Anti-(human immunodeficiency virus) activity of polyoxotungstates and their inhibition of human immunodeficiency virus reverse transcriptase

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Heteropolyoxotungstates of the Keggin class containing different heteroatoms were tested for inhibition of two strains of human immunodeficiency virus 1 (HIV-1); they exhibited varying anti-viral activity. Compounds containing boron were inactive, only one of those containing phosphorus showed selective anti-viral activity, whereas all silicon-containing compounds exhibited significant anti-viral activity in C8166 cells infected with the IIIB strain. Their effectiveness was some 10-fold higher in JM cells with selectivity indices of about 2000. The silicotungstates were effective inhibitors of HIV reverse transcriptase, showing greater

inhibition with RNA/DNA template primers than with DNA/DNA template-primer. Kinetic analysis demonstrated that they inhibit the enzyme by different mechanisms, as, of the four compounds examined, two competed with template-primer and two competed with deoxynucleoside triphosphate. Inhibition of DNA polymerase activity by these compounds was compared using polymerases from different sources, including human; although not necessarily most specific for HIV-1 reverse transcriptase, they did not inhibit all DNA polymerases to a similar degree.

INTRODUCTION

Polyoxometalates represent a class of compounds with a broad spectrum of anti-viral activity. HPA-23 ($\text{NH}_4[\text{NaSb}_9\text{W}_{21}\text{O}_{86} \cdot 14\text{H}_2\text{O}]$) has been shown to inhibit, both *in vitro* and *in vivo*, a number of viruses including scrapie (Kimberlin and Walker, 1983), murine leukaemia (Jasmin et al., 1973), sarcoma virus (Jasmin et al., 1973) and human immunodeficiency virus (HIV) (Rozenbaum et al., 1985). The Keggin polyoxotungstates (in particular $\text{H}_4\text{SiW}_{12}\text{O}_{40}$) also inhibit the replication of rubella (Jasmin et al., 1973), rubeola (Bonissol et al., 1972) and Moloney virus (Raynaud et al., 1971) as well as HIV-1 (Hill et al., 1990).

Heteropolytungstates are members of a family of compounds known collectively as polyoxometalates. These are formed by the condensation of metal-oxo units, typically MO_6 , to form cluster ions such as $\text{M}_6\text{O}_{18}^{z-}$ (if $\text{M} = \text{Mo}$ or W , $z = 2$; if $\text{M} = \text{Nb}$ or Ta , $z = 8$) [for review see Pope (1989)]. These compounds contain metal ions embedded in a lattice of oxygen atoms which may, in some cases, constitute closely packed layers. The incorporation of heteroatoms leads to a variety of heteropolyions among which the heteropolytungstates $\text{EW}_{12}\text{O}_{40}^{z-}$ ($\text{E} = \text{Be}^{2+}$, $z = 6$, $\text{E} = \text{B}^{3+}$, $z = 5$; $\text{E} = \text{Si}^{4+}$ or Ge^{4+} , $z = 4$; $\text{E} = \text{P}^{5+}$ or As^{5+} , $z = 3$ for example) are probably the best known. These compounds contain a central heteroatom, E surrounded by linked WO_6 octahedra and may adopt any of several isomeric structures, among which the more symmetrical α -Keggin structure is well established. Lacunary ions of formulae $\text{EW}_{11}\text{O}_{39}^{(z+4)-}$ can also be formed by removal of a $\{\text{WO}_4\}^{4+}$ unit. The vacancy formed in the cluster structure can be filled by other metal ions to give $\text{MEW}_{11}\text{O}_{39}^{(z+4)-}$ (where M can be any of a variety of metals including Cr^{3+} , Fe^{3+} , Co^{2+} , Ni^{2+} , Cu^{2+} and Zn^{2+} , and z depends on the nature of

E and M). These compounds are structurally similar to $\text{EW}_{12}\text{O}_{40}^{z-}$ but have one surface metal site occupied by M. A wide variety of heteropolyions are known with different compositions and structures. Hill et al. (1990) carried out a comprehensive study of different structural categories of polyoxometalates with respect to their anti-HIV-1 activity and toxicity and found that only the Keggin class shows significant selective anti-HIV-1 effects. This finding may reflect the labile nature of the heteropolyions, the structures and states of oligomerization of which are pH-dependent, and the fact that the Keggin heteropolytungstates are among the most stable of these species.

In the present study a series of structurally related compounds [$\text{K}_z\text{EMW}_{11}\text{O}_{39}$, OH_2 ($\text{M} = \text{Cu}$, Ni , Zn , $\text{E} = \text{Si}$ and $z = 6$; $\text{E} = \text{P}$ and $z = 5$; $\text{E} = \text{B}$ and $z = 7$)] were synthesized and evaluated for anti-HIV-1 activity. This series provides nine compounds with different charges, surface heteroatoms M and lacunary ions with a surface $\{\text{WO}\}$ moiety removed. The silicotungstates exhibited higher selectivity against HIV replication than any previously reported. The activities of these compounds were also commensurate with inhibition of reverse transcriptase (RT). Kinetic studies of this inhibition are reported together with a comparison of inhibition of other DNA polymerases.

MATERIALS AND METHODS

Materials

$[^3\text{H}]\text{dTTP}$ and $[^3\text{H}]\text{dGTP}$ were purchased from Amersham. Template-primers were from Pharmacia. Recombinant HIV-1 RT was a gift from Dr. Nick Cammack, Glaxo Group Research, MRC Collaborative Centre, Mill Hill, London, U.K. Human DNA polymerase α was kindly provided by Dr. Fred Perino,

Abbreviations used: HIV, human immunodeficiency virus; RT, reverse transcriptase; AMV, avian myeloblastosis virus; MMLV, Moloney murine leukaemia virus; TIBO, tetrahydroimidazo [4,5,1-*jk*] [1,4]-benzodiazepin-2(1H)-one and -thione derivatives; AZT, 3'-azido-2'-deoxythymidine.

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Bowman-Gray School of Medicine, Winston-Salem, NC, U.S.A. RT from avian myeloblastosis virus (AMV) and Moloney murine leukaemia virus (MMV) and *Escherichia coli* DNA polymerase I were purchased from New England Biolabs.

Compounds

Silicotungstic acid ($H_4SiW_{12}O_{40}$) and phosphotungstic acid ($H_3PO_{12}O_{40}$) were obtained by diethyl ether extraction of acidified solutions containing the appropriate proportions of $Na_2WO_4 \cdot 2H_2O$ and $Na_2SiO_3 \cdot 5H_2O$ (Jolly, 1960) or $Na_2HPO_4 \cdot 2H_2O$ (Bailar, 1939). The lacunary ions $K_6SiW_{11}O_{39}$ and $K_7PW_{11}O_{39}$ were obtained by base degradation of the respective Keggin ion precursors (Tourné and Tourné, 1969). The Keggin ions $K_zEMW_{11}O_{39}$, OH_2 ($M = Cu, Ni, Zn, E = Si, z = 6; E = P, z = 5$) were synthesized by degradation of the appropriate heteropoly acid in the presence of a salt M^{2+} (Weakley and Malik, 1967; Tourné et al., 1970). The compounds $K_7BMW_{11}O_{39}$, OH_2 ($M = Ni, Cu, Zn$) were prepared by reaction between the appropriate proportions of $Na_2WO_4 \cdot 2H_2O$ and H_3BO_3 in the presence of a salt of M^{2+} and KO_2CMe (Tourné et al., 1970). The compounds were characterized by i.r. spectroscopy. Compounds 3–5 and 7–12 (see Table 1) are hydrates cited as $EMW_{11}O_{39} \cdot nH_2O$ where $n = 10$ –20.

Anti-viral assays

The anti-HIV-1 activities and toxicities of compounds were assessed in two different cell lines: C8166 cells infected with the IIIB strain, and JM cells infected with the GB8 strain of HIV. Cells were grown in RPMI 1640 with 10% fetal calf serum. Cells (4×10^4 /microtitre-plate well) were mixed with 5-fold dilutions of compound before addition of 10 CCID₅₀ (50% cell culture infectious dose) units of virus and incubated for 5–7 days. Formation of syncytia was examined from 2 days after infection. Supernatant was collected at 5–7 days and p24 antigen production measured by e.l.i.s.a. Viability of uninfected control cells was measured by the 1-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazon (MTT-formazan) method as described by Pauwels et al. (1988).

Assay of p24 antigen

A microtitre antigen-capture e.l.i.s.a. was used in which wells were coated with a mixture of anti-p24 monoclonal antibodies. After washing and blocking with 10% BSA, dilutions of viral supernatants in 0.25% emipgen (Albright and Wilson, Whitehaven, U.K.) were added to the wells, and plates were incubated at room temperature for 12–16 h. Bound p24 antigen was detected using anti-HIV-1 human serum, and finally visualized with anti-human IgG coupled to horseradish peroxidase.

Polymerase assays

HIV-1 RT reactions were carried out as described (Moore and Pizza, 1992) in a final volume of 25 μ l containing 50 mM Tris/HCl, pH 8.0, 100 mM KCl, 6 mM MgCl₂, 5 mM dithiothreitol, 0.05% Triton X-100 and, unless otherwise indicated, 10 μ M appropriate dNTP (4 Ci/mmol) and 1.6 μ g/ml template-primer [p(rA)·(dT)_{12–18}, unless otherwise indicated]. The final enzyme concentration was 1.4 μ g/ml (0.05 unit/ml). For assay of DNA polymerase α , reaction mixtures contained 20 mM Tris/HCl, pH 7.5, 2 mM dithiothreitol, 10 mM MgCl₂, 0.1 mg/ml BSA, 10 μ M dNTP, [³H]dTTP (4 Ci/mmol), 72 μ g/ml activated calf thymus DNA and 0.0086 unit/ μ l enzyme, and were incubated for 1 h at 37 °C. For assay of AMV RT, MMLV RT

and DNA polymerase I, conditions used were those recommended by the supplier, the enzyme being used at a concentration of 0.05 unit/ml. Reactions were started by addition of the enzyme. At the end of the incubation time, aliquots of the reaction mixture were spotted on to DEAE-cellulose paper, and dropped immediately into ice-cold 5% trichloroacetic acid/50 mM sodium pyrophosphate, followed by washing with water and finally with ethanol. Filters were then air-dried and counted for radioactivity by liquid scintillation.

RESULTS

Inhibition of virus replication

Anti-HIV activity was evaluated in C8166 cells infected with the IIIB strain and in JM cells infected with the GB8 strain (Table 1). Compounds 1, 2 and 6 have been reported previously as having anti-HIV-1 activity (Hill et al., 1990) and were thus used as reference compounds in addition to 3'-azido-3'-deoxythymidine (AZT). In the former system, none of the compounds containing boron displayed any appreciable selective anti-viral activity, and only one phosphorus-containing compound, 7, showed significant inhibition of viral replication at non-toxic concentrations. In contrast, all of the polyoxotungstates containing silicone as a heteroatom showed anti-HIV-1 activity with selectivity indices (EC_{50}/TC_{50}) greater than 50. The compounds containing Cu, Zn or Ni as the other heteroatom all showed somewhat greater inhibition of viral antigen p24 production (EC_{50} values are improved 2-fold or more) than those with only one heteroatom. Compound 5 had the highest selectivity index of 250 in C8166 cells. These compounds exhibited similar toxicity in JM and C8166 cells, but were 5- to 10-fold more effective at inhibiting HIV replication in JM cells. Hill et al. (1990) reported TC_{50} and EC_{50} values similar to those found in JM cells for compounds 1, 2 and 6 in peripheral blood mononuclear cells, with complete protection against virus-induced cytopathicity at 10 μ M. The four compounds selected for kinetic analysis showed similar cell viability profiles (results not shown). Inhibition of the replication of HIV-2_{ROD} in CEM cells was to a similar extent as that found

Table 1 Anti-HIV-1 activity of polyoxotungstates

Anti-HIV-1 activity was determined by assay of p24 as described in the Materials and methods section. IIIB/C8166, C8166 cells infected with the IIIB strain of HIV-1; GB8/JM, JM cells infected with the GB8 strain of HIV-1. EC_{50} values are the concentrations that inhibited p24 production by 50%; TC_{50} values are the concentrations that resulted in 50% viability of uninfected control cells.

Compound	IIIB/C8166		GB8/JM	
	EC_{50} (μ M)	TC_{50} (μ M)	EC_{50} (μ M)	TC_{50} (μ M)
1 $H_4SiW_{12}O_{40}$	2	100	0.16	100
2 $K_6SiW_{11}O_{39}$	1.6	200	0.20	200
3 $K_6SiCuW_{11}O_{39} \cdot OH_2$	1	50	0.08	> 50
4 $K_6SiZnW_{11}O_{39} \cdot OH_2$	0.8	> 50	0.08	> 50
5 $K_6SiNiW_{11}O_{39} \cdot OH_2$	0.8	200	0.10	200
6 $K_7PW_{11}O_{39}$	8	40	—	—
7 $K_5PCuW_{11}O_{39} \cdot OH_2$	1	50	—	—
8 $K_5PZnW_{11}O_{39} \cdot OH_2$	25	> 25	12.5	> 25
9 $K_5PNiW_{11}O_{39} \cdot OH_2$	> 40	40	—	—
10 $K_7BCuW_{11}O_{39} \cdot OH_2$	> 25	25	—	—
11 $K_7BZnW_{11}O_{39} \cdot OH_2$	> 50	50	2	> 25
12 $K_7BNiW_{11}O_{39} \cdot OH_2$	20	100	—	—
AZT	0.016	> 1000	100	> 1000

Figure 1 Lineweaver-Burk analysis of inhibition of HIV RT by compounds 1 and 3

(a) and (b), compound 1; (c) and (d), compound 3. (a) and (c) were performed with the indicated template-primer as the variable substrate, (b) and (d) with dTTP as the variable substrate. ∇ , Reactions carried out in the absence of inhibitor; $\square$, concentrations of 1 μ M for compound 1 and 2 μ M for compound 3; $\blacksquare$, 2 μ M for compound 1 and 3 μ M for compound 3; $\triangle$, 3 μ M for compound 1 and 4 μ M for compound 3. Insets show the respective slope and intercept replots: $\bullet$, slope; $\circ$, intercept.

in JM cells with strain GB8 (results not shown). A time-of-addition experiment performed in C8166 cells with compounds 4 and 5 and dextran sulphate showed that, if the compounds are added 3 h after infection there is only a slight reduction in efficacy of about 10–15% (results not shown).

Inhibition of RT

Inhibition of RT by these compounds was initially demonstrated using poly(ethylene glycol)-precipitated supernatant from virus-infected cells (results not shown) and was further characterized using a recombinant enzyme. As shown in Figures 1 and 2, compounds 1, 3, 4 and 5 inhibited recombinant HIV-1 RT equally well. The Lineweaver-Burk analysis yielded K_i values for compounds 1 and 3 of 1.9 ± 0.5 and 0.9 ± 0.15 μ M respectively. Primary double-reciprocal plots were linear, as were the slope replots. These two compounds are strictly competitive with the template-primer as the lines converged to a common point on the ordinate (Figure 1). This suggests that they interact with the same form of the enzyme as the template-primer. An analysis of inhibition using dTTP as the variable substrate with these two compounds showed non-competitive inhibition as the lines converged to a common point on the abscissa. This is compatible with competitive inhibition seen with the template-primer, as the reaction mechanism is processive (Majumdar et al., 1988), and therefore the K_m for dTTP would not be expected to change in

the presence of the inhibitor. Intercept and slope replots were linear for both compounds 1 and 3 with deoxynucleotide triphosphate as the variable substrate.

An analysis of substrate kinetics and product inhibition by compounds 4 and 5 showed that these compounds are not competitive inhibitors with respect to template-primer (Figure 2). The double-reciprocal plots were linear and the lines formed a converging pattern above the abscissa, with different intercepts on the ordinate. Intercept replots were linear whereas slope replots were parabolic. This suggests that the inhibition is uncompetitive at lower concentrations of inhibitor and mixed at higher. With dTTP as the variable substrate, both of these compounds showed competitive inhibition, with slightly parabolic slope replots. K_i values were 1.05 ± 0.1 and 0.36 ± 0.08 for compounds 4 and 5 respectively. Although unexpected, this finding is compatible with the inhibition observed using template-primer as the variable substrate and suggests that compounds 4 and 5 can inhibit the processive catalytic mechanism at more than one site.

As further evidence that polyoxotungstates can inhibit RT by different mechanisms, a mixed inhibition experiment was performed with compounds 3 and 5 using template-primer as the variable substrate. In the presence of increasing concentrations of compound 5 the slope would be expected to remain constant in the presence of a fixed concentration of compound 3, if the two inhibitors acted by the same mechanism. It was found, however,

Figure 2 Lineweaver-Burk analysis of inhibition of HIV RT by compounds 4 and 5

(a) and (b) compound 4; (c) and (d) compound 5. (a) and (c) were performed with the indicated template-primer as the variable substrate, (b) and (d) with dTTP as the variable substrate. ▼, Reactions carried out in the absence of inhibitor; □, concentrations of 1 μ M for compound 4 and 0.6 μ M for compound 5; ■, 2 μ M for compound 4 and 0.8 μ M for compound 5; △, 3 μ M for compound 4 and 1 μ M for compound 5. Insets show the respective slope and intercept replots: ○, slope; ●, intercept.

Table 2 Inhibition of synthesis of HIV-1 RT by polyoxotungstates with RNA/DNA and DNA/DNA template-primers

Template-primer	IC ₅₀ (μ M)			
	1	3	4	5
p(rC)·(dG) ₁₂₋₁₈	0.9	0.8	0.6	0.25
p(dC)·(dG) ₁₂₋₁₈	6	3.8	3.5	0.75

that the slope increased, as expected from the experiments analysing the inhibitors separately (results not shown).

Inhibition of RNA- and DNA-dependent polymerase functions

To determine if the RNA- and DNA-dependent polymerase activities of HIV-1 RT showed differential sensitivities to inhibition by the polyoxotungstates, IC₅₀ values were obtained for compounds 1, 3, 4 and 5 using either a p(rC)·(dG)₁₂₋₁₈ or a p(dC)·(dG)₁₂₋₁₈ template-primer. As shown in Table 2, the RNA-dependent polymerase activity was more sensitive to inhibition than the DNA-dependent activity. IC₅₀ values were 3- to 6-fold less with the RNA/DNA template-primer than with the DNA/DNA template-primer. In addition, as can be seen

Table 3 Inhibition of various DNA polymerases by polyoxotungstates

Compound	IC ₅₀ (μ M)				
	HIV-1 RT	MMLV RT	AMV RT	DNA polymerase α	DNA polymerase I
1	3	0.035	28	0.5	43
3	2.5	0.013	18	0.4	27
4	2	0.010	22	0.65	18
5	0.6	0.006	8	0.7	4.5

from a comparison of Tables 2 and 3, RT activity with the p(rC)·(dG)₁₂₋₁₈ template-primer was more sensitive to inhibition than with the p(rA)·(dT)₁₂₋₁₈ template-primer, indicating that the nature of the template-primer influences the inhibitory activity of the polyoxometalates.

Inhibition of other DNA polymerases

The specificity of these compounds with respect to inhibition of a variety of DNA polymerases was examined (Table 3). IC₅₀ values were about 100-fold lower with MMLV RT. Also human

DNA polymerase α was slightly more sensitive to inhibition by the polyoxotungstates than HIV-1 RT. This observation does not reflect the selective anti-viral action in cell culture. *E. coli* DNA polymerase I and AMV RT were relatively insensitive to inhibition by these compounds, having IC_{50} values about 10-fold higher than HIV-1 RT. These compounds thus exhibited some specificity for the active centres of different polymerases, but did not display any particular specificity for RNA-dependent DNA polymerase activity.

DISCUSSION

Differences in the heteroatoms influenced anti-HIV activity at two levels, the overall inhibition of virus replication and in the mechanism of inhibition of RT. The differing behaviour observed within this series of related compounds cannot be attributed solely to charge effects, as compounds with charges ranging from -4 to -8 (compounds 1–5 and 7) have anti-HIV-1 activity. Furthermore, variations in the surface metal group M, which might be expected to be important, have little effect on activity within the silicotungstate series, although a slight effect is seen in the phosphotungstate series. The borotungstates were virtually inactive towards IIIB infection in C8166 cells.

The main difference between the active and non-active systems is the nature of the primary heteroatom E in $EMW_{11}O_{30}, OH_2^-$, yet this heteroatom is buried in the centre of the heteropolyion and is inaccessible to intermolecular interaction. However, the primary heteroatom does affect the overall stability of the heteropolyion and the relative stability of its structural isomers. It is possible that the exact isomeric structure of a Keggin ion, or its rate of reorganization to a structure required for activity, is the factor that determines its overall inhibitory activity against HIV replication. This structure or reorganization rate is affected by the nature of the primary heteroatom E and probably by the microenvironment at the site of biological action. The structures of the heteropolyions are highly pH-dependent and the possibility that structural reorganizations occur on transfer from the bulk phase to the site of interaction with the biological substrate cannot be discounted. More detailed studies of the speciation of heteropolyions and their interactions at the biological level are needed before any definitive conclusions can be made about the chemical basis of their anti-viral activity.

The silicotungstates were some 10-fold more effective in JM than in C8166 cells, indicating the importance of the cell type in eliciting anti-viral action. Whether this is due to differences in uptake, stability or responses at the cell surface is not known. With regard to stability, we have found no difference in the inhibition of RT between freshly prepared compounds and those incubated for 3 days at 37 °C in cell-culture medium (results not shown). It is apparent that they can act as polyanions and, like dextran sulphate, inhibit early events in infection (Take et al., 1991) as well as being potent inhibitors of RT. The relative contributions of these two activities may vary in different cells, but were difficult to assess in the present series of experiments.

In accord with their broad spectrum of anti-viral activity, these compounds inhibited a wide variety of DNA polymerases but to widely differing extents. The RT of MMLV was much more sensitive than that of HIV-1. It is interesting to note that MMLV RT is composed of only a single subunit (Dickson et al., 1982), whereas those of AMV (Varmus and Swanstrom, 1984) and HIV-1 (DiMarzo Varonese et al., 1986; Lightfoote et al., 1986) are heterodimers. Other factors such as the rate of processivity *in vitro* could also account for these differences. It has been reported that MMLV RT is not a very processive enzyme *in vitro* (Varmus

and Swanstrom, 1984), which might account in part for the extreme sensitivity observed. Also, DNA polymerase α exhibited a sensitivity of the same order of magnitude as HIV-1 RT, but much greater than that of the *E. coli* enzyme. The basis of the observed selective anti-viral activity is thus not clear.

A previous report has shown that HPA-23 competes with template·primer to inhibit MMLV RT (Chermann et al., 1975). However, we find that all polyoxometalates do not inhibit HIV-1 RT by the same kinetic mechanism. The inhibition appears to be complex, however, as some compete for template·primer and some for nucleotide triphosphate. This result was unexpected as compounds 1, 3, 4 and 5 are all chemically very similar. The observation that they can compete for different substrates was confirmed by a mixed inhibition experiment. Similar inhibition kinetics to those found for compounds 4 and 5 have been reported for pyrophosphate with respect to competition with template·primer (Majumdar et al., 1988).

Inhibition of HIV RT was dependent on the template·primer used, in the order $p(rC) \cdot (dG)_{12-18} > p(rA) \cdot (dT)_{12-18} > p(dC) \cdot (dG)_{12-18}$, the DNA-directed synthesis being the least sensitive. A similar finding was reported for the TIBO compounds (Debyser et al., 1991) and suramin (Onu et al., 1988). The significance of this is unclear for either class of compounds, but indicates, at the least, that the choice of substrate can influence the degree of inhibition observed at a fixed concentration of compound. This may also be due to differential processivity or template·primer affinity for different substrates. Different homopolymers undoubtedly have dissimilar structural conformations. At any rate it is curious that a similar substrate effect on inhibition was found with compounds that act by entirely different mechanisms. BI-RG-587 and the TIBO drugs selectively inhibit HIV-1 RT via a non-substrate-binding site present on the enzyme (Wu et al., 1991). Other DNA polymerases, including that from HIV-2, are not affected (Merluzzi et al., 1990; Debyser et al., 1991).

In summary, these results demonstrate that these polyoxometalates show more specific anti-viral activity and are less toxic than any previously studied. However, the mechanism by which they inhibit RT activity is not as simple as previously believed: similar compounds inhibit HIV-1 RT by dissimilar modes. It is also possible that they inhibit more than one step in the viral replication process. Although the relative contributions of the individual inhibitory effects to overall anti-viral activity are not yet clear, consideration should be given to these compounds for further study and development as anti-viral agents.

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