(2,2′:6′,2″-Terpyridine)platinum(II) Complexes Are Irreversible Inhibitors of *Trypanosoma cruzi* Trypanothione Reductase But Not of Human Glutathione Reductase

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(2,2′:6′,2″-Terpyridine)platinum(II) complexes possess pronounced cytostatic activities against trypanosomes and leishmania. As shown here, the complexes are irreversible inhibitors of trypanothione reductase (TR) from *Trypanosoma cruzi*, the causative agent of Chagas' disease. The most effective derivatives are the (4′-chloro-2,2′:6′,2″-terpyridine)platinum(II) ammine and the (4-picoline)(4′-*p*-bromophenyl-2,2′:6′,2″-terpyridine)platinum(II) complexes which in the presence of NADPH inhibit TR with second-order rate constants of about 1.3×10^4 M^{-1} s^{-1}. The modified enzyme species possess increased oxidase activities. The inhibition is not reversed upon dialysis or treatment with low-molecular-mass thiols. Kinetic and spectroscopic data suggest that Cys52 in the active site has been specifically altered. Inhibition of this key enzyme of parasite thiol metabolism probably contributes to the antitrypanosomal activity of the compounds. In contrast to the parasite enzyme, most (terpyridine)platinum complexes interact only reversibly with human glutathione reductase and an initial inhibition is completely abolished during the course of the assay.

Introduction

Trypanosomes and leishmania are the causative agents of tropical diseases such as African sleeping sickness (*Trypanosoma brucei gambiense*, *Trypanosoma brucei rhodesiense*), Chagas' disease (*Trypanosoma cruzi*), Nagana cattle disease (*Trypanosoma congolense*, *Trypanosoma brucei brucei*), Kala-azar (*Leishmania donovani*), and Oriental sore (*Leishmania tropica*). These parasitic protozoa possess a unique thiol metabolism. In contrast to nearly all eukaryotes and prokaryotes, they lack the enzyme glutathione reductase (GR; GSSG + NADPH + $H^+ \rightarrow$ 2GSH + $NADP^+$). Their main thiols are covalent conjugates of glutathione and spermidine, namely monoglutathionylspermidine and bis(glutathionyl)spermidine (trypanothione, $T(SH)_2$),[1] which are kept reduced by the flavoenzyme trypanothione reductase (TR; TS_2 + NADPH + $H^+ \rightarrow T(SH)_2$ + $NADP^+$).

Mechanistically and structurally TR and GR are closely related FAD disulfide oxidoreductases. During catalysis the enzymes change between two stable forms (E and EH_2). E is characterized by an active site disulfide bridge between Cys52 and Cys57 (in *T. cruzi* TR; Cys58 and Cys63 in human GR). Binding of NADPH leads to formation of the two-electron reduced EH_2 which contains the two cysteines as thiols.[2,3] In the EH_2 state, Cys57 (Cys63 in GR) forms the characteristic charge-transfer complex with the prosthetic group FAD, and Cys52 (Cys58 in GR) reacts with the respective disulfide substrate forming a mixed disulfide reaction intermediate.[2−4]

The known sensitivity of trypanosomatids toward oxidative stress and the absence of the enzyme from the mammalian host make TR an attractive target molecule for the development of new antiparasitic drugs.[5,6] Genetic studies on bloodstream *T. brucei* revealed that parasites lacking TR are avirulent and show increased sensitivity to oxidative stress.[7] Despite an overall sequence identity of 41%, human GR and *T. cruzi* TR have mutually exclusive specificities for their respective substrates which should allow the design of selective inhibitors. The availability of the recombinant enzyme[8] and the knowledge of the three-dimensional structure of TR form the basis for a rational inhibitor design. The structures of TR[9−11] and of complexes with its substrates[11−13] as well as with the reversible inhibitor mepacrine[14] have been elucidated.

Different classes of compounds are selective ligands of TR, whereby most of them are competitive inhibitors (for recent reviews, see refs 5, 6, 15). Another class is the so-called turncoat inhibitors (subversive substrates) which induce an oxidase activity (oxidation of NADPH in the absence of the disulfide substrate) in the enzyme. Examples are nitrofuran and naphthoquinone derivatives.[16−18] The one-electron reduction of turncoat inhibitors leads to the formation of reactive oxygen species. The concomitant increase of the intracellular oxidative stress is an interesting mechanism for antiparasitic and antitumor drugs.[19]

Irreversible inhibitors are another type of drug candidate. In contrast to competitive inhibitors, accumulation of substrate due to the blockage of the pathway cannot overcome inhibition. In addition, irreversibly binding ligands may be effective at much lower concentrations. Known covalent inhibitors of TR are the nitrosourea drug carmustine (BCNU),[17] and ajoene, a

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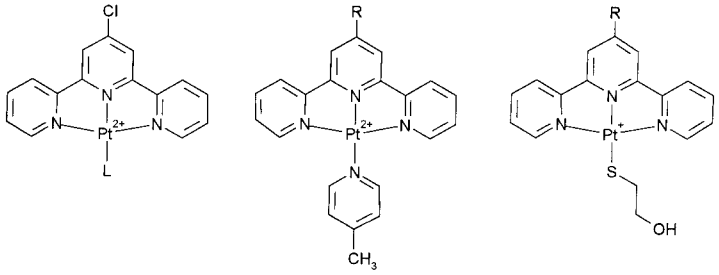
Table 1. Inhibition of *T. cruzi* TR by (2,2′:6′,2″-Terpyridine)platinum(II) Complexes[a]



1 **2** **3**

compd	L or R	$t_{1/2}$ for inhibition (min) in the presence of NADPH at a fixed inhibitor concentration (μM)	$t_{1/2}$ for inhibition (min) in the absence of NADPH at a fixed inhibitor concentration (μM)	k_{obs} for inhibition of the NADPH-reduced enzyme (M^{-1} s^{-1})
1a	NH_3	8 (0.11)	none (0.11)[b]	12200 ± 1100
1b	OH	9 (20)	60 (20)	109 ± 14
1c	$C_{10}H_{16}N_3O_6S$	no inhibition (20)[b]	nd[c]	no inhibition (20)[b]
2a	H	11 (20)	215 (20)	85 ± 24
2b	Cl	90 (20)	453 (20)	10 ± 3
2c	2-C_5H_4N	73 (20)	nd[c]	15 ± 9
2d	*p*-C_6H_5-C_6H_4	20 (4)	74 (4)	382 ± 63
2e	*p*-Br-C_6H_4	11 (0.10)	none (0.10)[b]	13300 ± 1500
3a	H	35 (20)	453 (20)	40 ± 15
3b	OCH_2CH_3	11 (20)	none (20)[b]	83 ± 27

[a] 100 nM *T. cruzi* TR (10 nM TR in the case of **1a** and **2e**) was incubated in the presence and absence of 100 μM NADPH and varying concentrations of inhibitor. At different time intervals 10−20-μL aliquots were removed and the remaining activity was determined in a standard TR assay. [b] ≤10% inhibition which did not increase with time was observed at the respective inhibitor concentrations. [c] nd, not determined.

garlic-derived natural compound,[20] but both compounds also inhibit human GR.[20,21] These covalent inhibitors modify Cys58 in human GR and most probably Cys52 in *T. cruzi* TR.

Platinum(II) complexes have trypanocidal activity. *cis*-Platinum(II) pentamidine complexes were effective against *T. brucei* in vitro as well as in vivo.[22] Recently a series of (2,2′:6′,2″-terpyridine)platinum(II) complexes were revealed as cytotoxic agents against *T. cruzi*, *T. brucei*, and *L. donovani* in culture.[23] Besides their ability to intercalate and/or platinate DNA, platinum(II) complexes can react with cysteinyl and histidinyl residues of proteins.[24−26]

To gain insight into the probable targets of (terpyridine)platinum(II) complexes in trypanosomatids, we studied their interaction with *T. cruzi* TR and human GR. Here we show that (2,2′:6′,2″-terpyridine)platinum-(II) complexes are irreversible inhibitors of parasite TR but most of them are only weak reversible ligands of mammalian GR. The kinetic and spectroscopic data will be discussed in light of the known structures of the enzymes and the antiparasitic activity of these compounds.

Results

In the Presence of NADPH *T. cruzi* TR Is Rapidly Inactivated by (Terpyridine)platinum(II) Complexes. A series of (2,2′:6′,2″-terpyridine)platinum(II) complexes were studied as inhibitors of *T. cruzi* TR (Table 1). The compounds inhibit the NADPH-reduced enzyme in a concentration- and time-dependent manner. The (4′-chloro-2,2′:6′,2″-terpyridine)platinum(II) ammine complex (**1a**) and the (4-picoline)(4′-*p*-bromophenyl-2,2′:6′,2″-terpyridine)platinum(II) complex (**2e**) react most rapidly. Incubation of 10 nM TR with 108 nM **1a** or 100 nM **2e** leads to 50% inactivation within about 10 min. The bimolecular rate constants are 2−3 orders of magnitude higher than those observed with the other complexes. **1a** is not stable in aqueous solution since the ammine is displaced by water.[23] The resulting hydroxy complex (**1b**) reacts with TR much more slowly. A 200-fold higher concentration is needed to inactivate the enzyme in comparable time (Table 1). The p*K* value for the protonation of the hydroxyl group of **1b** in water is about 5.2.[27] At low pH values the complex is converted into the aquo complex. Incubation of TR with **1b** at pH 6.5 did not speed up inhibition compared to that at pH 7.5 (data not shown). Lower pH values could not be used because of the very low activity of TR under these conditions. The glutathione complex **1c** is not an inhibitor of TR.

In the absence of NADPH, only very weak inhibition (≤10%) which does not increase with time is observed with **1a**, **2e**, and **3b**. The other complexes cause a slow inactivation of TR also in the absence of NADPH (Table 1). Maybe in the oxidized enzyme, platinum(II) complexes react with a histidinyl residue as has been described for several proteins.[25,28,29]

The fast inhibition of the reduced enzyme by the platinum complexes is indicative of an active site cysteinyl residue being modified. In the first half-reaction of the catalytic cycle, TR is reduced by NADPH resulting in the formation of EH_2, the two-electron reduced enzyme species. To study if free EH_2 or the EH_2·NADPH complex is the reacting species, 3 nM TR was incubated with 108 nM compound **1a** in the presence of 100 μM NADPH or the nonphysiological coenzyme NADH. After different times, the remaining activity was determined by adding 130 μM TS_2 directly to the incubation mixture. The rate of inhibition was indepen-

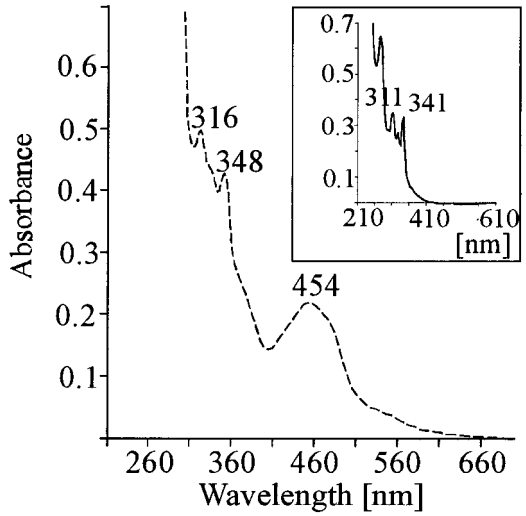


Figure 1. Visible absorption spectrum of TR modified by **1a**. TR was inhibited by complex **1a** in the presence of NADPH as described under Experimental Procedures. The spectra were recorded in TR assay buffer, pH 7.5. Inset: Spectrum of free complex **1a**.

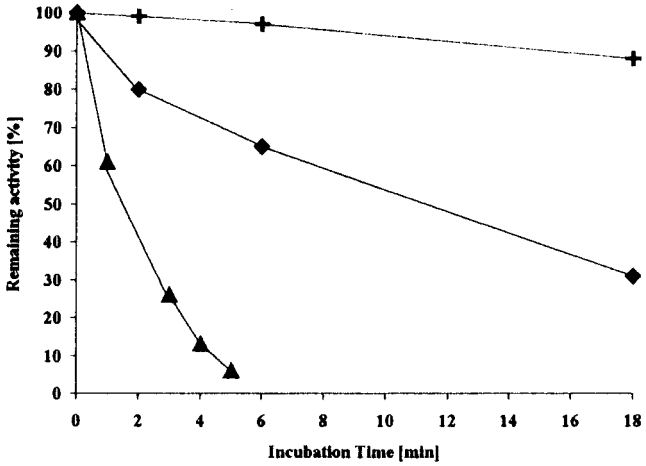


Figure 2. Reversible and irreversible inhibition of TR by **2e**. Two reaction mixtures containing 100 μM NADPH, 100 nM **2e**, and 3 nM (▲) or 10 nM (◆) TR in TR assay buffer, pH 7.5, were incubated at 25 °C. The control sample (+) contained NADPH and 10 nM TR but no inhibitor. At the indicated time points, the remaining activity was determined by adding 130 μM TS_2 to 800 μL of the incubation mixture (▲) corresponding to the sum of reversible and irreversible inhibition. To follow only the irreversible inhibition, a 100-μL aliquot of the incubation mixture (◆, +) was added to a standard TR assay. The complete data set was measured three times. The values at the different time points differed by less than 5%.

dent of the nature of the reducing coenzyme in accordance with EH_2 and EH_2·NADPH being equally sensitive toward the platinum complexes.

Inhibition of NADPH-Reduced TR Is Not Reversed by Dialysis or Thiols. The modification of NADPH-reduced TR by the (terpyridine)platinum(II) complexes is stable. No activity was recovered after dialyzing the inactivated enzyme against TR assay buffer for 14 h at 4 °C. Inhibition was also not abolished by treatment with thiols. Incubation of the modified enzyme for 1−3 h with 1−5 mM DTE, 3 mM glutathione, or 2 mM trypanothione at 25 °C did not recover any activity.

As already mentioned, with several platinum(II) complexes a slow inactivation of TR occurred also in the absence of NADPH. Dialyzing TR that was inhibited approximately 70% by **1b** and **2d**, for 14 h at 4 °C against TR assay buffer, restored up to 8% activity. Incubation of TR that was 90% inhibited by **1d** with 3 mM DTE for 10 min at 25 °C recovered about 25% activity, but no further increase of activity was observed when continuing the incubation for 6 h with 5 mM DTE. Obviously quite stable complexes can also be formed between the oxidized enzyme and the platinum(II) complexes.

The Absorption Spectrum of TR Inhibited by 1a Shows Characteristics of the Two-Electron Reduced Enzyme Species. Modification of TR by compound **1a** causes a slight decrease and shift of $\lambda_{max,visible}$ from 463 to 454 nm (Figure 1) and a concomitant absorbance increase around 530 nm. The extinction coefficients of TR modified by **1a** are 10.9 and 2.42 mM^{-1} cm^{-1} at 454 and 540 nm, respectively. The long wavelength absorption is lower than that of free EH_2.[30] It is comparable to that of yeast GR forming a mixed disulfide with glutathione.[4] The broad long wavelength band is characteristic of a charge-transfer interaction between Cys57 and the flavin cofactor[2,30,31] and is evidence for Cys52 being covalently modified. Compound **1a** has absorption maxima at 311 and 341 nm (Figure 1, inset) which are slightly shifted to longer wavelengths in the modified enzyme. The molar ratio between TR and inhibitor was estimated as described under Experimental Procedures. Seven independent measurements yielded a ratio of about 1.3 platinum(II) complex per protein monomer which fits well the expected 1:1 ratio if Cys52 is specifically modified.

(Terpyridine)platinum(II) Complexes Are Also Reversible Inhibitors of TR. As described above, the fast inhibition of TR in the presence of NADPH by the platinum(II) complexes is probably irreversible (Table 1). To study if the enzyme is also reversibly inhibited, TR was incubated with **2e** in the presence of NADPH (Figure 2). After different time intervals, the degree of irreversible inhibition was determined by transferring an aliquot of the incubation mixture into a standard TR assay. In a parallel assay, the remaining activity was measured by adding TS_2 directly to the incubation mixture. At all time points, the inactivation was much higher when the remaining activity was determined without prior dilution indicating that, in addition to the irreversible inactivation, TR is also reversibly inhibited. This effect has been observed with all complexes except **1c** which does not inhibit TR.

Figure 3 shows the progress curves of TR that has been preincubated with compound **3a** in the presence of NADPH. After 2 and 10 min, aliquots of the incubation mixture were removed and the remaining enzyme activity was determined in a standard TR assay. The initial velocity is less than the steady-state velocity, and a lag period at the beginning of the reaction is observed. To get an insight in the mechanism underlying the lag phase, two identical reaction mixtures containing 100 nM TR, 100 μM NADPH, and 20 μM **3a** were incubated at 25 °C. After 2 min, 10-μL aliquots were removed and diluted in TR assay buffer. One reaction was started immediately by adding NADPH and TS_2, to the other one the substrates were added 3 min later. The identical

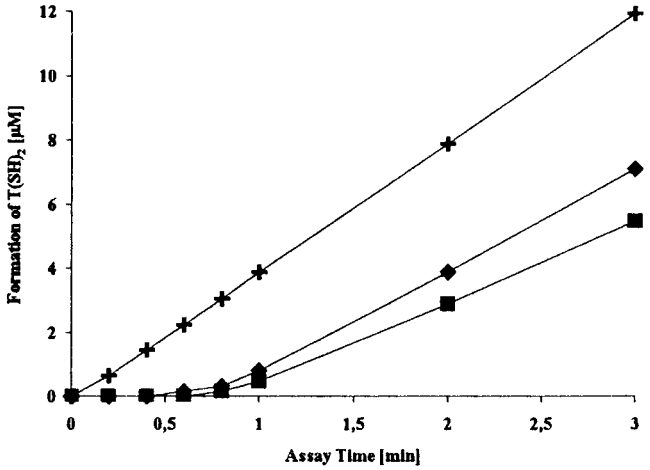


Figure 3. Inhibition of TR by **3a**. Progress curves for the recovery of activity after incubating the enzyme with inhibitor and starting the reaction with substrate. In a total volume of 1 mL, 100 nM TR was incubated with 100 μM NADPH and 20 μM compound **3a** (◆, ■) in TR assay buffer, pH 7.5. The control sample (+) contained NADPH and enzyme but no inhibitor. After 2 min (◆, +) and 10 min (■), a 10-μL aliquot was removed and the remaining enzyme activity was determined in a standard TR assay. NADPH consumption was followed at 340 nm and the concentration of formed $T(SH)_2$ calculated. The kinetics were measured two times and differed by less than 5%.

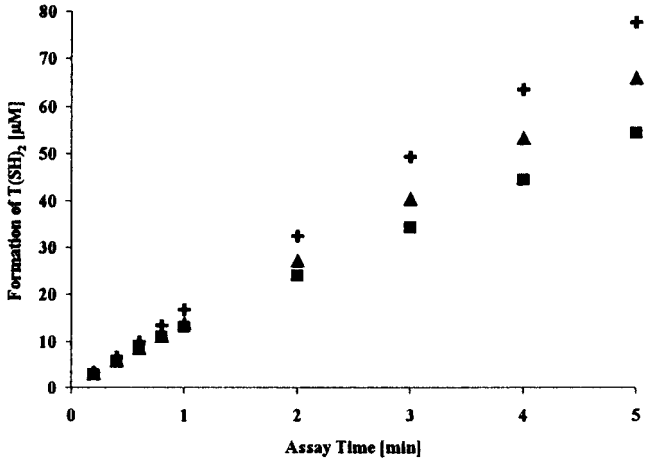


Figure 4. Inhibition of TR by **2d**. Progress curves for the slow onset of inhibition when starting the reaction with the enzyme. The assay contained 200 μM NADPH, 200 μM TS_2, and none (+), 0.8 μM (▲), or 4 μM (■) inhibitor in 1 mL of TR assay buffer, pH 7.5. The reaction was started by adding 3 nM TR. NADPH consumption was followed and the concentration of formed $T(SH)_2$ calculated. The kinetics were recorded two times and differed by less than 5%.

progress curves obtained rule out the possibility that a slow dissociation of EI into E and I upon dilution is responsible for the lag phase. Probably inhibitor (I) and disulfide substrate (S) compete for the same binding site, and establishment of the new equilibrium (EI ↔ E ↔ ES) is a relatively slow process. $T(SH)_2$ accelerates the setup of the equilibrium. Two identical reaction mixtures containing TR, NADPH, and **3a** were incubated for 1 min at 25 °C, and the reaction was started with TS_2. To one assay, $T(SH)_2$ was given immediately after adding TS_2 which resulted in a much faster establishment of the final steady-state activity. Probably $T(SH)_2$ reacts with the free platinum(II) complex leading to inactive derivatives (see Discussion). The control sample without inhibitor is not influenced by the addition of $T(SH)_2$. The progress curves with nearly all platinum(II) complexes showed an initial lag phase. The reactions started immediately and with constant activity, only with **2c**,**d**.

Progress curves obtained when starting the reaction with enzyme were recorded for **2d**,**e**. As shown in Figure 4, the initial velocities in the presence and absence of **2d** are very similar indicating that the EI complex is also formed slowly. The activity of TR decreases with time reflecting the slow onset of inhibition during the course of the assay. The same effect was observed with **2e**.

Thiols Interfere with Inhibition. The presence of 200 μM DTE in the incubation mixture prevented inhibition of TR by 100 nM **2e** as well as 4 μM **2a**,**b** and **3a** under conditions where, without added dithiol, the enzyme was inhibited to 50% within 1−2 min. In the presence of 20 μM $T(SH)_2$, inactivation of TR by 4 μM **2a** and **3a**, respectively, was abolished. 5 μM **2b** and 200 nM **2e** still showed some inhibition that did not increase with time. 100 μM 2-mercaptoethanol caused a much slower inactivation of TR by 4 μM **2a** and 100 nM **2e**. A simple substitution of the fourth ligand of the platinum(II) ion is unlikely as the reaction would convert **2a** into the mercaptoethanol complex **3a,** being also an inhibitor of the enzyme. But in the presence of 2-mercaptoethanol, inhibition of TR by **3a** is also prevented.

The Modified TR Shows Increased Oxidase Activity. TR possesses a very low intrinsic oxidase activity. The substrate-independent oxidation of NADPH occurs at a rate of about 1/3000 of the physiological reductase activity.[18] Modification of the enzyme by the (terpyridine)platinum(II) complexes causes a strong increase in oxidase activity. When 100 nM NADPH-reduced TR is inactivated by 10 μM **2b**,**c** and 20 μM **1b** and **3a,b**, respectively to 30−40%, the oxidase activity rises by a factor of about 5. Complete inactivation of TR by **1a** leads to a 10-fold higher rate of NADPH oxidation when compared with the unmodified enzyme. The oxidase activity increases with incubation time and is not abolished by dialysis in accordance with covalently modified enzyme being the reactive species.

Interaction of Human GR by (Terpyridine)-platinum(II) Complexes Is Mainly Reversible. Several platinum(II) complexes were also studied as inhibitors of human GR. 50 nM GR (5 nM in the case of **1a** and **2e**) was incubated with 108 nM **1a**, 100 nM **2e**, 50 μM **1c**, and 4 μM **3b** in the presence and absence of 100 μM NADPH. After different time intervals, an aliquot was removed and the remaining enzyme activity was determined in a standard GR assay. Only up to 5% inhibition is observed independent of the presence of NADPH and the incubation time. In the case of **2c**,**d**, without NADPH inhibition of GR is also negligible, but in the presence of the reducing cofactor inactivation slowly increases with time. Incubation of 50 nM GR with 100 μM NADPH and 4 μM **2c** or **2d** leads to 50% inactivation within 1.5 h (65−70% within 4 h). No activity is recovered after dialyzing the 70% inhibited enzyme against GR assay buffer for 14 h at 4 °C. Probably compounds **2c**,**d** slowly modify an active site cysteinyl residue of GR as observed with TR.

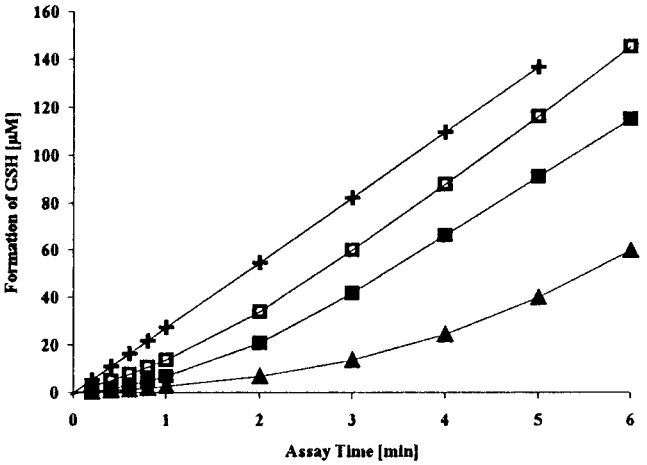


Figure 5. Interaction of human GR with **2e**. The reaction mixtures contained, in a total volume of 1 mL, 1.6 nM GR, 100 μM NADPH, 200 μM GSSG, and 20 nM (□, ■) or 60 nM (▲) **2e** in GR assay buffer, pH 6.9, and were started with enzyme (□) or with substrate (■, ▲), respectively. The control sample contained NADPH and GR but no inhibitor and was started with 200 μM GSSG (+). NADPH consumption was followed and the concentration of formed GSH calculated. The kinetics were measured two times and differed by less than 5%.

Figure 5 shows the kinetics for the interaction of GR with compound **2e** in the presence of NADPH. The progress curves have an initial lag phase, but the reaction rate rapidly increases to a final activity which is nearly identical with that of the control. The lag phase may be caused by the slow displacement of the inhibitor by GSSG analogously to the mechanism proposed for TR. Corresponding progress curves have been obtained with **1a**,**c** and **3b** independent of the presence or absence of NADPH and the incubation time. Obviously the platinum(II) complexes undergo a reversible interaction with the mammalian enzyme which is easily abolished under assay conditions. In contrast to TR (Figures 3 and 4), the progress curves obtained when starting the reaction with GSSG are very similar to those started by the enzyme (Figure 5). In both cases, the initial velocity is lower than the steady-state velocity indicating that an EI complex is formed more rapidly than in the case of TR (see above). Interestingly, the initial activity is slightly higher and the increase of activity is faster when the reaction is started with enzyme. This might indicate that the complex between enzyme and inhibitor is not formed as rapidly as expected for a classical reversible inhibitor. In the assays started with substrate, EI can be formed before the reaction is started by GSSG, whereas in assays started with enzyme, no preformed EI complex is present.

As outlined above, the pronounced initial inhibition of GR observed with **1a**,**c**, **2e**, and **3b** is abolished during the assay. Addition of GSH causes an even faster increase of enzyme activity. As expected, the control assay is not influenced by glutathione. Formation of an inactive thiol complex by substitution of the fourth ligand is not likely as the progress curves obtained for the interaction of GR with the glutathione complex **1c** and NADPH and starting the reaction with 1 mM GSSG also show strong initial inhibition.

Discussion

(2,2′:6′,2″-Terpyridine)platinum(II) complexes are irreversible inhibitors of *T. cruzi* TR, but most of them do not inhibit human GR. The crucial role of positive charges for ligands binding in the active site of TR has been outlined by Faerman et al.[32] The complexes studied here carry an overall charge of +1 or +2 depending on the fourth ligand at the metal ion. The (4′-chloro-2,2′:6′,2″-terpyridine)platinum(II) ammine complex (**1a**) and the (4-picoline)(2,2′:6′,2″-terpyridine)platinum(II) complexes (**2a**−**e**) have an overall charge of +2, whereas the complexes with hydroxide (**1b**) or 2-hydroxyethanethiolate (**3a**,**b**) as the fourth ligand carry a charge of +1 (Table 1). In the group of (4′-chloro-2,2′:6′,2″-terpyridine)platinum(II) complexes (**1**), the ammine complex **1a** reacts 2 orders of magnitude faster with NADPH-reduced TR than the hydroxyl complex **1b** and the uncharged glutathionato complex **1c** does not interact with the enzyme at all. Another pair of complexes differing by the overall charge due to the nature of the fourth ligand is **2a** and **3a**. Again the double positively charged complex **2a** reacts faster with the reduced enzyme than the respective singly charged 2-mercaptoethanol complex **3a**. The overall charge of the complexes, however, is only one factor affecting the reactivity of the complexes. The group of (4-picoline)(2,2′:6′,2″-terpyridine)platinum(II) complexes (**2**) varying in the 4′-substituent on the central pyridine ring includes both the most and the least reactive derivative (see below).

Irreversible inhibition of TR by the (terpyridine)platinum(II) complexes implies the coordinative replacement of the fourth ligand by a protein residue. In complexes of the cisplatin type *cis*-$Pt(NH_3)_2X_2$, NH_3 is a stable ligand whereas an aquo group is easily exchanged.[33] In the platinum(II) complexes studied here, the *trans* influence of the terpyridine moiety and the steric interaction with the 6,6″-hydrogens of the 2,2′:6′,2″-terpyridine ligand render the ammine a leaving group which is easily replaced by water, yielding complex **1b**.[23] Toward two-electron reduced TR, **1a** is one of the two most reactive complexes. In comparison to **1b**,**c** and **2b** which differ from **1a** only by the nature of the leaving group, **1a** reacts by orders of magnitude faster. Interestingly, toward TR also the (2-mercaptoethanol)(terpyridine)platinum(II) complexes (**3**) act as irreversible inhibitors. Obviously the high nucleophilicity of Cys52 is responsible for the displacement of 2-mercaptoethanol from the metal ion (see below). Thiolate ligands possess a very high affinity for platinum(II) and are usually resistant to substitution.[23] In contrast to picoline, ammonia, or water, 2-mercaptoethanol as a fourth ligand of (terpyridine)platinum(II) complexes is not easily exchanged resulting in intercalation but not covalent binding to DNA.[23,34,35] In addition, chloride as a fourth ligand of (terpyridine)platinum(II) complexes is rapidly displaced by cysteine and reduced glutathione.[36] The high affinity of platinum(II) for protein sulfhydryl groups is probably the cause of the nephrotoxicity of cisplatin.[24]

A detailed discussion of structure−activity relationships (SARs) would be premature, but in the series of picoline complexes (**2**) the reaction rates vary by 3 orders of magnitude depending on the 4′-substituent on the

central pyridine ring. Obviously there are no steric constraints imposed by the protein since the most bulky complexes **2d**,**e** rapidly inhibit the enzyme. This observation is in accordance with the probable fixation of the platinum ion to Cys52 and the terpyridine moiety pointing into the outer region of the active site. The disulfide substrate binding site of TR is lined by Leu17, Glu18, Trp21, Ser109, Met113, and Phe114. This hydrophobic and negatively charged region fixes trypanothione disulfide[13] as well as the acridine drug mepacrine.[14] Probably the hydrophobic substituents in **2d**,**e** can interact with this region enhancing their reactivity toward the enzyme.

The sulfhydryl group of Cys52 in the active site of TR (Cys58 in GR) is a highly reactive nucleophile and is selectively modified by compounds such as iodoacetamide,[3] the nitrosourea drug BCNU,[17,21] and ajoene, a garlic-derived natural compound.[20] Kinetic and spectroscopic studies indicate that the (terpyridine)platinum(II) complexes react with Cys52 of reduced TR. Obviously this residue substitutes the fourth ligand of the platinum ion leading to very stable enzyme−inhibitor complexes. (Terpyridine)platinum(II) complexes with low-molecular-mass thiols as a fourth ligand exhibit a low absorption band around 475 nm.[34] In the TR−**1a** complex such a band is not resolved since it would coincide with the flavin absorption of the enzyme.

Trivalent organic arsenicals and antimonals are other time-dependent metal inhibitors of TR and GR which only react with the NADPH-reduced enzymes.[37,38] The reaction leads to the loss of the charge-transfer absorption around 530 nm probably due to the coordination of the metal to both redox-active cysteinyl residues. In contrast to the platinum complexes studied here, inhibition by the arsenical and antimonal drugs is reversible upon dilution, and thiols and the compounds do not show significant selectivity for the parasite enzyme. In the case of the (terpyridine)platinum complexes, complexation with both cysteines is not likely. Such a species would necessitate formation of a pentacoordinate platinum or displacement of one of the pyridine ligands of the terpyridine. In addition, the inhibited enzyme species retains the long wavelength absorption that arises from the interaction of Cys57 with the flavin cofactor. This is strong evidence that only Cys52 is involved in binding the (terpyridine)platinum(II) complex.

Several complexes slowly inactivate TR also in the absence of NADPH leading to stable enzyme−inhibitor complexes. The nature of the modification is not yet known, but it is tempting to speculate that His460′ may be modified. In all FAD disulfide oxidoreductases, this residue contributed by the second subunit is essential for catalysis.[2,39] In addition, in several proteins histidine residues have been shown to be selectively modified by (terpyridine)platinum(II) complexes.[25,28,29] Reaction of the compounds with the active site disulfide bridge in the oxidized enzyme is very unlikely. In contrast to complexes of the cisplatin type,[40] (terpyridine)platinum(II) complexes are unreactive with cystine or glutathione disulfide due to steric constraints caused by the 6,6″-hydrogens on the 2,2′:6′,2”-terpyridine ligand.[28]

TR irreversibly modified by the (terpyridine)platinum(II) complexes in the presence of NADPH shows an increased oxidase activity. Blocking of Cys52 in the two-electron reduced enzyme is expected to shift the electron density toward the flavin ring promoting reduction of molecular oxygen. A high oxidase activity leads to the generation of reduced oxygen species, consumption of NADPH, and lowering of the thiol/disulfide quotient. As a consequence, the oxidative stress of the cell is increased. Comparable observations have been made for GR inhibited by ajoene[20] and for human thioredoxin reductase modified by 1-chloro-2,4-dinitrobenzene.[41] In both enzymes only the NADPH-reduced enzyme is time-dependently modified leading to the covalent modification of the distal cysteine in the active site corresponding to Cys52 in TR.

In addition to the irreversible modification developing with time, the (terpyridine)platinum(II) complexes also reversibly inhibit TR, but the type of reversible inhibition could not be determined. When starting the reactions with substrate, the equilibrium between enzyme, inhibitor, and substrate is not established immediately as expected for a classical reversible ligand. In assays started with enzyme, no initial inhibition is observed even at very high inhibitor concentrations. These findings indicate that also the reversible complex is formed slowly and that this complex is converted into the irreversible complex. Such a mechanism has been reported for inhibition of caspase-1 by aryl- and acyloxymethyl ketones.[42]

Free thiols such as DTE, $T(SH)_2$, and 2-mercaptoethanol interfere with inhibition of TR by the (terpyridine)platinum(II) complexes. DTE readily displaces the terpyridine moiety from **2a** to form a bis(DTE)platinum(II) complex, and several other dimeric dithiolato-linked platinum complexes have been described.[43] 2-Mercaptoethanol rapidly displaces the 4-picoline ligand from **2a**,[44] but this is not sufficient to explain the abolition of the inhibitory activity of **2a** since the hydroxyethanethiolato complexes **3a**,**b** also inhibit the enzyme. We have shown, however, that in the presence of a large excess of 2-mercaptoethanol the 2,2′:6′,2″-terpyridine ligand is displaced by thiolate ligands. Excess $T(SH)_2$ presumably also displaces the 2,2′:6′,2″-terpyridine ligand from **2a**.

In mammalian cells GSH does not influence the activity of cisplatin when applied simultaneously.[40] On the other hand, the GSH concentration is increased in tumor cells which are resistant to this drug.[45] Even if under in vivo conditions $T(SH)_2$ retards the reaction with TR, the equilibrium will shift toward the modified protein species since the inhibition is not abolished by $T(SH)_2$.

The antiparasitic activity of **1a**, **2a**,**b**,**e**, and **3a** has been studied in cultures of *T. cruzi*, *T. brucei,* and *Leishmania donovani*.[23] (2,2′:6′,2″-Terpyridine)platinum(II) complexes are known to intercalate into DNA.[34] In addition, hydroxy[46] and picoline complexes[44,47] also platinate DNA. The most effective compounds against *T. cruzi* are **1a** and **2e** with ED_{50} values lower than 1 μM. As shown here, these complexes also react most rapidly with NADPH-reduced TR. Thus, in addition to the interaction with DNA,[23] irreversible inhibition of the key enzyme of the parasite thiol metabolism could be responsible for the antiparasitic activity of the complexes.

Most of the (terpyridine)platinum(II) complexes (including **1a** and **2e** − the most reactive complexes toward TR) interact only reversibly with human GR. An initial strong "inhibition" is completely abolished within the first minutes of the assay not allowing to determine the type of inhibition. Addition of GSH accelerates the process. The fast recovery of the enzyme activity is probably not due to formation of an "inactive" terpyridine−GSH complex. This reaction would generate **1c** which behaves with GR like the other complexes. Most likely GSSG displaces the inhibitor from the active site and GSH reacts with the free complex replacing the terpyridine moiety.

2c,**d** are the only two derivatives which showed a time-dependent inhibition of GR in the presence of NADPH. Thus they probably also modify an active site cysteine residue. This observation is quite surprising as these two complexes have very large hydrophobic substituents at the central pyridine ring. The GSSG binding site in GR is significantly smaller than in TR and has an overall positive charge.[11]

Conclusions

(2,2′:6′,2″-Terpyridine)platinum(II) complexes which are known to be cytostatic against trypanosomes and leishmania are irreversible inhibitors of *T. cruzi* TR, but most of them do not inhibit human GR. The kinetic data for the inhibition of NADPH-reduced TR are in accordance with the slow formation of a reversible EI complex which more slowly converts into the irreversibly inhibited enzyme. Most probably Cys52 in the active site is specifically altered. The modification is not reversed upon dialysis or treatment by low-molecular-mass thiols and yields enzyme species with increased oxidase activity. Interestingly **1a** and **2e** which react most rapidly with NADPH-reduced TR are also the most effective compounds against *T. cruzi* in vitro.[23] These findings indicate that besides the interaction with DNA, irreversible inhibition of the key enzyme of the parasite thiol metabolism contributes significantly to the antitrypanosomal activity of these compounds.

Experimental Procedures

Materials. Recombinant *T. cruzi* TR[8] and human GR[48] were prepared according to published procedures. Trypanothione disulfide was purchased from Bachem, Switzerland. Stock solutions (1−4 mM) of the (2,2′:6′,2″-terpyridine)platinum(II) complexes were made in DMSO and stored at −20 °C.

TR Assay. TR activity was measured spectrophotometrically at 25 °C in TR assay buffer (40 mM Hepes, 1 mM EDTA, pH 7.5) as described.[17] The standard assay mixture (1 mL) contained 100 μM NADPH and 5−10 mU (1−2 nM) *T. cruzi* TR. The reaction was started by adding 110 μM trypanothione disulfide (TS_2), and the absorption decrease at 340 nm due to NADPH consumption was followed. V_{max} was calculated using a K_m value of 18 μM for TS_2.[17]

GR Assay. The kinetics were carried out in GR assay buffer (20.5 mM KH_2PO_4, 26.5 mM K_2HPO_4, 200 mM KCl, 1 mM EDTA, pH 6.9) at 25 °C as described.[48] The standard assay mixture (1 mL) contained 100 μM NADPH and 5−10 mU (0.5−1 nM) GR. The reaction was started by adding 1 mM glutathione disulfide (GSSG), and the absorption decrease at 340 nm was followed.

Inhibition of TR by (2,2′:6′,2″-Terpyridine)platinum(II) Complexes. To follow the time-dependent irreversible inactivation, in a total volume of 1 mL, 100 nM TR (10 nM TR in the case of compounds **1a** and **2e**) was incubated in the presence and absence of 100 μM NADPH and varying concentrations of inhibitor in TR assay buffer, pH 7.5 at 25 °C. A control sample contained all components except that the inhibitor was replaced by DMSO. At different time intervals, aliquots of 10 and 20 μL (100 and 200 μL in the case of **1a** and **2e**) were removed and assayed for remaining activity in a standard TR assay. As the resulting volume activities were independent of the size of the aliquot, reversible inhibition is not recorded under these conditions.

In a second assay reversible and irreversible inhibition was followed simultaneously. In a total volume of 5 mL, 3 nM TR was incubated at 25 °C with varying concentrations of inhibitor in the presence and absence of 100 μM NADPH. After different time intervals, 800 μL was removed, 100 μM NADPH was added to those samples without NADPH, and the remaining enzyme activity was determined by starting the reaction with 130 μM TS_2. The effect of thiols on the inhibition of TR was studied by adding 200 μM DTE, 100 μM 2-mercaptoethanol or 20 μM $T(SH)_2$ to the incubation mixture.

The reversibility of inhibition was tested by dialyzing the modified enzyme for 12−14 h against TR assay buffer at 4 °C and by incubating the inhibited enzyme with 1−5 mM DTE, 3 mM glutathione or 2 mM trypanothione, respectively, for 1−6 h at 25 °C. Recovery of enzyme activity was determined in a standard TR assay.

Oxidase Activity of Modified TR. 100 nM TR was incubated with 100 μM NADPH and 10−20 μM **1b**, **2b**,**c**, and **3a,b**, respectively. When inhibition had reached 30−40%, 100 μM NADPH was added to 800 μL of the incubation mixture and oxidation of NADPH was followed at 340 nm. In the case of **1a**, 1 μM TR was incubated with 200 μM NADPH and 11 μM inhibitor. When TR was completely inactivated the reaction mixture was diluted 1:5 in TR assay buffer, 100 μM NADPH was added, and the absorption decrease at 340 nm was followed.

Interaction Between GR and the (2,2′:6′,2″-Terpyridine)platinum(II) Complexes. A probable irreversible inhibition was studied by incubating 50 nM GR (5 nM GR in the case of **1a** and **2e**) in 1 mL of GR assay buffer, pH 6.9 at 25 °C, with varying concentrations of inhibitor in the presence and absence of 100 μM NADPH. A control sample contained all components but the inhibitor was replaced by DMSO. At different times, aliquots of 25 μL (100 and 200 μL in the case of **1a** and **2e**) were removed and assayed for remaining activity in a standard GR assay. Reversible plus irreversible inhibition was measured by incubating 1−2 nM GR with varying concentrations of inhibitor in the presence and absence of 100 μM NADPH in a total volume of 5 mL as described for TR. After different time intervals, 950 μL was removed, 100 μM NADPH was added to the samples without NADPH, and the remaining enzyme activity was determined by starting the reaction with 1 mM GSSG. The reversibility of inhibition was studied by dialyzing modified GR for 12−14 h against GR assay buffer at 4 °C. Recovery of enzyme activity was determined in a standard GR assay.

Spectroscopic Studies. For spectroscopic studies and crystallization purposes, 1 μM TR was incubated with 200 μM NADPH and 11 μM compound **1a** in a total volume of 100 mL. After 15 min − when TR was completely inhibited − the reaction mixture was washed with TR assay buffer and concentrated in a Centriprep-30 concentrator (Amicon). The protein was precipitated by 90% ammonium sulfate, washed with 90% ammonium sulfate in 50 mM potassium phosphate, 1 mM EDTA, pH 7.0, and the pellet was dissolved in TR assay buffer. The protein concentration was determined by the Micro BCA-Assay (Protein Assay Reagent Kit, Pierce). Absorption spectra were recorded in microcuvettes (90 μL) in a Beckman DU-65 spectrophotometer. The molar ratio between inhibitor and enzyme was estimated by determining the concentration of **1a** from the spectrum of the modified enzyme. The total absorption at 348 nm was corrected for the absorption of the reduced enzyme at this wavelength.[30] The molar extinction coefficient of **1a** at 341 nm is 10.5 $mM^{-1}\,cm^{-1}$.

Chemical Synthesis. Thin-layer chromatography (TLC), electrospray ionization (ESI) mass spectra, and proton mag-

netic resonance spectra were performed as described.[23] Chemicals were purchased from Sigma-Aldrich Co. (Poole) or Lancaster Synthesis Ltd. (Lancaster) and used without further purification. Solvents were obtained from the British Drug Houses (BDH) or Fisons at reagent grade and used without distillation.

(4′-Chloro-2,2′:6′,2″-terpyridine)platinum(II) Complexes 1. (4′-Chloro-2,2′:6′,2″-terpyridine)platinum(II) Ammine Bis(nitrate) (1a). Silver nitrate (71.4 mg, 0.42 mmol) was dissolved in demineralized water (0.1 mL) and added to a suspension of diiodocyclooctadienylplatinum(II) (111.4 mg, 0.2 mmol) in dioxane (0.75 mL). The reaction mixture was vigorously shaken until no dark yellow color could be seen in solid or solution and the precipitated silver iodide removed by centrifugation. The supernatant was added to a suspension of 4′-chloro-2,2′:6′,2″-terpyridine (42.8 mg, 0.16 mmol) in dioxane (0.25 mL) and the resulting suspension mixed vigorously then left to stand for 15 min. The precipitate was isolated by centrifugation, washed with dioxane:diethyl ether (1:1, 2 × 1.5 mL) and resuspended in dioxane saturated in ammonia gas. The resulting suspension was vigorously mixed then incubated at room temperature for 1.5 h. The solid material was isolated by centrifugation, washed liberally with dioxane: diethyl ether (1:1) and dried under vacuum to give **1a** as a brown solid (65.0 mg, 67.3%): mp > 230 °C; $\delta 1_H$ (400 MHz; D_2O:DMSO-d_6 (7:1)) 8.69 (2H, d, $J = 5.5$ Hz, H6,6″); 8.52 (2H, s, H3′,5′); 8.47 (2H, t, $J = 7.5$ Hz, H4,4″); 8.36 (2H, d, $J = 8.0$ Hz, H3,3″); 7.93 (2H, m, H5,5″); *m*/*z* (ESI+) = 239.8 ($[M]^{2+}$). (Note: The complex is highly unstable toward solvolysis by MeOH:H_2O diluant used in ESI mass spectrometry and solvolyzed species were also seen unless the sample was prepared immediately prior to analysis.)

(4′-Chloro-2,2′:6′,2″-terpyridine)platinum(II) Hydroxide Nitrate (1b). Silver nitrate (35.7 mg, 0.21 mmol) was dissolved in aqueous acetone (4:1 acetone:water, 0.5 mL) and added to a suspension of diiodo-1,5-cyclooctadienylplatinum-(II) (55.7 mg, 0.1 mmol) in aqueous acetone (0.75 mL). The mixture was vigorously shaken until the dark yellow color had subsided then the precipitated silver salt removed by centrifugation. The supernatant containing the active platinum species was added to a suspension of 2,2′:6′,2″-terpyridine (18.7 mg, 0.08 mmol) in aqueous acetone (0.25 mL). After standing for ca. 5 min the yellow precipitate formed was isolated by centrifugation, washed with ether:acetone (3:1, 3 × 1.5 mL) and redissolved in demineralized water saturated with triethylamine (1 mL, ca. 20% TEA). The mixture was incubated at room temperature for 1.5 h then added to an excess of acetone:diethyl ether (5:3, 40 mL). The precipitated product was isolated by centrifugation, washed successively with acetone:diethyl ether and diethyl ether alone and dried under vacuum to leave **1b** as a powdery yellow solid (18.7 mg, 43.1%): mp > 230 °C; $\delta 1_H$ (500 MHz; D_2O) 8.53 (2H, d, $J = 5.0$ Hz, H6,6″); 8.37 (2H, td, $J = 8.0$, 1.0 Hz, H4,4″); 8.28 (2H, s, H3′,5′); 8.17 (2H, d, $J = 8.0$ Hz, H3,3″); 7.88 (2H, m, H5,5″); *m*/*z* (ESI+) = 480.2 ($[M]^+$).

(*S*-Glutathionato)(4′-chloro-2,2′:6′,2″-terpyridine)platinum(II) Nitrate (1c). The complex was prepared by a modification of the general method described previously[49] analogous to the preparation of **3a** (see below) but using 4′-chloro-2,2′:6′,2″-terpyridine and reduced glutathione on a 0.1-mmol scale. Precipitation from water:acetone (1:3) afforded the product as a gray-green solid (dark red in solution) (34 mg, 41%): mp > 230 °C; $\delta 1_H$ (250 MHz; D_2O) 8.83 (2H, d, $J = 5$ Hz, H6,6″); 8.29 (2H, s, H3′,5′); 8.22 (2H, t, $J = 8$ Hz, H4,4″); 8.05 (2H, d, $J = 8$ Hz, H3,3″); 7.72 (2H, t, $J = 5$ Hz, H5,5″); 4.38 (1H, dd, $J = 5.7$ Hz, Hα-Cys); 3.54 (1H, t, $J = 4$ Hz, Hα-Glu); 3.44 (2H, s, H-Gly); 2.71 (2H, m, Hβ-Cys); 2.16 (2H, m, Hγ-Glu); 1.81 (2H, m, Hβ-Glu); *m*/*z* (ESI+) = 767.2 ($[M]^+$). Anal. Found: C, 36.0; H, 3.2; N, 11.9. $C_{25}H_{26}ClN_7O_9PtS$ requires C, 36.1; H, 3.1; N, 11.8.

(4-Picoline)(4′-substituted-2,2′:6′,2″-terpyridine)platinum(II) Bis(tetrafluoroborate) Complexes 2. The complexes were prepared by the general method described previously.[49] The synthesis of (4-picoline)platinum(II) complexes of 2,2′:6′,2″-terpyridine (**2a**), 4′-chloro-2,2′:6′,2″-terpyridine (**2b**) and 4′-*p*-bromophenyl-2,2′:6′,2″-terpyridine **(2e)** has been reported.[49]

(4-Picoline)(4′-(2-pyridyl)-2,2′:6′,2″-terpyridine)platinum(II) Bis(tetrafluoroborate) (2c). 2c was prepared from 4′-(2-pyridyl)-2,2′:6′,2″-terpyridine (77.6 mg, 0.25 mmol)[50] following the published procedures.[49] Recrystallization from acetonitrile gave **2c** as a yellow solid (136.4 mg, 70.7%): mp > 230 °C; $\delta 1_H$ (300 MHz; DMSO-d_6) 9.35 (2H, s, H3′,5′); 9.06 (2H, d, $J = 6.5$ Hz, picH2,6); 8.98 (2H, d, $J = 7.8$ Hz, H3,3″); 8.93 (1H, d, $J = 3.8$ Hz, H6‴); 8.59 (3H, m, H4,4″ + H3‴); 8.43 (1H, td, $J = 7.8$, 1.8 Hz, H4‴); 7.9 (6H, m, H6,6″ + H5,5″ + picH3,5); 7.72 (1H, dd, $J = 7.8$, 4.0 Hz, H5‴); 2.65 (3H, s, picMe); *m*/*z* (ESI+) = 299.4 ($[M]^{2+}$).

(4-Picoline)(4′-(*p*-biphenyl)-2,2′:6′,2″-terpyridine)platinum(II) Bis(tetrafluoroborate) (2d). The (4-picoline)(4′-*p*-biphenyl-2,2′:6′,2″-terpyridine)platinum(II) bis(tetrafluoroborate) complex (**2d**) was prepared from 4′-(*p*-biphenyl)-2,2′:6′,2″-terpyridine (96.3 mg, 0.25 mmol)[50] as described.[49] Recrystallization from acetonitrile gave **2d** as a yellow solid (155 mg, 73%): mp > 230 °C; $\delta 1_H$ (300 MHz; DMSO-d_6) 9.16 (2H, s, H3′,5′); 9.05 (2H, d, $J = 6.0$ Hz, picH2,6); 8.99 (2H, d, $J = 8.0$ Hz, H3,3″); 8.62 (2H, t, $J = 7.5$ Hz, H4,4″); 8.37 (2H, d, $J = 8.5$ Hz, H3‴,5‴); 8.03 (2H, d, $J = 8.5$ Hz, H2‴,6‴); 7.9 (8H, m, H6,6″ + H2⁗,6⁗ + H3⁗,5⁗ + picH3,5); 7.56 (2H, m, H5,5″); 7.48 (1H, t, $J = 7.5$ Hz, H4⁗); 2.62 (3H, s, picMe): *m*/*z* (ESI+) = 337 ($[M]^{2+}$).

(2-Hydroxyethanethiolate)(4′-substituted-2,2′:6′,2″-terpyridine)platinum(II) Nitrate Complexes 3. (2-Hydroxyethanethiolato)(2,2′:6′,2″-terpyridine)platinum(II) Nitrate (3a). Compound **3a** was prepared by the method of Jennette et al.[35] For comparison, **3a** was also prepared by a modification of the general method described for the preparation of (4-picoline)(4′-substituted-2,2′:6′,2″-terpyridine)platinum(II) bis(tetrafluoroborate) complexes.[49] Silver nitrate (35.7 mg, 0.21 mmol) was dissolved in aqueous acetone (4:1 v/v acetone: water, 0.5 mL) and added to a suspension of diiodo-(1,5-cylooctadienyl)platinum (II) (55.7 mg, 0.1 mmol) in aqueous acetone (0.75 mL). The mixture was vigorously shaken until the dark yellow color had subsided, then the precipitated silver salt was removed by centrifugation. The supernatant containing the active platinum species was added to a suspension of 2,2′:6′,2″-terpyridine (18.7 mg, 0.08 mmol) in acetonitrile (0.25 mL). After standing for ca. 5 min, the yellow precipitate formed was isolated by centrifugation, washed with ether:acetonitrile (3:1, 3 × 1.5 mL) and redissolved in 1 mL demineralized water. To this solution was added mercaptoethanol (7.54 μL = 8.6 mg, 0.11 mmol). After standing for 1 h the product was isolated by precipitation from excess acetone: ether (5:3, 40 mL), washed successively with acetone:ether then ether alone and dried in a vacuum desiccator to leave **3a** as a red powdery solid (35.9 mg, 79.1%). The electrospray mass spectrum and 500 MHz proton NMR precisely matched those of material made via the literature method.

(2-Hydroxyethanethiolate)(4′-ethoxy-2,2′:6′,2″-terpyridine)platinum(II) Nitrate (3b). Compound **3b** was prepared by the modified general method given above[49] using 4′-ethoxy-2,2′:6′,2″-terpyridine[23] in place of 2,2′:6′,2″-terpyridine. The product was a dark red powdery solid (55.0%): *m*/*z* (ESI+, ex. MeOH:H_2O) 549 (M^+); δH (500 MHz, D_2O, referenced to dioxan (3.75 ppm) 8.96 (2H, d, $J = 5.1$ Hz, broadened, H6.6″), 8.23 (2H, t, $J = 7.3$ Hz, H4.4″), 8.03 (2H, d, $J = 7.9$ Hz, H3.3″), 7.69 (2H, m, H5.5″), 7.60 (2H, s, H3′/5′), 4.30 (2H, q, $J = 7.0$ Hz, OCH_2CH_3), 3.86 (2H, t, $J = 6.9$ Hz, OCH_2CH_2S), 2.53 (2H, s, broadened, OCH_2CH_2S), 1.53 (3H, t, $J = 6.5$ Hz, OCH_2CH_3).

All compounds were judged pure by the absence of nonassignable peaks in high-field ^{1}H NMR spectrum and the molecular ion peak in the ESI mass spectrum with the correct isotope distribution.

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Supporting Information Available: Figure 6, time-dependent inactivation of *T. cruzi* TR by **1b**; Figure 7, effect of $T(SH)_2$ on the interaction of TR with **3a**; Figure 8, effect of GSH on the interaction of human GR with **3b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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