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CORRELATION BETWEEN STRUCTURE OF POLYOXOTUNGSTATES AND THEIR INHIBITORY ACTIVITY ON POLYMERASES

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The 21-tungsto-9-antimonate (TA, HPA 23), a polyoxotungstate, has shown a significant antiviral activity in vivo and in vitro. It inhibits viral and bacterial DNA polymerases. In this paper, several compounds of two polyoxotungstic families, tungstoantimonates and tungstoarsenates, have been used to specify the mechanism of polymerase inhibition. It has been demonstrated that the inhibitory activity of polyoxotungstates is not related to the occupation of their coordinative sites by cations, nor to the nature of these bound cations. Kinetic studies and binding assays have shown that polyoxotungstates bind to the polymerases in competition with the nucleic acid template. This result seems to be related to their polyanionic nature. Furthermore, the size and charge of these compounds may play a prominent part in their affinity for the polymerases.

Polyoxotungstates (or tungstic heteropolyanions, HPA) are large anionic polyelectrolytes with negative charges on all oxygen atoms. Their general structure is schematically represented in figure 1. HPA have coordinative sites which can bind metallic cations. Two types of sites can be distinguished: a central one in which are often included alkaline or alkaline-earth cations and external sites where transition metallic cations can be fixed.

The 21-tungsto-9-antimonate ammonium salt (TA, HPA 23) is an antiviral agent which has shown a significant activity in vitro at non cytotoxic doses against many DNA and RNA viruses (1-4). In vivo, TA protects mice against lethal infections induced by oncornaviruses, Friend leukemia virus, mammary tumor virus, and non oncogenic viruses such as vesicular stomatitis and encephalomyocarditis viruses (5-8). Furthermore, it is the first antiviral compound which has a significant in vivo activity against experimental scrapie (9).

The mechanism of action of TA is not known but it has been shown that the product has an inhibitory effect on cellular DNA polymerases and on retrovirus transcriptases (1,4). Enzymatic kinetic studies have demonstrated that the in-

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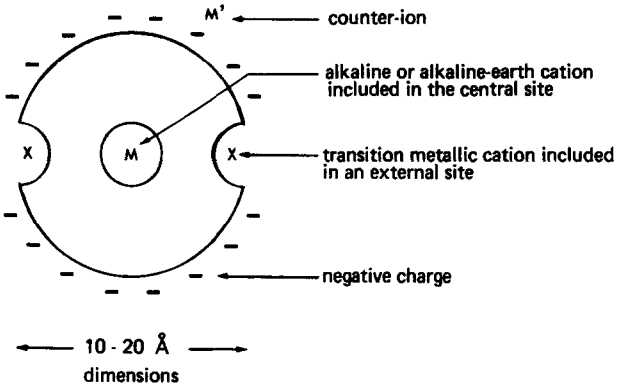

Figure 1. Schematic representation and principal characteristics of polyoxotungstates. The external circle represent the globular shape of polyoxotungstates, with negatives charges (-) distributed on all oxygen atoms of the W-O-W frame (not represented).
Some oxygen atoms define coordination sites which can be vacant or occupied by metallic cations. Chemical studies (10, 11) have shown that, when these sites are in central position (represented by a small circle), alkaline and alkaline-earth cations M are preferentially bound. On the opposite, when they are external, on contact with the solvent, transition metal cations X, such as Co^{2+} or Fe^{3+}, are preferentially bound.

hibition of the reverse transcriptase of MLV by TA was due to a competition between the heteropolyanion and the template (1). In order to specify the mechanism of polymerases inhibition by HPA and the structure activity relationship, we have selected two families of HPA: the tungstoantimonates (TA) and tungstoarsenates (TAs) and compared their inhibitory activity on E. coli DNA and RNA polymerases and on two reverse transcriptases.

MATERIAL AND METHODS

Polyoxotungstates.

Tungstoantimonates $Sb_9W_{21}O_{86}^{19-}$ can be isolated as sodium, potassium or ammonium salts as previously described (10). The central site is occupied by a cation such as Na^+, K^+, Sr^{2+} or Ca^{2+}, noted M. External sites can be occupied by a transition metal such as Fe^{3+}, noted X. The various tungstoantimonates reported below will be shortened MTA or MTAX.

Tungstoarsenates $As_4W_{40}O_{140}^{28-}$ were synthetized as sodium or potassium salts as described before (11). They will be shortened as MTAs (M= Na^+, K^+ or Ba^{2+}) and MTAsX (X= Ag^+ or Co^{2+}).

Tungstosilicate $SiW_{11}O_{39}^{8-}$ has been used in binding assays and was prepared according to Tézé (12). It will be shortened as TS.

Polymerases and nucleotides.

Unlabeled triphosphate nucleotides were obtained from Calbiochem and unlabeled poly or oligonucleotides from Boehringer Mannheim Corp.

^{3}H-dTTP and ^{3}H-UTP were purchased from Amersham.

^{3}H-poly d(A-T) was synthetized with E. coli DNA polymerase I using ^{3}H-dTTP. The enzyme was removed by extraction with a phenol-chloroform mixture and ^{3}H-poly d(A-T) purified by elution from a sephadex G50 column, precipitated with absolute alcool at -20°C and then dissolved in water (1µl gave 6000 cpm).

E. coli DNA polymerase I and E. coli RNA polymerase were purchased from Boehringer Mannheim Corp.

Reverse transcriptase from murine Moloney leukemia virus (M,MLV) and from mouse mammary tumor virus (M.MTV) were prepared according to the methods previously described (7).

Assay procedures for polymerases.

M,MLV reverse transcriptase assays. The reaction mixture contained, in a final volume of 50 µl: 50 mM tris pH 7.9, 20 mM KCl, 0.5 mM $Mn(CH_3COO)_2$ 1mM dithiotreitol (DTT), 0.1% triton X100, 3.3 µM ^{3}H-TTP (2.10^4 cpm/p mole), 0.05 OD unit/ml poly A, 0,05 OD unit/ml oligo dT_{12-18} and 5 µl of viral solution (in NTE containing 10 mM tris pH 7.4, 100 mM NaCl, 1 mM EDTA).

After incubation at 37°C for 1h, the reaction was stopped and the radioactivity incorporated into TCA precipitable material was measured.

M.MTV reverse transcriptase assays. The protocol was the same, but the reaction mixture contained 50 mM tris buffer pH 7.6, 3 mM NaCl, 12 mM $MgCl_2$, 10 mM DTT, 0,1% triton X100, 10 µg/ml poly C, 4 µg/ml oligo dG_{10}, 9 µM ^{3}H-dGTP (8.10^3 cpm/ p mole) and 5 µl of viral solution.

E. coli DNA polymerase assays. The reaction mixture contained, in a final volume of 60 µl: 77 mM tris buffer pH 7.9, 0.77 mM $MgCl_2$, 0.77 mM DTT, 0.004% triton X100, 96 µM of each dXTP, 3.3 µM of ^{3}H-dTTP, 77 µg/ml of E. coli native DNA and 0.32 unit of DNA polymerase. The mixture was incubated at 37°C for 30 mn.

E. coli RNA polymerase assays. The reaction mixture contained, in a final volume of 50 µl, 40 mM tris buffer pH 7.9, 1 mM $Mn(CH_3COO)_2$, 4 mM $MgCl_2$, 20 mM DTT, 0.005% triton X100, 4 mM ATP, 8.3 µM ^{3}H-UTP ($7\ 10^3$ cpm/p.mole), 0.25 OD unit/ml poly d(A-T) and 0.4 unit of RNA polymerase. The reaction mixture was incubated at 37°C for 30 mn.

The inhibitory effect of HPA was determined from reactions in which they were added to the standard mixture at varying amounts before the enzyme addition. The activity was expressed in 50% inhibitory doses determined by the log-probit method (13).

Binding assays.

The binding of HPA to the polymerases was evidenced by competition with ^{3}H-poly d(A-T) in excess (in order to saturate the enzyme) using the nitrocellulose filter binding technique.

Binding assays with DNA polymerase. The reaction mixture contained, in a final volume of 50 µl, 67 mM Hepes buffer pH 7.4, 1 mM 2-mercaptoethanol, 10 µl of ^{3}H-poly d(A-T) solution, 7.14 pmoles of DNA polymerase I and HPA at different concentrations. After incubation at 37°C for 15 mn, 40 µl were picked up, directly filtered on a Millipore filter (type HA 24 mm diameter) and very quickly washed with 1 ml of cold buffer (10 mM hepes buffer pH 7.4, 5 mM 2-mercaptoethanol and 50 mM NaCl).

Binding assays with RNA polymerase. The same procedure was used with 50 µl of the following mixture: 40 mM tris buffer pH 8.0, 4 mM $MgCl_2$, 12 mM 2-mercaptoethanol, 10 µl ^{3}H-poly d(A-T) solution, 7.14 pmoles of RNA polymerase. The mixture was incubated for 5 mn before filtration. Washing buffer contained 10 mM tris buffer pH 8.0 and 50 mM NaCl.

RESULTS

Relation between structure and activity of HPA on the polymerases.

The results obtained with TA and TAs compounds upon the different polymerases are summarized in Table I.

With TA compounds, no significant differences of inhibitory activities were found except when the central site was occupied by a potassium: KTA was less active on all the polymerases and specially on the reverse transcriptase of M.MTV than the other MTA.

Table I. Effect of the central site cation of tungstoantimonate and tungstoarsenate compounds on the polymerases inhibition. ID_{50} values, given in mol. l^{-1}, are defined as the concentration of HPA giving 50% inhibition under the conditions described in Material and Methods.

HPA	Reverse transcriptase M MLV	Reverse transcriptase M MTV	E. coli DNA POL	E. coli RNA POL
NaTA	$7.3\ 10^{-8}$	$8.8\ 10^{-7}$	$1.9\ 10^{-6}$	$7.3\ 10^{-9}$
KTA	$3.1\ 10^{-7}$	$1.8\ 10^{-5}$	$9.6\ 10^{-6}$	$5.9\ 10^{-8}$
CaTA	$1.0\ 10^{-7}$	$4.4\ 10^{-7}$	$1.9\ 10^{-6}$	$1.2\ 10^{-8}$
SrTA	$1.0\ 10^{-7}$	$1.0\ 10^{-6}$	$1.6\ 10^{-6}$	$5.9\ 10^{-9}$
NaTAs	$1.7\ 10^{-8}$	$5.8\ 10^{-7}$	$3.3\ 10^{-9}$	$5.0\ 10^{-9}$
KTAs	$1.7\ 10^{-8}$	$5.0\ 10^{-7}$	$3.3\ 10^{-9}$	$4.2\ 10^{-9}$
BaTAs	$1.7\ 10^{-8}$	$5.8\ 10^{-7}$	$3.3\ 10^{-9}$	$5.0\ 10^{-9}$

No difference was observed between Na, K and BaTAs activities, whatever the polymerase.

Comparison of the TA and TAs families (except KTA) have shown that their inhibitory activities are similar on reverse transcriptases and on E. coli RNA polymerase, while on E. coli DNA polymerase I, TAs compounds have a stronger effect than TA ones (ratio of ID_{50} = 200).

The occupation of external sites by transition metal cations did not result in an appreciable effect on the ID_{50} values for any of the polymerases studied (results not shown).

Mechanism of the polymerases inhibition by TA and TAs.

DNA polymerases. Lineweaver-Burk plots of the results obtained with the reverse transcriptase of M.MLV (fig.2) show that the inhibition pattern for TAs is consistent with competitive inhibition as for TA (1). It suggest that TAs as well as TA, prevent the template binding to the polymerase. The inhibition constant K_i for TAs is very small (10^{-8} M) and has the same order of magnitude than TA ($2.\ 10^{-8}$M).

By the same way, a competitive inhibition of E. coli DNA polymerase I has been demonstrated for both TA and TAs with regard to the template. The inhibitory constant values are: $K_i(TA) = 3.10^{-7}$M and $K_i(TAs) = 2.10^{-9}$ M.

Binding of HPA to E. coli DNA polymerase I was evidenced by binding assays (fig.3): they strongly reduce the amount of poly d(A-T) complexed. If we compare the three HPA, their effectiveness is in the order TAs > TA > TS. Quantitatively, a concentration of TAs just slightly superior to the enzyme one was sufficient to completely prevent the formation of the enzyme-poly d(A-T) complex, whereas a large excess of NaTA was needed.

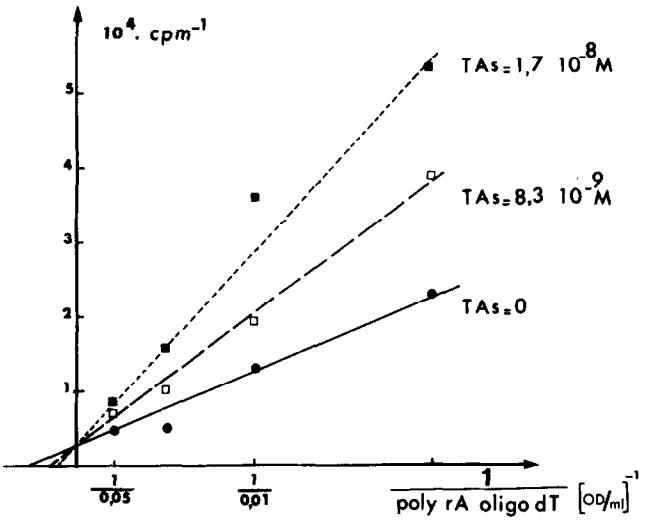

Figure 2. Lineweaver-Burk representation of the influence of poly A-oligo dT template concentration on the inhibition by TAs of M.MLV reverse transcriptase activity. The reaction conditions were as described in Material and Methods.

E. coli RNA polymerase. Lineweaver-Burk plots obtained for the inhibition by TAs are shown fig.4. Binding assays have shown that, as for E. coli DNA pollymerase, the percentage of bound poly d(A-T) was reduced in presence of HPA. Moreover, it was dependent of the order of addition of products: there was more poly d(A-T) complexed when it has been incubated with the enzyme before TAs addition than when it was the reverse order of mixing products.

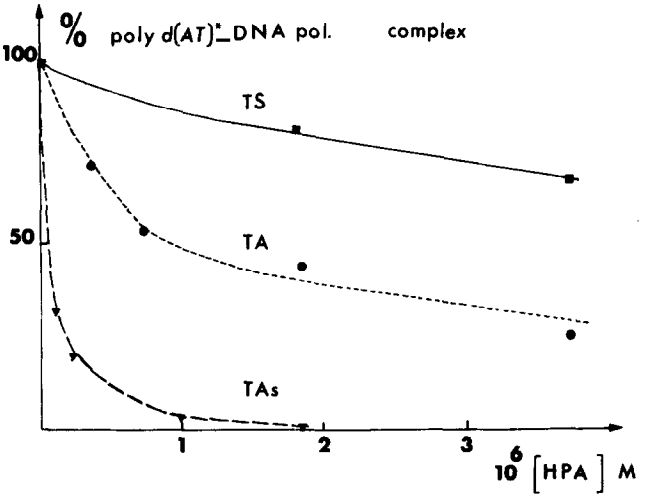

Figure 3. Influence of HPA concentration on the quantity of poly d(A-T)-DNA polymerase complex retained on nitrocellulose filter. Enzyme concentration: 1.4 10^{-7} M. Poly d(A-T) was in excess in order to saturate the polymerase.

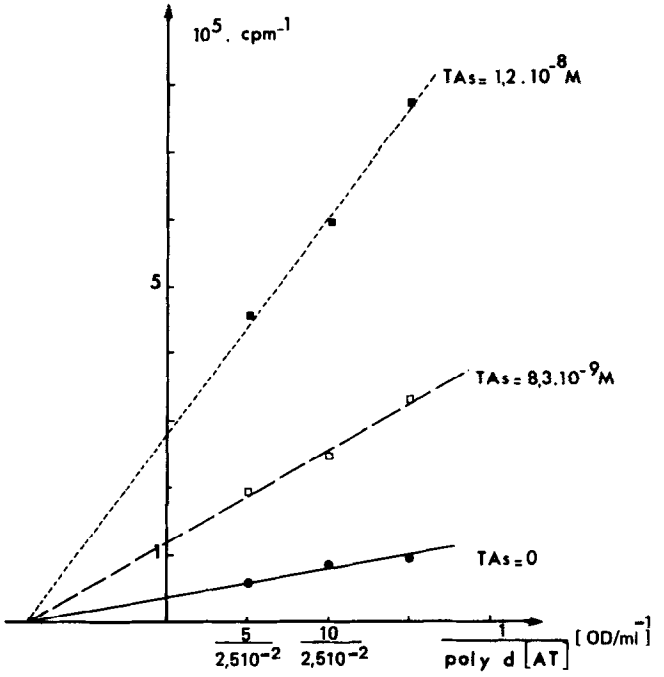

Figure 4. Lineweaver-Burk representation of the influence of template concentration on E. coli RNA polymerase inhibition by TAs.

DISCUSSION

This study has shown that all the tungstoarsenates and tungstoantimonates are very good inhibitors of all DNA and RNA polymerases used. TA compounds inhibit less strongly E. coli DNA polymerase I than the other ones and it is the reverse for TAs compounds. But a marked specificity for one type of polymerase has not been observed.

The nature of the cation M^{n+} included in the central site of the tungstoarsenates does not take a part in their activity on polymerase. On the other hand, the activity significantly decreases when Na^+ is replaced by K^+ at the center of TA compounds (table I). With regard to chemical reactivity, the central cation can act following two ways: the first one is an exchange with the cations present in the solution. It has been previously reported that the exchange is fast with TAs compounds (11) but does not occur with TA compounds (10). Thus, the differences observed between KTA and NaTA cannot be correlated with cations exchanges. The second way is the effect of M^{n+} on the stability of the polyoxotungstic structure. All the MTAs compounds are stable (11) but KTA is less stable than the other MTA (10). The half-lives of KTA and NaTA have been determinated by polarography in conditions near those used in enzymatic experiments (37°C, pH 7.8, conc. = 2.10^{-5} M). They are respectively 0.7 and 7 hours. Both products having the same structure, the lower stability of

KTA may explain that it is less active on polymerase. This difference of stability may also be related to the difference of action observed in vivo (6,14).

A second chemical property of TA and TAs compounds is to act on transition metal cations (Mn^{2+}, Zn^{2+}...) as chelating reagents. The inhibitory activity of HPA with external sites occupied by transition metals is approximatively the same than those of free HPA. So, the chelating action of HPA is not implicated in the inhibition of the polymerases.

Inhibition mechanism. Kinetic experiments and binding assays have allowed us to establish that TAs inhibit the M MLV reverse transcriptase by competition with the template as it has been previously reported for TA (1) and that the same type of inhibition is observed with the other DNA polymerases studied .

For E. coli RNA polymerase, binding assays show also a competitive mechanism. Kinetic results must be interpreted by an irreversible inhibition of the enzyme, probably due to a low rate of dissociation of the RNA pol-HPA complex. This phenomenon has been already observed with other polyanionic ligands (15, 16,17). Polyoxotungstates have a rigid structure with no other chemical reactivity than to complex metallic cations. As this property is not implicated in the inhibition, it can be expected that the binding of HPA to the polymerases is due to their polyanionic nature.In this case, the sites of fixation of HPA and template on the enzyme are probably the same. For instance, 11 electrostatic bonds are established between a double strand DNA and the holoenzyme of E. coli RNA polymerase (18). Thus, there are positively charged residues of aminoacids at the binding site of the polymerase, which can explain the formation of ionic bonds with HPA. The affinity of an HPA for polymerases is probably in relation with its structural characteristics (shape, size, charge repartition). In fact, both charge (8^-, 18^-, 27^-) and size (about 10, 18 and 20 Å) increase in the sequence TS-TA-TAs and the same order is observed in the affinity of these compounds for E. coli DNA polymerase.

In conclusion, the inhibition of the DNA and RNA polymerases have been frequently invoked to design antiviral drugs. It can be pointed out that in vivo studies are necessary to check up their effective usefulness. For instance, TAs have not yet shown an antiviral activity in vivo (unpublished results). In vitro, it has a very low cytotoxicity. So, other properties, such as absorption and penetration into cells may play an important role on the biological activity of polyoxotungstates.

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