A potential role for protein tyrosine phosphatase inhibition by a Ru^{III}–edta complex (edta = ethylenediaminetetraacetate) in its biological activity

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$[Ru^{III}(edta)(OH_2/OH)]^{1-/2-}$ (edta = ethylenediaminetetraacetate) inhibits protein tyrosine phosphatase (PTP) at physiological pH values in a mechanism that involves binding of the Cys residue of the catalytic domain of the enzyme and similar interactions may be important in the anti-cancer properties of the active forms of many Ru pro-drugs.

Ruthenium complexes containing polyaminocarboxylate (pac) ligands are promising with regard to potential biological applications, including their potential as chemotherapeutic agents.[1] For example, they show good antitumor activities, inhibit DNA recognition and DNA lysis, and stimulate NADPH oxidase and a respiratory burst in phagocytic neutrophils.[1] This arises through the binding of bio-molecules *via* rapid and facile aqua-substitution reactions and a range of accessible oxidation states. They also possess catalytic properties that mimic the enzymatic hydrocarbon oxidations catalysed by cytochrome P-450 under homogeneous conditions. The pac ligands are somewhat comparable in their donor characters to many proteins of metallo-enzymes that make use of carboxylate and amine donors from amino acids to bind to the metal centre, and form very stable 1 : 1 (metal : ligand) complexes with Ru. It was shown earlier,[2] and later confirmed by crystallographic studies,[3] that pac ligands function as pentadentates in Ru-pac complexes. The sixth coordination site of the ruthenium centre is occupied by an aqua ligand at low pH values or by a hydroxo ligand at high pH values. A structural representation and abbreviations for various $[Ru(pac)(OH_2)]^{n-}$ complexes are shown in Fig. 1.

The feature that dominates the chemistry of $[Ru^{III}(edta)(OH_2)]^-$ is its lability towards aqua-substitution reactions, which affords an advantage of facile and straightforward syntheses of mixed-ligand complexes.[1,2] Recent studies have shown that cysteine (Cys) binds rapidly to Ru^{III}–edta complexes (on the stopped-flow timescale) to inhibit cysteine protease activity.[4] In the present work, we have explored the ability of $[Ru^{III}(edta)(OH_2/OH)]^{1-/2-}$ to inhibit a protein tyrosine phosphatase (PTP) at physiological pH values. The PTPs are critical regulators of signal transduction under normal and pathophysiological conditions.[5] Defective or inappropriate regulation of PTP activity leads to abnormal tyrosine phosphorylation, which contributes to the development of many human diseases.[6] A number of PTPs have been identified as important therapeutic targets for the treatment of various diseases, including cancer.[7–13] We report herein the ability of Ru–edta to inhibit a PTP (Yop51*)† that contains a $Cys(X_5)Arg$ catalytic domain (X is any amino acid), which is common for all the microbial and mammalian PTPs.[14]

$K[Ru^{III}(Hedta)Cl]\cdot 2H_2O$ was prepared by following the published procedure and was characterized as before.[15] The micro-analysis and spectral data‡ are in good agreement with those reported in the literature.[15] The complex rapidly hydrolyses to the aqua complex when dissolved in water. The pK_a values related to the acid-dissociation equilibria of the pendant carboxylic acid arm and the aqua ligand are 2.4 and 7.6, respectively, at 25 °C.[2]

At pH 7.4, the Ru–edta complex exists as a mixture of aqua- and hydroxo-species ($[Ru^{III}(edta)(OH_2/OH)]^{1-/2-}$).[2] The results of PTP inhibition studies by $[Ru^{III}(edta)(OH_2/OH)]^{1-/2-}$ are given in Table 1. The results of these inhibition studies clearly demonstrate that $[Ru^{III}(edta)(OH_2/OH)]^{1-/2-}$ is capable of reducing the hydrolysis of *p*-nitrophenylphosphate by PTP under specified conditions.† The observed ability of Ru^{III}–edta complex toward inhibition of PTP activity is attributed to the high affinity of the $[Ru^{III}(edta)(OH_2)]^-$ complex to the binding of the RS^- group of thio-amino acids.[4] The $[Ru^{III}(edta)(OH_2)]^-$ complex presumably binds the Cys residue in the catalytic domain of the PTP through a rapid aqua-substitution reaction to inhibit the phosphatase activity of the enzyme by forming a stable Ru(edta)–enzyme complex. Addition of glutathione (GSH, 10 mM) to the reaction system substantially reduced the inhibitory activity of the Ru^{III}–edta complex (Run 5 in Table 1), which further supports the above

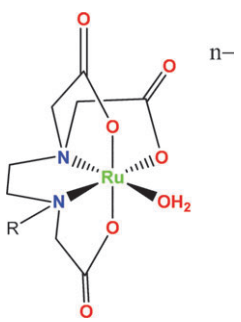

Fig. 1 Structures of Ru-pac complexes. **1** $R = CH_2COO^-$: pac = $edta^{4-}$ (ethylenediaminetetraacetate) **2** $R = CH_2CH_2OH$: pac = $hedtra^{3-}$ (*N*-hydroxyethylethylenediaminetetraacetate).

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Table 1 Results of inhibition of a recombinant *Yersinia enterocolitica* PTP by Ru^{III}–edta at 37 °C

Run No.	$[Ru^{III}]$/mM	[Glutathione]/mM	% Inhibition
1	0.1	0	37 ± 3
2	0.5	0	56 ± 5
3	1.0	0	67 ± 6
4	1.5	0	76 ± 8
5	1.0	10	7 ± 1

Experiments were carried out in tris(hydroxymethyl)aminoethane buffer (Tris, 50 mM, pH 7.0) containing NaCl (0.1 M), edta (0.2 mM), bovine serum albumin (BSA, 1 mg mL^{-1}), and Brij35 (0.001%). The above buffer solution is designated as *Buffer A* in the text. The buffer *A* used for the PTP reactions, as well as for dilutions of the enzyme, corresponded to that recommended by Sigma, except for the absence of added DTT.

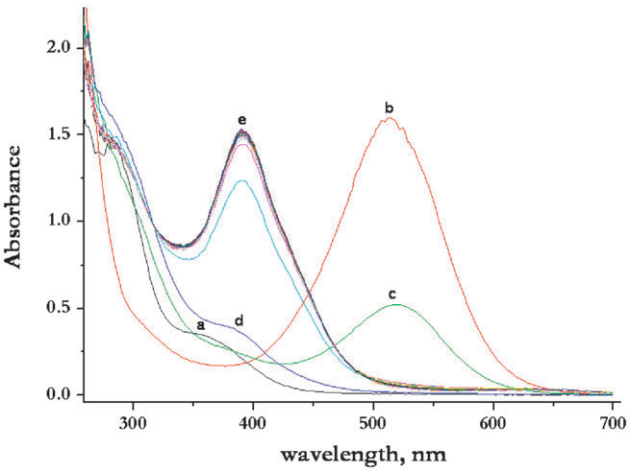
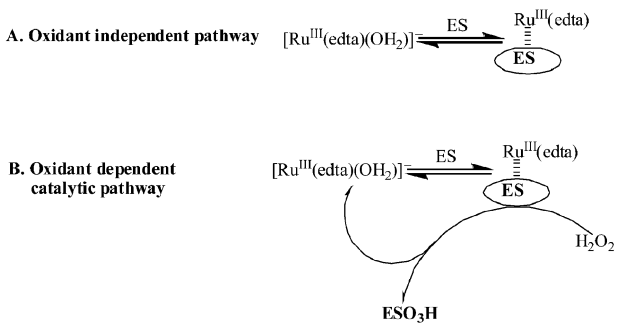


Fig. 2 Spectra of: (a) Ru–edta (4×10^{-4} M); (b) a + GSH ([GSH] = 4×10^{-4} M); (c) b + H_2O_2 (1×10^{-3} M); (d) spectrum of c after 3 min; (d–e) progressive changes in spectra of the product from c until the end of the reaction (after 1 h). pH = 7.0, temp. = 37 °C.

argument. However, the fact that the inhibition is not complete, shows that PTP can compete with GSH under physiological conditions and, hence, the inhibition has direct biological relevance. This is supported by the fact that preformed $[Ru(edta)(SR)]^{2-}$ (RSH = GSH) species cannot bind to the Cys residue of PTP. By contrast, $[Ru^{III}(hedtra)(OH)]^{-}$, which is structurally almost identical to $([Ru^{III}(edta)(OH)]^{2-}$ was almost ineffective (7% inhibition of PTP activity at $[[Ru^{III}(hedtra)(OH)]^{-}]$ = 1.5 mM) towards inhibition of the activity of PTP under similar experimental conditions. In order to gain more insight into the kinetics of the reactions of the Ru(III) complexes with thiolato groups (RS^{-}), stopped-flow kinetic studies§ were performed for the reaction of both Ru^{III}–edta and Ru^{III}–hedtra with the thiol-containing biomolecules, GSH or Cys, in buffer *A*.¶ For the Ru^{III}–edta complex, the values of the pseudo-first-order constants (k_{obs}) were 2.05 ± 0.02 s^{-1} and 2.35 ± 0.04 s^{-1} for GSH and Cys, respectively, and for the Ru^{III}-hedtra complex the values were 0.033 ± 0.004 s^{-1} and 0.065 ± 0.007 s^{-1} for GSH and Cys, respectively, at 37 °C ($[Ru^{III}] = 1.0 \times 10^{-4}$ M, [RSH] = 1.0×10^{-3} M, pH = 7.0). The $[Ru^{III}(hedtra)(OH)]^{-}$ complex was much less labile towards aqua substitution than $[Ru^{III}(edta)(OH_2)]^{-}$. Thus, the lower efficacy of Ru–hedtra complex towards inhibition of PTP, as compared to that of Ru–edta complex, is most likely linked with the lesser affinity of Ru-hedtra complex to bind the RS^{-} group of the Cys residue of the catalytic domain of PTP. It is also hypothesized that the negatively charged pendant carboxylate group in the $[Ru^{III}(edta)(OH_2/OH)]^{1-/2-}$ complex might favor an enzyme–metal complex interaction through electrostatic attraction, since the natural substrates for the enzyme are anionic at pH 7.0.

It is worth mentioning here that although vanadate and pervanadate (a complex of vanadate and H_2O_2) compounds are well-known to modulate insulin metabolic effects by inhibiting protein tyrosine phosphatase (PTP) activity, vanadate and pervanadate inhibit PTPs by completely different mechanisms. PTP inhibition by vanadate takes place through an oxidant-independent pathway and inhibition is reversible with edta.[16] By contrast, pervanadate inhibits PTP by irreversibly oxidizing the catalytic cysteine of PTP.[16] In the present case, Ru^{III}–edta inhibits PTP, like vanadate, through an oxidant-independent pathway. However, oxidation of thiolato ligands in $[Ru^{III}(edta)(SR)]^{2-}$ occurs readily, as shown typically in Fig. 2c–d by the loss of spectral features at 510 nm of $[Ru^{III}(edta)(GSH)]^{2-}$ upon addition of H_2O_2 to the solution of $[Ru^{III}(edta)(GSH)]^{2-}$ (spectrum of $[Ru^{III}(edta)(GSH)]^{2-}$ is shown in Fig. 2b). Excess H_2O_2 present in the system further oxidizes Ru(III) species to $[Ru^{V}(edta)O]^{-}$, as demonstrated by the change in the spectral features (growth at 391 nm)[17] shown in Fig. 2d–e. The above results also point to the possibility of a catalytic pathway for the inhibition of PTP with Ru^{III}–edta in the presence of extra- or intra-cellular H_2O_2 through oxidation of the catalytic Cys residue, as suggested in Scheme 1. Pathway B may be of general importance in the anti-cancer properties of Ru complexes under oxic conditions, whereas pathway B would be more likely with hypoxic solid tumours.[18] Such a mechanism also imparts selectivity for cancer cell cytotoxicity as opposed to that for normal cells since the lower pH values in tumours[19] will favour the labile aqua form of the complexes and, hence, provide selectivity for reactivities with PTPs.

In summary, the observed interactions of the title biologically active complex with PTP may not only be important in understanding its biological activity, but may be of more general applicability in understanding the activities of Ru anti-cancer drugs. The drugs currently in clinical trials are pro-drugs and undergo extensive interactions with proteins before they reach the target tumours.[18] Thus, the pac



Scheme 1 Proposed mechanisms of PTP inhibition by Ru–edta complexes.

complexes are likely to be better biomimetic models for studies of the *in vivo* activities of Ru complexes in clinical trials than are the parent pro-drugs. The reactivities of the Ru-pac complexes can provide clues as to possible mechanisms by which the general class of Ru drugs exert their anti-cancer activities. Most of the studies on the anti-cancer properties of the Ru drugs have focussed on their interactions with DNA, but since PTP inhibition is known to exert anti-cancer properties,[7–13] this may be an important alternative factor in their activities.

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Notes and references

† The recombinant protein tyrosine phosphatase (Yop51* PTP) from *Yersinia enterocolitica* that contains the C235R mutation for enhanced stability was used in all studies. The activity of the Yop51* PTP was measured by its ability to hydrolyse *p*-nitrophenylphosphate (PNPP, 10 mM) to *p*-nitrophenol ($\varepsilon_{405} = 1.8 \times 10^4$ M^{-1} cm^{-1} at pH $\geq$ 12).[20] The reaction components were added on ice to the reaction buffer (buffer *A* in Table 1, 43 μL) in the following order: dilute enzyme solution (1.0 μL in buffer *A*), inhibitor (1.0 μL of aqueous solution), and PNPP (5.0 μL of 0.10 M aqueous solution). After incubation at 37 °C for 30 min, the reactions were stopped by the addition of NaOH (50 μL, 0.10 M) to the reaction mixtures on ice and the absorbances at 404 nm were measured immediately. For each experiment, a separate blank solution was used, which contained all the components of the reaction mixture (including Ru–edta complex), except for the enzyme, and was treated in the same way as the reaction mixture. Typically, 0.20 U enzyme per reaction was used, and this led to $A_{404} = 1.0 \pm 0.3$ ($l = 1$ cm) in the absence of inhibitors. Since the absolute values of A_{404} varied significantly between different preparations of the dilute enzyme, the experiments were performed in series, each of them included the reactions in the absence or presence of an inhibitor. Dithiothreitol (DTT, 2*R*,3*R*-1,4-dimercapto-2,3-butanediol), had to be added to the concentrated solution of the enzyme (57 U μl^{-1}, supplied by the manufacturer), to restore its activity, which was lost on storage (2–3 months at −70 °C). The PTP activity was fully restored after the reaction with DTT in buffer *C* for 3 h at 0 °C. The total concentration of RSH in the reaction mixtures determined with Ellman's reagent was ~7 μM.[21]

‡ Anal. Calculated for $K[Ru^{III}(Hedta)Cl]\cdot 2H_2O$: C 24.0, H 3.42, N 5.59; Found. C 23.8, H 3.45, N 5.63. IR, ν/cm^{-1}: 1720 (free –COOH), 1650 (coordinated $-COO^-$). UV-Vis in H_2O: λ_{max}/nm (ε_{max}/M^{-1} cm^{-1}): 283 (2800 ± 50), 350 sh (680 ± 10).

§ Kinetic measurements were conducted on an Applied Photophysics SX 17MV stopped-flow instrument coupled to Prokineticist software. The reaction was carried out under pseudo-first order condition of excess RSH concentration over the Ru complex, and the time course of the reaction was followed at 510 nm, where appreciable spectral changes between the reactant and product species exist. The instrument was thermostated at the desired temperature (±0.1 °C). The values of the observed rate constants (k_{obs}) are presented as the average value of the several kinetic runs (at least 8 to 10) with an average reproducibility of ±4%.

¶ The Tris buffer (50 mM, pH 7.0) containing NaCl (0.1 M), edta (0.2 mM), BSA (1 mg mL^{-1}), and Brij35 (0.001%) is designated as '*Buffer A*' in the text. The buffer *A* used for the PTP reactions, as well as for dilutions of the enzyme, corresponded to that recommended by Sigma, except for the absence of added DTT. There is no spectral evidence for the formation of complex with Tris-buffer. The reaction of buffer components with Ru-pac complexes is reported to be negligibly slow[2,4] as compared to the reaction of Ru-pac complexes with thio-ligands.

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