activity values is a consequence of the rapid primary inhibition event described above (Figure 2) whereas the subsequent exponential decay reveals a much slower secondary event that occurs after formation of the initial enzyme/inhibitor binary complex.

3.3 | P4 is a pseudoirreversible inhibitor of *Leishmania infantum* -trypanothione reductase

To assess the irreversible nature of this inhibition process, we incubated *Leishmania infantum*-trypanothione reductase (400 nM) with P4 (25 μM) for 16 h; 25-μM mepacrine was used as a control. After incubation, enzyme and inhibitors were diluted (∼2,500-fold) to final concentrations of 0.16 and 10 nM, respectively, before the reaction was started. It must be pointed out that when enzyme and inhibitor are mixed directly at these concentrations, none of the two inhibitors (mepacrine or P4) is able to noticeably reduce the speed of the reaction. The results shown in Figure 4 indicate that the enzyme incubated with P4 is completely inactive at the beginning of the assay. Only an almost negligible activity can be detected at the end of the 12-h period assayed, which might be indicative of an extremely slow recovery of the enzyme after removal of P4. In this case, the inactivation process could be considered reversible but with a very small k_{off} (k_6) value; hence, the process can be considered pseudoirreversible. Mepacrine, in contrast, behaves as a classical reversible inhibitor, allowing immediate recovery of the enzyme activity after the dilution process.

3.4 | Kinetic data can be adjusted to an enzyme isomerization mechanism of inhibition

The results obtained so far suggest that inhibition of *Leishmania infantum*-trypanothione reductase by P4 is a slow and pseudoirreversible process. Under these premises, we evaluated whether the reaction progress curves obtained could fit to those characteristic of isomerization mechanisms of inhibition (Copeland, 2000, 2013). In this inactivation model, the process of generating an inactive form of *Leishmania infantum*-trypanothione reductase after binding to P4 is governed by k_5 and k_6 first-order rate constants that describe an isomerization equilibrium between the initial enzyme/inhibitor (EI) complex and a second high-affinity complex (E*I) (Scheme 2). Similarly, k_{obs} is the first-order rate constant that defines the characteristic curvature of the progress curve so that its value describes the conversion from the initial reaction rate (v_i) to the final steady state velocity (v_s) (Equation 1). Under strictly irreversible conditions (e.g. due to covalent bond formation), v_s should reach the 0 value because, given enough time, there should not be any enzyme activity in the presence of inhibitor. All attempts to fit the progress curves to a model with $v_s = 0$ rendered poor fittings and this can be taken as an indication of the existence of a very small recovery of active enzyme from the inactive isomerized form (E*I); hence, the k_6 constant is different from 0.

Adjustment of the progress curves of the reactions catalysed by *Leishmania infantum*-trypanothione reductase to Equation 1 allowed us to obtain the k_{obs} values for each P4 concentration at a defined concentration of oxidized trypanothione (Figure 5a). Table 2 shows the k_{obs} values obtained at 6.25-μM oxidized trypanothione when P4 concentrations vary from 0.39 to 12.5 μM.

The different k_{obs} values obtained at a fixed oxidized trypanothione concentration can be used to determine the values of k_5, k_6 and K_i^{app} according to Equation 4 (Copeland, 2000, 2013). However, when all these three parameters were freely estimated during the fitting process, the confidence interval for k_6 contained the 0 value at all the substrate concentrations tested. Based on the relationship

TABLE 2 Rate constants for *Leishmania infantum*-trypanothione reductase time-dependent inhibition by P4 at 6.25-μM oxidized trypanothione

[P4] (μM)	k_{obs} (s^{-1})
0.39	$1.03 \times 10^{-4} \pm 2.78 \times 10^{-6}$
0.78	$1.53 \times 10^{-4} \pm 2.18 \times 10^{-6}$
1.56	$1.85 \times 10^{-4} \pm 2.29 \times 10^{-6}$
3.12	$2.06 \times 10^{-4} \pm 2.92 \times 10^{-6}$
6.25	$2.14 \times 10^{-4} \pm 3.51 \times 10^{-6}$
12.5	$2.21 \times 10^{-4} \pm 4.13 \times 10^{-6}$

Note: k_{obs} values for each P4 concentration were obtained by fitting to Equation 1 the progress curves shown in Figure 5a. Results are the estimated values from the non-linear regression ± the associated standard errors.

TABLE 3 Apparent inhibition constants and isomerization rate constants for *Leishmania infantum*-trypanothione reductase time-dependent inhibition by P4

[TS_2] (μM)	K_i^{app} (μM)	k_5 (s^{-1})
3.12	0.49 ± 0.13	$2.13 \times 10^{-4} \pm 1.23 \times 10^{-5}$
6.25	0.47 ± 0.04	$2.22 \times 10^{-4} \pm 4.30 \times 10^{-6}$
12.5	0.63 ± 0.21	$2.54 \times 10^{-4} \pm 1.96 \times 10^{-5}$
25	0.62 ± 0.20	$2.36 \times 10^{-4} \pm 1.78 \times 10^{-5}$
50	0.52 ± 0.10	$2.08 \times 10^{-4} \pm 8.58 \times 10^{-6}$

Note: K_i^{app} and k_5 values at different TS_2 concentrations were obtained by fitting to Equation 4 the k_{obs} values for every P4 concentration at the different fixed oxidized trypanothione (TS_2) concentrations assayed. Results are the estimated values of the non-linear regression ± the associated standard errors.

among k_{obs}, v_i and v_s ($k_6 = k_{obs} \times v_i/v_s$) at all the oxidized trypanothione and P4 concentrations assayed, k_6 varies between 2×10^{-6} s^{-1} and 4.5×10^{-5} s^{-1}. Taking into consideration that only a negligible recovery of activity could be observed after 12 h (Figure 4), a value of 1×10^{-5} s^{-1} was selected as a conservative upper value for k_6. Fixing this value for k_6 rendered very good estimations of k_5 and K_i^{app} after the fitting process. Nonetheless, it must be pointed out that re-analysis of the data for a k_6 value of 4.5×10^{-5} s^{-1} generated very similar estimations.

Adjustment to Equation 4 of the k_{obs} values found at 6.25-μM oxidized trypanothione (Figure 5b) generates a value of 2.22×10^{-4} s^{-1} for k_5 and 0.47 μM for K_i^{app} (Table 3). The non-zero intercept at the double-reciprocal plot (Figure 5b ; inset) indicates that, as already suggested by the results shown in Figure 3, the inhibition mechanism requires two sequential steps:- an initial binding step followed by a slower inactivation event (Copeland, 2000, 2013). The same process described for a oxidized trypanothione concentration of 6.25 μM was repeated at different oxidized trypanothione concentrations ranging from 3.12 to 50 μM to obtain the k_5 and K_i^{app} values at five different substrate concentrations (Figure 6, Table 3 and supporting information).

Even though most of the slow-binding inhibitors reported in the literature are active-site directed (and hence competitive) inhibitors, this kind of inhibitors may also interact with the enzyme through non-competitive or uncompetitive inhibition mechanisms. The inhibition modality may be determined from the effect of substrate concentration on the value of k_{obs} at any fixed inhibitor concentration (Copeland, 2000, 2013; Tian & Tsou, 1982). For a competitive

TABLE 4 Estimation of α values at different P4 concentrations

[P4] (μM)	α
0.39	1.52 ± 0.50
0.78	0.98 ± 0.18
1.56	1.05 ± 0.17
3.12	1.00 ± 0.17
6.25	0.93 ± 0.20
12.5	1.06 ± 0.14

Note: Estimated values ± standard error of α at different P4 concentrations were obtained by fitting to Equation 8 the k_{obs} values as a function of oxidized trypanothione (TS_2) concentration at five different P4 concentrations.

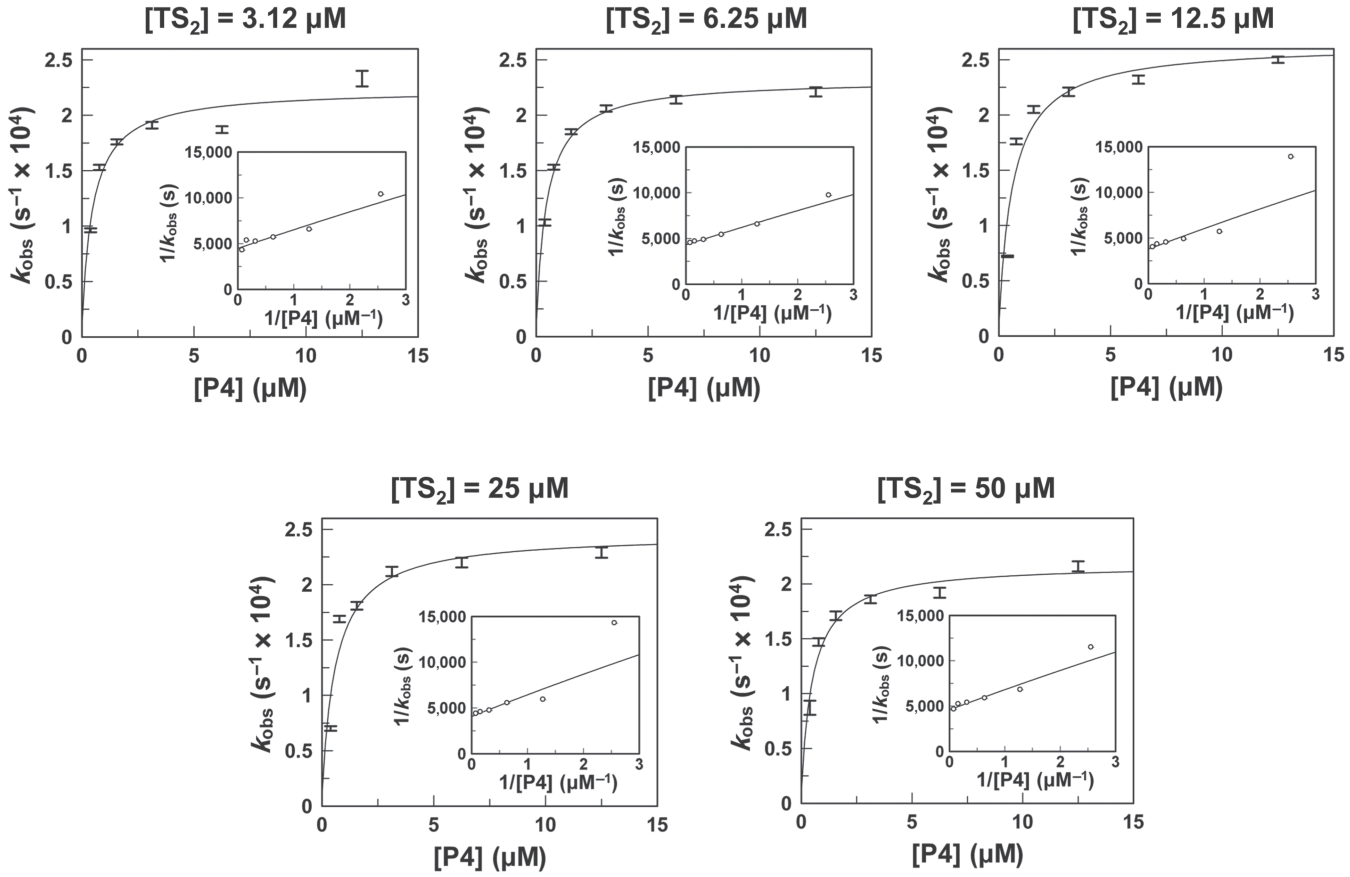


FIGURE 6 P4 concentration dependence of the observed rate constants of *Leishmania infantum* trypanothione reductase (*Li*-TryR) inactivation at different oxidized trypanothione (TS_2) concentrations. Plot of the k_{obs} values (± standard errors) as a function of P4 concentration at five different TS_2 concentrations (3.12, 6.25, 12.5, 25 and 50 μM) (k_{obs} determinations for every P4 concentration at the different fixed TS_2 concentrations assayed are shown in the supporting information). Double-reciprocal plots are shown in the insets. Curves were fitted using Equation 4 and results of the fits are shown in Table 3

inhibitor, the value of k_{obs} diminishes hyperbolically with increasing substrate concentrations according to Equation 7A. For uncompetitive inhibition, this value increases as a rectangular hyperbola according to Equation 7B. Finally, for non-competitive inhibition, the value of k_{obs} varies with substrate concentration depending on the value of α, which is the factor that transforms the dissociation constants K_i to K_i' and K_s to K_s' when the enzyme is bound both to the substrate and to the inhibitor. In the extreme case in which the inhibitor binds with equal affinity to the free enzyme and to the enzyme/substrate complex (α = 1), the value of k_{obs} remains constant at any substrate concentration (Equation 7C). Equations 7A–7C are particular cases of the general expression shown in Equation 8.

For each P4 concentration, the k_{obs} values obtained at the different oxidized trypanothione concentrations assayed could only be fitted to Equation 7C describing a pure non-competitive inhibition with an α value of 1. In fact, adjustment of these data to the general expression shown in Equation 8 allowed us to obtain six different estimations for α (one at every P4 concentration), all of them with an approximate value of 1 (Table 4). According to the results shown in Figure 7, P4 shows a non-competitive mechanism of inhibition and binds with similar affinity to *Leishmania infantum*-trypanothione reductase in the absence or in the presence of oxidized trypanothione (α = 1).

The relationships between the different K_i^{app} and K_i values in slow-binding inhibitors are the same as those for classical linear reversible inhibitors (Copeland, 2000, 2013). Applying Equation 6 to fit the K_i^{app} values obtained at the different oxidized trypanothione concentrations assayed (Figure 8), we could obtain an estimated value for α (1.29 ± 0.28) and K_i (0.46 ± 0.03 μM). As shown in Table 3, the estimated values for k_5 fluctuate between 2.08 × 10^{-4} s^{-1} and 2.54 × 10^{-4} s^{-1} (mean of 2.27 × 10^{-4} ± 1.7 × 10^{-5} s^{-1}). Based on the

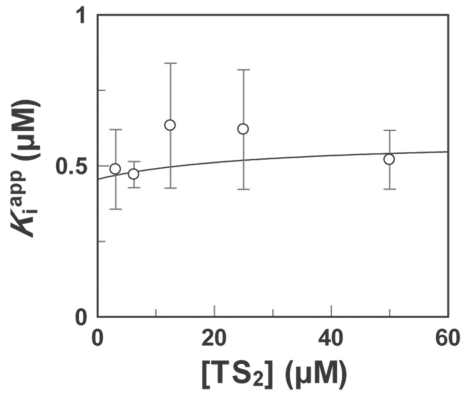


FIGURE 8 Effect of substrate concentration on the apparent inhibition constants of P4 over *Leishmania infantum*-trypanothione reductase. Plot of the estimated values of K_i^{app} (± standard errors) shown in Table 3 as a function of oxidized trypanothione (TS_2) concentration. The curve was fitted using Equation 6 and results of the fit are shown in Table 5

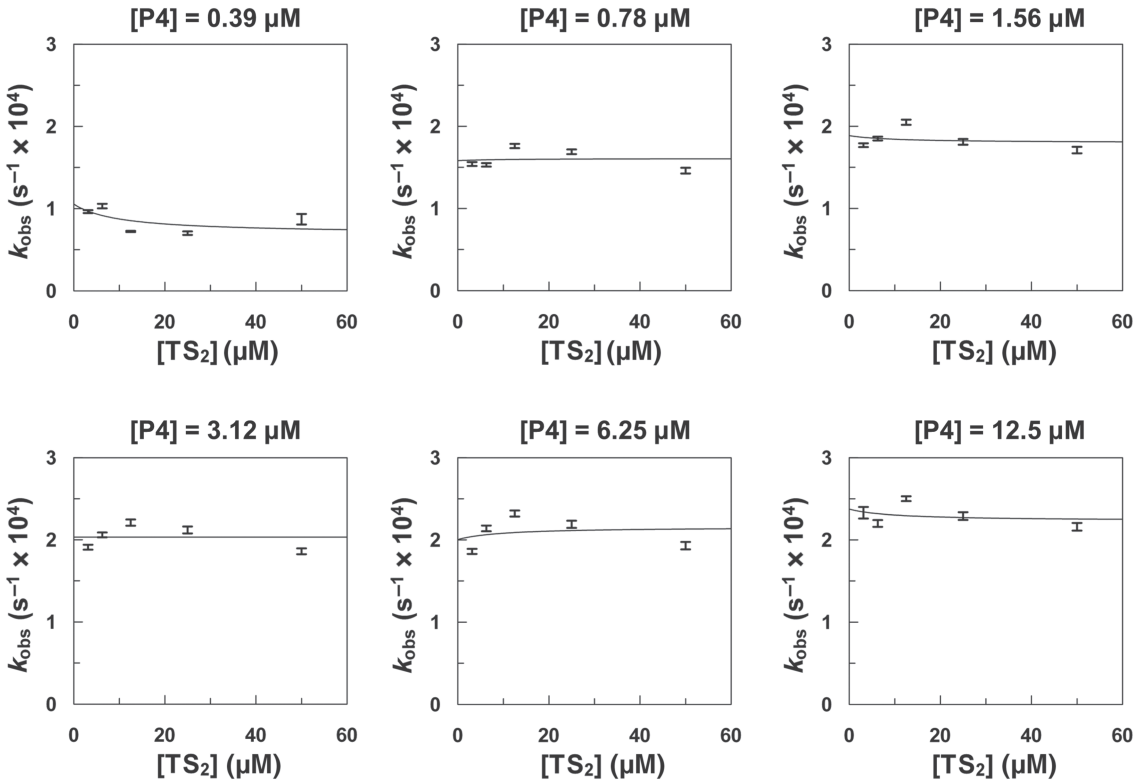


FIGURE 7 Effect of oxidized trypanothione (TS_2) concentration on the rate of *Leishmania infantum* trypanothione reductase inactivation at different P4 concentrations. Plots of the estimated values of k_{obs} (± standard errors; see supporting information) as a function of oxidized trypanothione (TS_2) concentration at six different P4 concentrations (0.39, 0.78, 1.56, 3.12, 6.25 and 12.5 μM). Curves were fitted using Equation 8 and results of fits are shown in Table 4

TABLE 5 Estimation of α, k_5 and K_i in the time-dependent inhibition mechanism

α	K_i (μM)	k_5 (s^{-1})
1.29 ± 0.28	0.46 ± 0.03	$2.27 \times 10^{-4} \pm 1.87 \times 10^{-5}$

Note: Estimated values ± standard error of α and K_i were obtained by fitting to Equation 6 the K_i^{app} values obtained for every oxidized trypanothione concentrations assayed. k_5 is the mean ± SD of all k_5 values obtained by fitting to Equation 4 the k_{obs} values for every P4 concentration at the different fixed oxidized trypanothione concentrations assayed.

assumption of an upper value of 1×10^{-5} s^{-1} for k_6 and an estimation of 0.46 μM for K_i, the maximum value obtained for the overall equilibrium constant K_i^* is 0.02 μM (Equation 5).

4 | DISCUSSION AND CONCLUSIONS

Non-competitive inhibitors are especially interesting as potential drugs because their activity is not affected by the presence of the substrate. This is especially relevant for enzymes such as trypanothione reductase, whose substrate may reach intracellular concentrations above 1 mM. For this reason, since trypanothione reductase is only active in its dimeric form, we chose its dimerization interface as the target for inhibitor design, in an attempt to obtain a non-competitive mechanism of enzyme inactivation. We have previously reported the promissory activity of the prototype peptide P4 to inhibit the oxidoreductase activity of *Leishmania infantum*-trypanothione reductase by means of its capacity to disrupt the dimeric structure of this enzyme (Toro et al., 2013). However, up to now, determination of the true inhibitory constant (K_i) for P4 was unfeasible because, even under conditions in which substrate concentration was kept constant, reaction rates continuously decreased with time. The present work characterizes this progressive loss of activity of the enzyme in the presence of P4 and allows us to conclude that this lead molecule is a time-dependent inhibitor. To be able to select the right model of time-dependent inhibition, we determined whether enzyme activity could be rescued by removal of unbound inhibitor from the enzyme solution. Our results demonstrate that enzyme activity is only negligibly recovered following substantial dilution of a mixture of enzyme and inhibitor, which reveals a pseudoirreversible mode of action for P4.

Our experimental data very precisely adjust to a model in which the inactivation of the enzyme requires two sequential steps:- an initial binding event and a secondary inactivation event. First, the inhibitor binds to the enzyme in a reversible manner to form the enzyme/inhibitor (EI) complex and then a second slower inactivation event occurs that leads to generation of an inactive E*I complex (Scheme 3). The existence of a fast reversible first step is supported by a clear decrease in the fractional activity of the enzyme in the absence of preincubation with the inhibitor (Figure 3; 0-min time), which correlates with inhibitor concentration. This first step was characterized as a non-competitive (α = 1) hyperbolic process based on the analysis of the dependency of v_i on P4 and oxidized trypanothione concentrations. Our data reveal that the enzyme/substrate/inhibitor complex is still able to catalyse the reaction with half the velocity of enzyme/substrate complex (β = 0.5). A K_i value of 1.4 ± 0.3 μM was estimated for this process. Moreover, for every substrate concentration tested, a linear relationship is obtained between $1/k_{obs}$ and 1/[I] that intersects the *y* axis at a value greater than zero, a finding that strongly supports the proposed two-step mechanism of enzyme inactivation (Copeland, 2000, 2013).

Regarding the whole process (first and second steps), the mode of inhibition (competitive, non-competitive or uncompetitive) that takes place upon incubation of the enzyme with a time-dependent inhibitor can be defined by analysis of the effects of varying substrate concentrations on the values of k_{obs} at a fixed concentration of inhibitor (Copeland, 2000, 2013). Our experimental data points fit very precisely to a "pure" non-competitive model in which the inhibitor binds to the free enzyme and to the enzyme/substrate complex with similar affinities (α = 1). Additionally, the mode of inhibition can also be inferred from the relationship between K_i^{app} and substrate concentration. Data fitting to Equation 6 also generates an α value of 1 and a K_i of 0.46 ± 0.03 μM. Accordingly, two different data analyses (Figures 7 and 8) indicate that P4 binds to both the free enzyme (E) and the enzyme/substrate complex with similar affinities. Consequently, our results demonstrate that P4 is a non-competitive inhibitor of *Leishmania infantum*-trypanothione reductase.

Even though the K_i value of 0.46 μM does not exactly match with that obtained using only the initial velocities of the reactions in the hyperbolic model of inhibition (1.34 ± 0.3 μM), we consider that the higher complexity of the analysis and larger number of data points used for its calculation make the 0.46 ± 0.03 μM value a much more accurate estimate of the real K_i for P4.

The second step of the process consists on the slow isomerization of the enzyme inhibitor (EI) complex to an inactive form of the enzyme (E*I) defined by the k_5 (2.27×10^{-4} s^{-1}) and k_6 (upper value of 1×10^{-5} s^{-1}) first-order constants that characterize this pseudoirreversible inhibition mechanism. Under these premises, recovery of half the activity of the enzyme is expected only after almost 20 h in the absence of inhibitor. In practice, this time is longer than that expected for recovery of the enzyme in the cells by new protein synthesis.

Drug–target interactions are classically evaluated in terms of parameters related to the binding properties of the drug such as IC_{50} or K_d. Screening procedures usually are in vitro measurements in which the target is incubated with the drug at different concentrations that remain constant throughout the whole assay. However, these conditions are quite different from those characteristic of an *in vivo* system in which the concentration of ligand to which a target is exposed varies with time because of drug clearance. In this real-life scenario, the efficacy of a drug is related to the rate of receptor–ligand association, but it is particularly dependent on the dissociation rate constant (k_6 in this manuscript). k_6 values essentially depend on specific interactions between the ligand and its binding pocket in the target. Optimization of these interactions reduces dissociation rates

and is a powerful strategy to improve drug activity (Copeland, Pompliano, & Meek, 2006). Moreover, target selectivity is highly improved when dissociation rates are reduced and strategies aimed at decreasing these rates are proposed to be more relevant in terms of increasing efficacy and selectivity than determination of IC_{50}, K_d or K_i at fixed concentrations of the compounds during short periods of time (Copeland et al., 2006).

In summary, our kinetic data nicely fit to a model in which P4 acts as a pseudoirreversible time-dependent non-competitive inhibitor. The long dissociative half-life of P4 relates to a two-step enzyme isomerization mechanism of binding that is responsible for the good efficacy of this compound and its predictable high selectivity against trypanothione reductase. This type of inhibition strongly matches the expected activity of our prototype as a dimerization disruptor directed to the monomer–monomer interface of *Leishmania infantum*-trypanothione reductase.

ACKNOWLEDGEMENTS

The authors thank the Spanish Government (MINECO/FEDER Project SAF2015-64629-C2), the Comunidad de Madrid (PLATESA2-CM ref. S-2018/BAA-4370) and the Consejo Superior de Investigaciones Científicas (CSIC Project 201980E028) for financial support.

AUTHOR CONTRIBUTIONS

A.J.-R., H.d.L. and M.A.T. designed the study and interpreted the kinetic experiments. H.d.L. and M.A.T. conducted the kinetic experiments. A.J.-R. and H.d.L. wrote the first draft of the manuscript. M.-J.C., S.V. and F.G. contributed to the analysis of the kinetic data and revised the first draft of the manuscript. The final version of the manuscript was critically appraised and approved by all authors.

CONFLICT OF INTEREST

The authors declare no competing interests.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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SUPPORTING INFORMATION

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How to cite this article: de Lucio H, Toro MA, Camarasa M-J, Velázquez S, Gago F, Jiménez-Ruiz A. Pseudoirreversible slow-binding inhibition of trypanothione reductase by a protein–protein interaction disruptor. *Br J Pharmacol*. 2020;177:5163–5176. https://doi.org/10.1111/bph.15250