Mechanism of polyoxometalate-mediated inactivation of DNA polymerases: an analysis with HIV-1 reverse transcriptase indicates specificity for the DNA-binding cleft

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The anti-DNA polymerase activity of a structural family of polyoxometalates has been determined. Two representative compounds of this family, possessing a saddle-like structure $[(O_3POPO_3)_4W_{12}O_{36}]^{16-}$ (polyoxometalate I) and $[(O_3PCH_2PO_3)_4W_{12}O_{36}]^{16-}$ (polyoxometalate II) were found to inhibit all the DNA polymerases tested, with IC_{50} values ranging from 2 to 10 μ M. A comparative study with HIV-1 reverse transcriptase (RT) and Klenow polymerase as representative DNA polymerases indicated that protection from inactivation was achieved by inclusion of DNA but not by deoxynucleotide triphosphates (dNTPs). Kinetic analysis revealed that the mode of HIV-1 RT inhibition is competitive with respect to DNA, and non-competitive with respect to dNTP binding. Cross-linking experiments confirmed that the inhibitors interfere with the

DNA-binding function of HIV-1 reverse transcriptase. Interestingly, a number of drug-resistant mutants of HIV-1 RT exhibit a sensitivity to polyoxometalate comparable to the wild-type HIV-1 RT, suggesting that these polyoxometalates interact at a novel site. Because different polymerases contain DNA-binding clefts of various dimensions, it should be possible to modify polyoxometalates or to add a link to an enzyme-specific drug so that more effective inhibitors could be developed. Using a computer model of HIV-1 RT we performed docking studies in a binary complex (enzyme–polyoxometalate I) to propose tentatively a possible interacting site in HIV-1 RT consistent with the available biochemical results as well as with the geometric and charge constraints of the two molecules.

INTRODUCTION

HIV 1 reverse transcriptase (RT) is the sole enzyme required for the production of a double-stranded DNA copy of the single-stranded RNA genome of HIV-1. It has three distinct enzymic activities: an RNA-directed DNA polymerase, a DNA-directed DNA polymerase and an RNase H activity [1]. HIV-1 RT isolated from virions consists of a heterodimer containing two subunits of molecular masses 66 and 51 kDa with the same N-terminal sequences. The 15 kDa C-terminal portion of a p66 subunit is enzymically cleaved by HIV protease to yield the p51 subunit. The remaining C-terminal 15 kDa fragment of p66 contains the RNase H active site. The solution of X-ray crystal structures of HIV-1 RT has yielded detailed atomic models showing the two subunits to be strikingly asymmetrical. The RT polymerase domain shares many common features with other DNA polymerases, including a large cleft that accommodates the template-primer (TP), formed by structurally similar three-dimensional motifs (palm, finger and thumb) [2,3]. The nucleotide-binding site is part of the polymerase active site and contains three highly conserved aspartic acid residues (Asp-110, Asp-185 and Asp-186) that seem to be involved in the binding of metal-chelated deoxynucleotide triphosphate (dNTP) as well as its addition in the primer extension reaction [4]. The DNA-binding cleft of HIV RT is rather large and includes residues of the distant RNase H active site.

RT is essential for viral replication yet is not required for normal functioning of the host cell. Therefore it has been an attractive target for drug therapy. The available HIV-1 RT

inhibitors are of two general types and are targeted at two sites of the enzyme. Specifically, these are: (a) nucleoside antagonists, which are directed at the dNTP-binding site of the viral RT and act as chain terminators of proviral DNA synthesis; they are currently the main resource in the anti-RT chemotherapy arsenal; and (b) non-nucleoside HIV-1 RT inhibitors, such as nevirapine and other pyridinones, which are directed to a hydrophobic pocket at the base of the ‘thumb’ subdomain of the HIV-1 RT enzyme [2]. Nevertheless HIV-1 quickly develops resistance to the available inhibitors by producing drug-resistant mutants, presumably taking advantage of the low fidelity of HIV-1 RT [5,6]. The recognition of these compounds is based on interactions limited to few amino acid residues within the enzyme. This is suggested by the extremely small number of mutated residues (often no more than one) in the active site of resistant phenotypes [7,8]. However, the interaction of HIV-1 RT with DNA–DNA hybrids, as well as with DNA–RNA hybrids, involves a relatively large DNA-binding cleft [2,3]. Binding of nucleic acids in the cleft is effected mostly by the generation of an electropositive field formed by a number of amino acids [9]. It therefore seems that the electropositive cleft of HIV-1 RT could be an excellent target for drug development. We considered this site as a target for potential HIV-1 RT inhibitors because it is distinct from the nucleoside and non-nucleoside drug-binding sites, and on the basis of the speculation that potential inhibitors directed towards the DNA-binding cleft of polymerases (instead of the dNTP-binding site) might be more difficult for mutant phenotypes to escape. Suramin, a polysulphonated naphthylurea, has been reported to inhibit HIV-1 RT by targeting the TP-binding site of

Abbreviations used: AMV, avian myeloblastosis virus; AZT, azidothymidine; dNTP, deoxynucleotide triphosphate; MuLV, murine leukaemia virus; polyoxometalate I, $[(O_3POPO_3)_4W_{12}O_{36}]^{16-}$; polyoxometalate II, $[(O_3PCH_2PO_3)_4W_{12}O_{36}]^{16-}$; RT, reverse transcriptase; TP, template-primer.

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the enzyme [10,11]. However, BSA, a non-specific protein, readily reverses the inhibitory action of suramin. To explore HIV-1 RT inhibitors that target the TP-binding site, we searched for compounds that possess a strong negative charge, are less than 18 Å in diameter and are able to inhibit HIV-1 RT activity even in the presence of non-specific proteins. We were attracted by the polyoxometalate and polytungstate class of compounds, which seem to fit the first two criteria, while we determined their anti-RT activity.

Polyoxometalates [12–15] are anionic complexes that have high thermodynamic and/or kinetic stability in aqueous solutions at biological pH. A large variety of structural types of these metal–oxygen cluster anions, based predominantly on edge- and corner-shared MO_6 octahedra ($M = \text{W}^{\text{VI}}$, Mo^{VI} or V^{V}), have been characterized. In addition to well-developed applications in catalysis, separations, analysis and as electron-dense imaging agents, these complexes have been shown to exhibit biological activity ranging from selective enzyme inhibition to antitumour/antiviral properties [14,16–23]. We report here that two representatives of a new structural type of heteropolyoxometalate are inhibitors of a number of DNA polymerases, including HIV-1 RT. Furthermore we investigated the mechanism of inhibition with a combination of biochemical and computer-modelling techniques. Our results suggest that these compounds inhibit HIV-1 RT by interacting at the TP-binding cleft of the enzyme. Most interestingly, a number of drug-resistant phenotypes of HIV-1 RT also displayed a sensitivity to these compounds equivalent to that of the wild-type enzyme.

MATERIALS AND METHODS

Chemicals and enzymes

HPLC-purified dNTPs were obtained from Boehringer Mannheim. All other reagents were of the highest purity grade and were purchased from Fisher, Millipore, Boehringer Mannheim and Bio-Rad. Synthetic TPs were purchased from Pharmacia, and ^{32}P -labelled dNTPs and ATP were produced by DuPont/New England Nuclear Corp.

The cloning of wild-type HIV-1 RT has been described previously [24–26]. Site-directed mutagenesis, as well as preparation and purification protocols for the R72A, R72K, R78A, R78K, Q151A, Q151N, D113A and F116A HIV-1 RT mutants were as described previously [24–26]. The reconstitution of the hybrid subunit-specific mutants was performed as described previously [4].

Murine leukaemia virus (MuLV) RT was prepared by K. Chowdhury, V. N. Pandey and M. J. Modak (unpublished work); Klenow fragment was prepared as described previously [27]. The β -pol enzyme was a gift from Dr. S. H. Wilson (Sealy Cancer Center, Galveston, TX, U.S.A.). Avian myeloblastosis virus (AMV) RT was a gift from Life Sciences Inc. All enzymes were more than 98% pure as judged by SDS/PAGE.

Synthesis of inhibitors

The synthesis of $[(\text{O}_3\text{POPO}_3)_4\text{W}_{12}\text{O}_{36}]^{16-}$ (polyoxometalate I) and $[(\text{O}_3\text{PCH}_2\text{PO}_3)_4\text{W}_{12}\text{O}_{36}]^{16-}$ (polyoxometalate II) has been described previously [28]. The potassium salt of polyoxometalate I and the sodium salt of polyoxometalate II were used in the present study. The purity of both starting materials was checked by ^{31}P -NMR and IR. The NMR experiments were done on a Bruker AM300 WB spectrometer and the IR spectra were obtained with a Mattson FTIR instrument. The crystal structures of the polyoxometalates have been determined [28]. They both

seem to have a saddle-like structure, with a high degree of symmetry [28].

Enzymic assays

RT activities of HIV-1 RT, MuLV RT and AMV RT were assayed by standard methods [24,29,30]. Briefly, the assay mixture contained 0.5 A_{260} unit/ml homopolymeric TP, 0.02 mM $[\text{H}]$ dNTP (0.5 μCi /assay), 80 mM NaCl, 1 mM dithiothreitol, 50 mM Hepes, pH 8.0, and 5 mM MgCl_2 (0.5 mM MnCl_2 for MuLV RT). Typically, an aliquot containing approx. 50 ng of wild-type or 150 ng of mutant enzyme was incubated in 0.1 ml of assay solution at 37 °C. The reaction was initiated by the addition of MgCl_2 and terminated by the addition of 5% (w/v) ice-cold trichloroacetic acid after 15 min. The trichloroacetic acid-precipitable materials were collected on Whatman GF/B filters and counted for radioactivity in a liquid-scintillation counter as described previously [31].

The DNA polymerase activity of the Klenow fragment, HIV-1 RT and β -pol was assayed as above but with $(\text{dC})_n \cdot (\text{dG})_{15}$ and $(\text{dA})_n \cdot (\text{dT})_{15}$ as the respective TPs [32].

The reaction mixture for RNase H assay contained, in a final volume of 0.1 ml, 2–5 μM (as total nucleotides) $[\text{rA}-^3\text{H}](\text{rA})_n \cdot (\text{dT})_n$ (100 000 d.p.m. per assay), 5% (v/v) glycerol, 50 mM Hepes, pH 8.0, 0.1 mg/ml BSA, 0.02% Nonidet P40, 5 mM MgCl_2 and 100 ng of enzyme. Reactions were incubated at 37 °C and processed as described for the RT activity assays.

Steady-state kinetics of polymerization

The kinetic studies were performed as described by Majumdar et al. [33] with homopolymeric $(\text{rA})_n \cdot (\text{dT})_{15}$ or $(\text{dC})_n \cdot (\text{dG})_{15}$ as TPs and dTTP or dGTP as nucleotide substrates. The kinetic constants were calculated by computer-generated linear regression analysis from plots of reciprocal velocities of polymerization against reciprocal substrate concentration in Lineweaver–Burk and Dixon plots. The IC_{50} values were calculated from the inhibition curves as a function of inhibitor concentration. Kinetic experiments were performed at least twice and values were averages of at least duplicate samples. Standard deviations for all K_i and IC_{50} values were less than 15%.

Cross-linking of enzyme to TP

We used the 18/47 DNA for cross-linking studies. The 18mer primer was 5' end-labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and T_4 polynucleotide kinase by a standard protocol (see pp. 5.68–5.72 of [34]). The labelled species was purified on a NAP-10 column (Pharmacia) as suggested by the manufacturer, and adjusted to the required specific radioactivity with unlabelled compound. The reaction mixture contained 50 mM Hepes, pH 8.0, 1 mM dithiothreitol, 2 mM EDTA, 5% (v/v) glycerol, 4 μg of enzyme and 45 pmol of TP. The samples were then exposed to UV radiation in a Spectrolinker (Spectronic Corporation) at a dose of 300 mJ/cm^2 . The cross-linked species were resolved by SDS/PAGE and the extent of cross-linking was quantified by excising the radioactive bands and measuring the Čerenkov counts associated with them. The cross-linked enzyme–TP complexes show the expected differences in gel mobility [24].

Computer modelling studies

Computer modelling studies were performed with the SYBYL 6.1 software package on an Indigo 2 workstation. The crystal

structure coordinates of the HIV-1 RT enzyme, co-crystallized with nevirapine, were downloaded from the Protein Database [2]. Similar results were obtained when the unliganded form of HIV-1 RT [35] was used for the docking studies. After addition of the hydrogen atoms, the Gasteiger–Hückel electrostatic potential of the protein was calculated (SYBYL manual and references therein). Similarly, the crystal co-ordinates of polyoxometalate I were used to prepare a molecular model of this inhibitor. Because the proper parameters for the calculation of atomic point charges are not available for the tungsten atom, we manually assigned a point charge of -0.57 for each of the 28 non-bridging oxygen atoms of the highly symmetrical polyoxometalate I, thus accounting for the total charge of -16 per molecule of polyoxometalate I. This approximation might affect the computations of precise charge distribution and thus the absolute values of the energy of interaction. However, the general position of the ligand in the active site should not change because it is based on differences in the energies of interaction and not on their absolute values. An assumption made in this study is that neither the enzyme nor the inhibitor undergoes major structural changes on binding. This assumption could be evaluated in spectrophotometric or crystal studies of the enzyme–inhibitor complex. More than 100 slightly different docking positions were examined across the length of the DNA-binding cleft of HIV-1 RT by using the docking sub-routines of SYBYL 6.1. These candidate positions satisfied the following criteria, dictated by our results from biochemical experiments (see the Results section): (a) polyoxometalate I does not bind at the dNTP-binding site, (b) polyoxometalate I binds at the DNA-binding cleft, and (c) binding of polyoxometalate I affects the RNase H activity. The enzyme–inhibitor complex with the lowest (most negative) energy was chosen for further examination. Specifically, some fine adjustments of the inhibitor's position in the active site were performed manually, with the criterion of negative overall energy of the enzyme–inhibitor complex. Finally, energy minimization of the resulting complex yielded the final presented modelled structure. Other manipulations of the computer model were as described previously [24,36].

RESULTS

Effect of polyoxometalates I and II on various DNA polymerases

Both polyoxometalates I and II are potent inhibitors of the RNA-directed DNA polymerization by HIV-1 RT, with IC_{50} values achieved at inhibitor concentrations of about 4.3 and 5.2 μM (Table 1). To assess whether these polyoxometalates

Figure 1 Effect of polyoxometalates on the polymerase activity of HIV-1 RT and of cellular polymerases

HIV-1 RT (50 ng) (A), Klenow fragment (25 ng) (B) and DNA polymerase β (50 ng) (C) were assayed for DNA polymerase activity as described in the Materials and methods section. The activity was followed with $(rA)_n \cdot (dT)_{15}$ and $(dC)_n \cdot (dG)_{15}$ as the TPs for the HIV-1 RT and Klenow fragment enzymes, and with $(dA)_n \cdot (dT)_{15}$ for the DNA polymerase β enzyme. Dose–response curves in the presence of varying amounts of polyoxometalate I are shown. An activity of 100% corresponds to 150, 245 and 95 pmol of TTP incorporated in $(rA)_n \cdot (dT)_{15}$ by the assayed amounts of HIV-1 RT, Klenow fragment and DNA polymerase β respectively in a reaction time of 15 min.

Table 1 Effect of polyoxometalates I and II on RNA-directed DNA polymerase activity of various retroviral RTs

Polymerase activity was measured as described in the Materials and methods section, with $(rA)_n \cdot (dT)_{15}$ as the TP. Experiments were performed at least three times and values were averages of at least duplicate samples. Standard deviations for the IC_{50} values were less than $\pm 15\%$. An activity of 100% corresponds to 150 pmol of dNTP incorporated in TP by 50 ng of HIV-1 RT enzyme in the reaction time of 15 min. * More than 300 μM .

Inhibitor	Reverse transcriptase					
	HIV-1		MuLV		AMV	
	IC_{50} (μM)	IC_{95} (μM)	IC_{50} (μM)	IC_{95} (μM)	IC_{50} (μM)	IC_{95} (μM)
I	4.3	48.0	3.5	28.5	63.0	*
II	5.2	81.0	6.2	45.7	*	*

have a general inhibitory effect on retroviral reverse transcriptases, we tested their effect on enzymes from two additional retroviruses, namely AMV and MuLV. We found that MuLV RT was strongly inhibited by both polyoxometalates (IC_{50} values of 3.5 and 6.2 μM respectively), whereas AMV RT was substantially less susceptible to inhibition (IC_{50} values of 63 and

Figure 2 Inhibition of HIV-1 RT RNase H activity by polyoxometalates

HIV-1 RT enzyme was assayed for RNase H activity as described in the Materials and methods section. The amount of non-hydrolysed $[rA\text{-}^3\text{H}](rA)_n \cdot (dT)_n$ substrate in the presence or absence of 10 μM polyoxometalate I or II is plotted against the time of reaction.

more than 300 μM , for inhibition by polyoxometalate I and II respectively). We therefore observed no significant differences in the inhibition of HIV-1 RT and MuLV RT by the two polyoxometalates. However, the more distant retrovirus AMV seems to be more resistant to these inhibitors.

Because reverse transcriptases catalyse both RNA- and DNA-directed DNA synthesis we also examined the effect of the two polyoxometalates on the DNA-directed polymerase function of HIV-1 RT. Polyoxometalates I and II are substantially less effective in inhibiting the extension of $(dC)_n \cdot (dG)_{15}$ by RT. Specifically, the dose-response inhibition curves of the HIV-1 enzyme (Figure 1) show that the RNA-directed DNA synthesis, compared with the DNA-directed DNA synthesis, is 2–3-fold more susceptible to inhibition by polyoxometalate I. A similar decrease in the TP-dependent sensitivity of HIV-1 RT was also observed with the methylene-bridged polyoxometalate II (results not shown). Furthermore, the same pattern was observed in the inhibition of MuLV RT by polyoxometalates I and II, with the RNA-directed DNA synthesis being approx. 5-fold more susceptible than the DNA-directed DNA synthesis (results not shown). Therefore our results suggest that there is no substantial difference in the efficacy of the inhibitor when the bridging group of the phosphate ions is changed from oxygen (polyoxometalate I) to methylene (polyoxometalate II).

Effect of polyoxometalates on the RNase H activity of HIV-1 RT

All retroviral reverse transcriptases, including HIV-1 RT, have RNase H activity, which degrades the RNA template on completion of its copying into RNA–DNA hybrid. The active sites for the polymerase function and the RNase H activity are clearly separated, although the polynucleotide-binding cleft is common to both [2,3]. If polyoxometalates I and II do indeed inhibit HIV-1 RT polymerization by preventing binding of the polynucleotide at the DNA-binding site, it follows that similar inhibition should be observed for the RNase H activity. In fact, our results show that the RNase H hydrolysis of $[rA\text{-}^3\text{H}](rA)_n \cdot (dT)_n$ by HIV-1 RT is inhibited by both polyoxometalates (Figure 2). Interestingly, the IC_{50} values for RNase H inhibition are similar to those for inhibition of polymerization, supporting our contention

Figure 3 Kinetic analysis of the inhibition of HIV-1 RT by polyoxometalate I

Double-reciprocal plots of various TTP concentrations as a function of initial velocity of polymerization, in the presence of various amounts of polyoxometalate I. Assays were performed as described in the Materials and methods section.

that the polyoxometalate-mediated inhibition is due to its binding at the DNA-binding site.

Effect of polyoxometalates on the DNA-dependent DNA polymerase activity of other polymerases

The high inhibitory activity of the two polyoxometalates led us to examine their potential inhibitory activity on cellular polymerases, including the prokaryotic Klenow fragment of the *Escherichia coli* DNA polII and the eukaryotic DNA polymerase β . The resulting inhibition curves are shown in Figures 1(B) and 1(C). Both DNA polymerases are sensitive to the polyoxometalate inhibitors. Interestingly, as with the various reverse transcriptases studied, these enzymes are more sensitive to polyoxometalate I than to polyoxometalate II (results not shown). The effectiveness of DNA polymerase β inhibition (IC_{50} 3 μM) is comparable with that of HIV-1 RT and of the Klenow fragment (Figures 1C, 1B and 1A), suggesting a common mechanism of action.

Specificity of inhibition: determination of steady-state kinetic constants

To improve our understanding of the nature of the inhibition of HIV-1 RT, a kinetic analysis of enzyme inhibition was conducted to determine the mechanism by which these polyoxometalates inhibit this enzyme. Initially we established that the inhibition is specific and not due to non-specific binding of the polyoxometalates to any protein. This was shown by increasing amounts of BSA, up to 3 mg/ml, in the polymerization reaction mixture, which did not change the inhibitory potential of the polyoxometalates (results not shown). The steady-state kinetics were then measured and the linear initial rates of $[^3\text{H}]\text{TTP}$ incorporation into $(rA)_n \cdot (dT)_{15}$ were used to construct Lineweaver–Burk plots at increasing concentrations of inhibitor. It is clear from Figure 3 that polyoxometalate I is a non-competitive inhibitor of HIV-1 RT with respect to dTTP. The apparent K_m is 8.9 μM irrespective of the presence of inhibitor. A secondary (Dixon) plot of inhibitor concentration against $1/v$ (where v is the polymerization reaction velocity) is shown in Figure 4. For

Figure 4 Dixon plot of the inhibition of HIV-1 RT by polyoxometalate I at various TTP concentrations

Effect of polyoxometalate I inhibitor concentration on the reciprocal velocity at various TTP concentrations. Assays were performed as described in the Materials and methods section.

Figure 5 Dixon plot of the inhibition of HIV-1 RT by polyoxometalate I at various TP concentrations

Effect of polyoxometalate I inhibitor concentration on the reciprocal velocity at various TP concentrations. Assays were performed as described in the Materials and methods section.

non-competitive inhibitors, this type of analysis results in an intercept at $(K_i, 0)$ [37]. The K_i values for polyoxometalates I and II (results shown only for polyoxometalate I) calculated in this manner were 18 and 21 μM respectively. A similar analysis of enzyme inhibition by polyoxometalates I and II, with various amounts of TP and a saturating concentration of dNTP, indicated a competitive mode of inhibition (results not shown). The K_i of approx. 5 μM was discerned from the Dixon plots constructed from the inhibition data (Figure 5).

Effect of polyoxometalates I and II on the binding of HIV-1 RT to TP

The results of the kinetic analysis suggest that both polyoxometalates inhibit HIV-1 RT by binding specifically to the active site, thereby precluding the binding of the DNA substrate. To verify this observation we directly examined the effect of the inhibitors on the ability of the enzyme to bind radiolabelled

Figure 6 Effect of polyoxometalate I on the binding of TP to HIV-1 RT

HIV-1 RT (4 μg) was incubated with 45 pmol of $[5'\text{-}^{32}\text{P}]18/47\text{mer}$ (2–100 nM) in a standard reaction mixture and exposed to UV radiation. The cross-linked species were resolved by SDS/PAGE and the extent of cross-linking was quantified by excising the radioactive bands and measuring the Čerenkov counts associated with them. Points in the graphs represent the amount of DNA bound to HIV-1 RT in the presence of the indicated amount of inhibitor, relative to the control sample with no inhibitor. The sequence of the 18/47 TP was 5'-CTCCATTCACACAGTGC-3'; 3'-GAAGTAAGTGTGTCACGATGTCTGACCTGTTTTGTGACATT-GAG-5'.

DNA TP. The binding ability was assessed by photochemical cross-linking of the HIV-1 RT enzyme with the ^{32}P -labelled 18/47 TP (Figure 6). The mobility of the labelled species, measured by means of SDS/PAGE, shows that cross-linking is associated only with the p66 subunit of HIV-1 RT [24]. UV irradiation brings about the formation of a 'zero length' covalent bond [38] between the enzyme and the associated TP, thereby freezing the interaction between them. The DNA binds at the active site of the enzyme in a specific and catalytically relevant manner, as demonstrated by the inability of either $(\text{dC})_n$ template or $(\text{dG})_{15}$ primer alone to prevent cross-linking, and also as by the competitive reduction in cross-linking by $(\text{dC})_n \cdot (\text{dG})_{15}$ [24]. When the cross-linking reaction is performed in the presence of increasing amounts of polyoxometalate I, a marked decrease in the cross-linking of TP to enzyme is observed (results not shown). The quantification of the complexes as a function of increasing inhibitor concentrations clearly demonstrates that polyoxometalate I inhibits the formation of DNA–RT complexes (IC_{50} 8 μM). Furthermore, polyoxometalate I seems to inhibit the Klenow fragment by a similar mechanism, as it also interferes with the formation of the Klenow–DNA complex (results not shown). Polyoxometalate II was also seen to interfere with the HIV-1 RT–DNA and Klenow–DNA interactions, as judged from similar decreases in DNA–RT cross-linking (results not shown).

Computer modelling studies

A computer-generated HIV-1 RT modelled structure has been constructed in our laboratory [39] based on the α -carbon co-ordinates of the crystal structure of HIV-1 RT co-crystallized with DNA [3]. However, the polyoxometalate inhibitors are proposed to bind the DNA-free form of HIV-1 RT. We therefore decided to use the published crystal structure co-ordinates [2] that correspond to HIV-1 RT crystallized in the absence of DNA. Similar results were obtained when we used the most recent structure of RT with no ligand bound [35]. Docking of the polyoxometalate I in the DNA-binding site of the HIV-1 RT enzyme is shown in Figure 7. The steric energy of the complex is slightly positive (109 kJ/mol; 26 kcal/mol), suggesting that the

Figure 7 A model of the binding interactions of polyoxometalate I at the DNA-binding cleft of HIV-1 RT

A distant view of the computer-generated DNA-binding cleft of HIV-1 RT, with polyoxometalate I docked at the interface of the p66 and p51 enzyme subunits. The HIV-1 RT model was generated by using co-ordinates from the Protein Database [2]. The polyoxometalate I crystal structure co-ordinates were taken from [28]. The inhibitor was docked in the DNA binding cleft of HIV-1 RT by using the standard docking protocols of SYBYL 6.1 (see the Materials and methods section) and in accordance with the biochemical data. The total energy of the HIV-1 RT–inhibitor complex was negative (-962 kJ/mol; -229 kcal/mol). Possible interactions of polyoxometalate I with HIV-1 RT include the basic residues (emphasized side chains on the enzyme) Arg-356, Arg-358, Lys-374 of the p66 enzyme subunit (in yellow) and Lys-1022, Lys-1395, Asn-1418 and Gln-1394 of the p51 subunit (in red). The inhibitor is shown in orange.

inhibitor is interacting with residues at the active site without, however, engaging in bad contacts that would substantially increase the energy of the complex. The electrostatic energy of the complex is negative (-1155 kJ/mol; -275 kcal/mol), suggesting that the highly negatively charged inhibitor is interacting with electropositive residues. Furthermore, the interaction is strong enough to overcome the unfavourable steric effects and thus yield a total negative energy (-1046 kJ/mol; -249 kcal/mol). The proposed model for the interaction of HIV-1 RT with polyoxometalate I is in agreement with the experimental results. Specifically, binding at this position also predicts RNase H inhibition. In addition this binding is in agreement with the observed modes of inhibition of polymerization with respect to dNTP (non-competitive) and DNA (competitive). This proposed working model is not intended to substitute for a crystal structure. In fact, the proposed mode of inhibitor binding is one of many possible binding modes. We are pursuing crystallographic studies of the RT–polyoxometalate complex to assess our computer modelling results.

Effect of polyoxometalates on the polymerase activity of drug-resistant HIV-1 RT mutants

A number of nucleotide (analogue) and non-nucleotide inhibitors of HIV-1 RT are known. However, the rapid emergence of resistant phenotypes, due mainly to the point mutation in the gene sequences coding for HIV-1 RT, has been well documented (reviewed in [5]). To understand the mechanism of drug resistance in HIV-1 RT, we prepared and extensively characterized a number of site-specific mutants of the enzyme [24–26]. As for the drug resistance of these mutants, we find that mutation at Gln-151 and Arg-72 induces resistance to dideoxynucleotides and phosphonoformate respectively [24,25]. The Met-184 mutant is found to have increased drug sensitivity [6], whereas Arg-78 → Ala seems to have a significantly decreased ability to bind TP [25]. Most of the above mutants exhibited impairment of their polymerase activity [24–26]. It was important to determine whether the mutant enzymes had an increase or decrease in their sensitivity to a different class of compounds, such as this structural family of polyoxometalates. No significant change in the IC_{50} values of any of the mutant enzymes, compared with the IC_{50} of the wild-type HIV-1 RT, is noted (results not shown). The unchanged sensitivity to polyoxometalate I shows that even upon mutation of all these residues the enzyme retains full ability to bind the inhibitor. Thus none of these amino acids is expected to participate in the binding of polyoxometalate I. This finding is in agreement with our proposed interaction, which takes place at a position distant from all these residues (Figure 7).

DISCUSSION

We have examined the anti-(DNA polymerase) activity of two polyoxometalates. The synthesis of the two polyoxometalates is performed in one step and is cost-effective [28]. The crystal structures of the two polyoxometalates revealed that they share a novel saddle-like symmetrical structure [28]. The high negative charge and the general dimensions of this unique structure led us to examine the inhibitory potential of these compounds against DNA polymerases in general, and HIV-1 RT in particular. Specifically, they have the high negative charge density of the natural DNA substrate and dimensions of $15 \text{ \AA} \times 15 \text{ \AA} \times 10 \text{ \AA}$, comparable to the diameter of B DNA (approx. $19 \text{ \AA}$). These characteristics are presumably responsible for their high potency as HIV-1 RT inhibitors and suggest that the site of interaction is at the DNA-binding cleft. Our kinetic analysis showed that the

polyoxometalates do not compete for the binding of dNTP substrate (non-competitive mode of inhibition). Furthermore, site-specific HIV-1 RT mutants, with conservative and non-conservative point mutations, mostly at or proximal to the dNTP-binding site, exhibited the same sensitivity as the wild-type enzyme, indicating a lack of effect of these inhibitors on the substrate-binding pocket of HIV-1 RT. However, the mode of HIV-1 RT inhibition with respect to the TP is competitive, further supporting a direct interaction of the polyoxometalates at the DNA-binding site. Additional support for this conclusion is obtained by direct assessment of the binding ability of HIV-1 RT to DNA TP in the presence of polyoxometalates (Figure 6). Results of photoaffinity labelling of HIV-1 RT in the presence of inhibitor clearly show that the binding of TP to enzyme is reduced in a dose-dependent manner.

Using computer-assisted molecular modelling technology we were able to dock the structure of polyoxometalate I at the DNA-binding cleft of HIV-1 RT with a favourable energy of interaction. Interestingly, this site in the model is distant from the dNTP-binding site as well as the nevirapine-binding site. The former site is the target of all nucleoside analogue inhibitors, including azidothymidine (AZT), dideoxycytosine, dideoxyhypoxanthine, 3-thiacytidine and others. Nucleoside-resistant phenotypes are reported to occur by the mutation of even a single amino acid in HIV-1 RT. The nucleoside-drug-resistance of these mutants permits them to distinguish between dNTP and dNTP-analogue inhibitors. The resistance of HIV-1 RT to many non-nucleoside HIV-1 RT inhibitors has also been found to appear after a few weeks of exposure of infected cells to the inhibitor. In this case the resistance phenotype is accompanied by mutations at the 181 and/or 188 position of HIV-1 RT. The region bounded by these residues forms a hydrophobic pocket [39] at the base of the thumb sub-domain. This hydrophobic cleft is not in itself essential for any vital function in the RT mechanism, but once it is distorted the resulting indirect effects are thought to interfere with the polymerase function. It is known that in the mechanism of polymerization by HIV-1 RT the binding of the enzyme to the TP precedes the binding to dNTP [33]. Therefore the interaction of the inhibitors described here at the HIV-1 RT DNA-binding site constitutes another facet of the interference of anti-AIDS drugs. In contrast with AZT and related nucleoside analogue inhibitors, whose binding depends heavily on only a few amino acids, or even only one [5], a number of potential contacts of the polyoxometalate I inhibitor at the active site of HIV-1 RT are seen in our computer model. Some basic residues in such a position are: Arg-356, Arg-358, Lys-1395, Lys-1022, Lys-374, Asn-1418 and Gln-1394 (residues with numbers higher than 1000 belong to the p51 subunit). However, in the absence of mutational data to support these specific interactions, we have to be aware of the limitations of the docking technology as well as those of the crystal structure that was the basis for this study ($3.5 \text{ \AA}$ resolution) [2]. Nevertheless, this potential variability suggests that these inhibitors would present more of a challenge to HIV-1 RT, as the depletion of inhibitor binding would require the mutation of numerous residues. Suramin, a polysulphonated naphthylurea, has also been reported to inhibit HIV-1 RT by a similar mechanism, i.e. by targeting the TP-binding site of the enzyme [10,11]. However, BSA, a non-specific protein, readily reverses the inhibitory action of suramin. Albumin has no effect on the inhibitory action of polyoxometalates I and II (results not shown), suggesting that the interaction of polyoxometalates with RT is different from that of suramin and is specific to the DNA-binding cleft.

A possible way of further increasing the binding of the inhibitors to HIV-1 RT would be to increase the size of the

inhibitor. Molecular models show that the DNA-binding cleft of HIV-1 RT [2,33,40,41] is able to accommodate molecules of even larger size. In fact, larger molecules that retain a high negative charge would presumably have better binding characteristics owing to the increased binding energy. Previously, compounds of similar charge and size have been used successfully against virus-infected cells [18,21]. Therefore the delivery of these compounds might not pose a serious problem. The main advantage of polyoxometalates I and II as potential HIV-1 RT inhibitors stems from their ability to inhibit the drug-resistant HIV-1 RT strains. These results raise the possibility of their use in combination with existing inhibitors. However, the clinical potential of these compounds will also depend on the extent of any side effects. If necessary, the specificity of the polyoxometalates could be increased by chemically linking them to HIV-1 RT-specific non-nucleoside inhibitors such as nevirapine, or to nucleoside analogue compounds such as dideoxynucleotides or AZT.

The two polyoxometalates inhibited other reverse transcriptases and also β -pol, a mammalian enzyme thought to be involved in DNA-damage repair. The production of β -pol is increased during chemotherapy of cancer patients [42]. This undesired (under such circumstances) DNA repair activity, which contributes to the survival of tumour cells, could conceivably be abolished by an inhibitor such as polyoxometalate I or II. A detailed investigation of the inhibitory effects of polyoxometalates on this enzyme is currently in progress.

The two inhibitors presented here can also be used to obtain information regarding the mechanism of polymerases. For example, the Klenow fragment, which is a DNA-dependent DNA polymerase, is also able to catalyse the extension of $(rA)_n \cdot (dT)_{15}$. However, it is not known whether the synthesis of DNA-DNA and DNA-RNA hybrids is catalysed by the same mechanism and by binding of the two types of polymer in a common manner. We find significant differences in the ability of polyoxometalates I and II to inhibit the polymerization of the two TPs. This result suggests that the Klenow enzyme binds the two polymeric substrates differently. In addition, comparisons of the inhibition profiles of the MuLV RT and AMV enzymes by polyoxometalates I and II can be used to infer their degree of similarity to the known HIV-1 RT structure.

In conclusion, we have introduced a new structural family of potent DNA polymerase inhibitors that target the DNA-binding cleft of these enzymes. Specific interactions based on kinetic analysis and molecular modelling of HIV-1 RT-inhibitor complexes have been proposed. The two compounds could help in the design of novel inhibitors of HIV-1 RT with increased specificity.

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