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A new strategy to inhibit the excision reaction catalysed by HIV-1 reverse transcriptase: compounds that compete with the template–primer

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Inhibitors of the excision reaction catalysed by HIV-1 RT (reverse transcriptase) represent a promising approach in the fight against HIV, because these molecules would interfere with the main mechanism of resistance of this enzyme towards chain-terminating nucleotides. Only a limited number of compounds have been demonstrated to inhibit this reaction to date, including NNRTIs (non-nucleoside RT inhibitors) and certain pyrophosphate analogues. We have found previously that 2GP (2-*O*-galloylpunicalin), an antiviral compound extracted from the leaves of *Terminalia triflora*, was able to inhibit both the RT and the RNase H activities of HIV-1 RT without affecting cell proliferation or viability. In the present study, we show that 2GP also inhibited the ATP- and PP_i-dependent phosphorolysis catalysed by wild-type and AZT (3′-azido-3′-deoxythymidine)-resistant enzymes at sub-micromolar concentrations. Kinetic and direct-binding analysis showed that 2GP was a non-competitive inhibitor against the nucleotide substrate, whereas it competed with the binding of RT to the template–primer ($K_d = 85$ nM). As expected from its mechanism of action, 2GP was active against mutations conferring resistance to NNRTIs and AZT. The combination of AZT with 2GP was highly synergistic when tested in the presence of pyrophosphate, indicating that the inhibition of RT-catalysed phosphorolysis was responsible for the synergy found. Although other RT inhibitors that compete with the template–primer have been described, this is the first demonstration that these compounds can be used to block the excision of chain terminating nucleotides, providing a rationale for their combination with nucleoside analogues.

Key words: 3′-azido-3′-deoxythymidine (AZT), combined chemotherapy, HIV, nucleoside excision, phosphorolysis, reverse transcriptase.

INTRODUCTION

Combined chemotherapy has dramatically reduced morbidity and mortality in patients with AIDS [1]. Nowadays the main target for the treatment of the HIV-1 is the RT (reverse transcriptase). In fact, 14 of the 24 approved drugs for the treatment of HIV infection are RT inhibitors [2]. They can be divided into two broad groups: nucleoside analogues and NNRTIs (non-nucleoside RT inhibitors) [3,4]. Nucleoside analogues include compounds such as AZT (3′-azido-3′-deoxythymidine), ddI or ddC, which inhibit DNA elongation in their triphosphate forms by acting as chain terminators. On the other hand, NNRTIs inhibit DNA polymerization by binding to a hydrophobic pocket close to the active site.

A major concern in HIV chemotherapy is the emergence of RT variants with reduced susceptibility to anti-retroviral drugs. This problem is more pronounced with NNRTIs, because a single mutation in the RT gene can cause cross-resistance against most of them [3]. For example, RT carrying the Y181C mutation shows > 30-fold resistance to nevirapine and delavirdine and 2- to -3-fold resistance to efavirenz [5–7]. Resistance to nucleoside analogues arises in two different ways [8]. Some mutations allow the enzyme to discriminate between the natural deoxynucleotide and the analogue at the time of incorporation. Other mutations do not affect the efficiency of incorporation, but increase the ability of the enzyme to excise the nucleotide analogue once it is incorporated into the primer. This is the case for AZT, in which the RT^{AZT} (AZT-resistant RT) efficiently catalyses the ATP-mediated excision of AZTMP (AZT-5′-monophosphate) from the terminated primer [8–10]. Currently the excision mechanism has become the dominant mechanism of nucleoside analogue resistance, and it seems that some mutants of RT are able to excise most nucleoside analogues with varying efficiencies.

Several strategies to block the excision reaction catalysed by HIV-1 RT have been proposed [8], but only a limited number of inhibitors are available to date. We and others have described that NNRTIs, in addition to blocking DNA polymerization, inhibit RT-catalysed phosphorolysis [11–13]. Recently, we have also found that some pyrophosphate analogues inhibit the excision of chain-terminating nucleotides by competing with ATP or PP_i, whereas RT uses other analogues as substrates for this reaction [14]. In addition, it has been suggested that compounds that interfere with the productive binding of ATP and analogues of the dinucleotide tetraphosphate product of the excision reaction, may also be suitable for this purpose [8]. Although these are attractive possibilities, the efficacy of such inhibitors remains to be proven. For this reason there is a need for new drugs that inhibit this process.

We have reported previously that aqueous leaf extracts from *Terminalia triflora* were active in inhibiting the DNA polymerase and RNase H activities of HIV-1 RT at concentrations that were not cytotoxic [15]. The bioassay-guided fractionation of these extracts lead us to the purification and characterization of 2GP

Abbreviations used: 4-arylmethylpyridinone, 3-dimethylamino-4-(3,5-dimethylbenzyl)-5-ethyl-6-methylpyridin-2(1*H*)-one; AZT, 3′-azido-3′-deoxythymidine; AZTMP, AZT-5′-monophosphate; AZTTP, AZT-5′-triphosphate; DTT, dithiothreitol; 2GP, 2-*O*-galloylpunicalin; NNRTI, non-nucleoside RT inhibitor; PP_i, inorganic pyrophosphate; RT, reverse transcriptase; RT^{5mut}, RT carrying mutations Y181C, D67N, K70R, T215Y, and K219Q; RT^{AZT}, AZT-resistant RT.

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(2-*O*-galloylpunicalin) [16]. This compound inhibited HIV-1 RT activity and viral replication at sub-micromolar concentrations. In the present study, we examined the mode of action of this compound and demonstrate that 2GP also inhibits the excision reaction catalysed by RT by competing with the template–primer. Consequently, 2GP synergistically inhibited the wild-type and the AZT-resistant enzymes when combined with AZT in the presence of the natural substrates of phosphorolysis. Taken together, these results provide a rationale for the combination of template–primer analogues with chain-terminating nucleotides.

EXPERIMENTAL

Enzymes and nucleic acids

Recombinant p66/p51 wild-type RT and RT^{AZT} were expressed and purified as described previously [12]. RT^{5mut} (RT carrying mutations D67N, K70R, T215Y, K219Q and Y181C) was obtained from the plasmid coding for p66-RT^{AZT} (which carries mutations D67N, K70R, T215Y and K219Q), by changing the TAT (codon 181) into TGT using a mutagenesis kit from Stratagene. The corresponding p51 subunit was obtained from the p66 plasmid by introducing two consecutive stop codons as described previously [12]. The presence of the mutations was verified by analysing the complete sequence of the gene. Poly(rA) and dNTPs were purchased from Amersham Biotech and PAGE-purified oligonucleotides were obtained from Proligo. In the following oligonucleotides: d refers to deoxynucleotides and r to ribonucleotides: r39 template, (5′-AAAAAAAAUAAAAGAA-CAGGUCGACUCUAGAGGAUCCCC-3′); r36 template, (5′-AAAAAAAAAAAAAAAGGUCGACUCUAGAGGAUCCCC-3′); d21 primer, (5′-GGGGATCCTCTAGAGTCGACC-3′). AZTMP-terminated d21 primer was obtained from d21 and purified by PAGE as described previously [14].

RT activity

Enzyme activity was measured as described previously [19]. Briefly, RT was incubated in a total volume of 50 μl for 10 min with the indicated amounts of enzyme, poly(rA)–dT_{20} and labelled dTTP in a buffer containing 50 mM Tris/HCl (pH 8.3), 100 mM KCl, 0.05 % Nonidet P-40, 7 mM $MgCl_2$, 2 mM EGTA, and 2 mM DTT (dithiothreitol). Initial rates were determined from the total radioactivity incorporated into the primer. In experiments where an excess of enzyme over template–primer were used, 10 nM RT was incubated in buffer A (50 mM Tris/HCl, pH 8.0, 1.25 mM EGTA, 0.5 mM EDTA, 0.05 % NP40, and 10 mM $MgCl_2$) containing 100 mM NaCl with 3 nM of the 5′-^{32}P-labelled d21 primer annealed to r36 template and 20 μM dTTP in a final volume of 50 μl. After 60 min, the reactions were quenched by the addition of an equal volume of loading buffer (90 % formamide, 10 mM EDTA, 0.025 % Bromophenol Blue and 0.025 % Xylene Cyanol). Samples were analysed by denaturing PAGE using 12 % (w/v) gels containing 7 M urea.

Phosphorolysis assay

d21-AZT annealed to r39 (1 nM of 5′-^{32}P-labelled) was incubated at 37 °C in a final volume of 50 μl with 25 nM RT and the inhibitor in buffer A containing 100 mM NaCl and 150 μM of PP_i or 3 mM ATP. The reactions containing PP_i were incubated for 60 min and those containing ATP were incubated for 150 min. After the stated time periods, the reactions were quenched by the addition of the same volume of loading buffer and the samples were analysed by denaturing PAGE [18]. Data were fitted by nonlinear regression to equation: $f = 1/[1 + (IC_{50}/D)^m]$, where f is the fractional inhibition, D represents the concentration (dose) of the inhibitor, IC_{50} the concentration of the inhibitor giving 50 % of inhibition, and m a parameter giving the sigmoidicity of the dose–response curve.

In some experiments designed to ascertain the mechanism of inhibition of RT-catalysed phosphorolysis by 2GP, the inhibitor was added after the formation of the complex between RT and r39–d21-AZT. In these experiments, RT was pre-incubated for 5 min either with the inhibitor or with the template–primer prior starting the reaction. Reactions were then incubated for 2 min in buffer A containing 20 mM NaCl with 1 nM of 5′-^{32}P-d21-AZT annealed to r39, 25 nM RT, 2.5 mM PP_i, and the indicated amount of the inhibitor in a final volume of 50 μl.

Filter binding assays

Oligo dT_{20} was labelled with polynucleotide kinase using [γ-^{35}S]ATP as substrate and hybridized with poly(rA) [14]. RT (60 nM) was incubated with 0.5 nM ^{32}P-labelled poly(rA)–dT_{20} and the indicated amounts of RT inhibitors in binding buffer (20 mM Tris/HCl, pH 8.0, 50 mM NaCl, 2 mM $MgCl_2$ and 2 mM DTT). After 30 min of incubation at 37 °C, the reactions were filtered under suction through a Multiscreen nitrocellulose filter plate (MAHA N45, Millipore) and rinsed with 50 μl of binding buffer. Under these conditions the filter binds the protein, but not the template–primer. Radioactivity retained on the filters was measured by scintillation counting. Apparent dissociation equilibrium constants were determined by fitting the data by nonlinear regression to the equation describing the competitive binding of a ligand to a single site on the enzyme [17].

Mobility shift assays

The ability of HIV-1 RT to form a stable complex with the template–primer was assessed by a gel shift assay [14]. For this purpose the 5′-^{32}P-labelled d21-AZTMP was annealed to the r39 template. The annealed template–primer (4 nM) was incubated for 5 min at 37 °C with 2GP and RT, in buffer A containing 20 mM NaCl. The reaction mixture was placed on ice for 5 min, and the putative complexes formed were challenged by the addition of 1.5 μM poly(rA)–dT_{20}. After 2 min of incubation, 5 μl of loading buffer 2 (30 % glycerol and 0.025 % Bromophenol Blue) was added, and the complexes that were resistant to this trap were resolved in a non-denaturing 5 % polyacrylamide gel. Apparent K_d values for 2GP were estimated from the fraction of template–primer detected in complex in the mobility shift assay as a function of 2GP concentration. Quantitation of the radioactivity and curve fitting were performed using ImageMaster software (Amersham-Pharmacia) and Grafit (Erithacus software) respectively.

Synergy analysis

To measure the combined effect of two inhibitors on RT activity, 25 nM RT was added to a mixture prepared in buffer A containing 100 mM NaCl, 3 nM poly(rA)–dT_{20}, 20 μM [α-^{33}P]dTTP and the indicated amounts of the inhibitors with or without 250 μM PP_i. Reactions were incubated at 37 °C for the indicated periods of time and quenched by the addition of 5 μl of 0.5 M EDTA. Aliquots (15 μl) were spotted on to DE81 paper (Whatman), washed three times with 0.5 M phosphate buffer (pH 7.5), dried and counted. Synergy analysis was performed both by the Yonetani–Theorell plot and by calculating the interaction indexes for each combination as previously described [18,20].

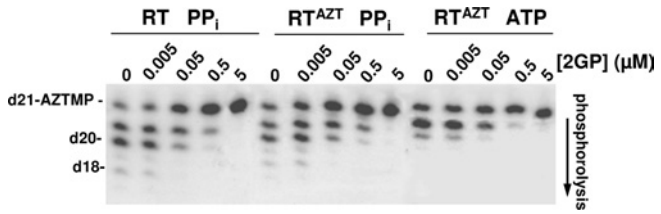

Figure 1 Inhibition of RT-catalysed phosphorolysis by 2GP

5′-^{32}P-labelled primer (d21-AZTMP; 1 nM) was annealed to the r39 template and incubated in buffer A containing 100 mM NaCl with 25 nM RT, the indicated amount of 2GP and 150 μM PP_i or 3 mM ATP. The reactions containing PP_i were incubated for 60 min and those containing ATP were incubated for 150 min. Reactions were then quenched and the products analysed by denaturing PAGE. Under these conditions, no ATP-dependent phosphorolysis was detected for wild-type RT.

Table 1 Inhibition of PP_i and ATP-dependent AZTMP-excision catalysed by RT and RT^{AZT}

The experiments were carried out as described in the legend for Figure 1. The results are shown as means $\pm$ S.D.

	IC_{50} (μM)		
	RT	RT^{AZT}	
Drug	PP_i	PP_i	ATP
Efavirenz	0.040 ± 0.002	0.020 ± 0.002	0.010 ± 0.001
4-Arylmethylpyridinone	0.010 ± 0.004	0.036 ± 0.01	0.001 ± 0.0001
Nevirapine	2.1 ± 0.15	3.4 ± 0.9	1.7 ± 0.41
9-Cl-TIBO	11 ± 0.54	12 ± 1.4	3.0 ± 0.5
2GP	0.06 ± 0.01	0.088 ± 0.01	0.079 ± 0.01

RESULTS

Inhibition by 2GP of RT-catalysed phosphorolysis

We tested, in the first instance, whether 2GP inhibited the ATP- and PP_i-dependent phosphorolysis catalysed by wild-type RT and RT^{AZT}. Figure 1 shows that 2GP effectively inhibited the pyrophosphorosis catalysed by both enzymes with similar potency. The amount of 2GP needed for half-inhibition of these reactions was 0.06 ± 0.01 μM and 0.088 ± 0.01 μM for RT and RT^{AZT} respectively, showing that the presence of AZT-related mutations did not affect the activity of this compound. Interestingly, 2GP inhibited the ATP-mediated excision catalysed by RT^{AZT} in the same range. Under the same conditions wild-type RT excised less than 5 % of the terminated primer with ATP as substrate, precluding the accurate determination of inhibition constants. We have reported previously that some NNRTIs effectively inhibited the phosphorolysis catalysed by HIV-1 RT. As shown in Table 1, 2GP was as effective as NNRTIs in inhibiting AZTMP excision from the terminated primer.

Kinetics of inhibition of DNA polymerase activity by 2GP

The ability of 2GP to inhibit the RNase H, DNA polymerase and phosphorolytic activities of HIV-1 RT lead us to study its mechanism of action. Steady-state kinetics was performed by measuring the effect of 2GP on RT activity at several concentrations of each substrate (dNTP or template–primer). Figure 2 shows kinetic data both in a direct Michaelis–Menten plot and in a double reciprocal plot. In the direct plot, all the experimental data were simultaneously fitted by nonlinear regression to the three-dimensional equations defining competitive, non-competitive or uncompetitive inhibition. The equation that fitted the experimental data best was plotted. Figure 2(A) shows that 2GP was a linear non-competitive inhibitor against dTTP in polymerization reactions. Analysis of the inhibition by 2GP with respect to template–primer was examined by varying the concentration of poly(rA)–dT_{20}, while maintaining dTTP at a fixed concentration of 10 μM. As shown in Figure 2(B), 2GP displayed a competitive inhibition pattern with respect to poly(rA)–dT_{20}. The calculated competitive constant was 0.031 ± 0.007 μM.

Effect of 2GP on the binding of HIV-1 RT to the template–primer

In order to verify the competitive nature of the interaction between 2GP and template–primer, we examined whether this compound interfered with the formation of the RT–template–primer binary complex in a filter-binding assay. As shown in Figure 3(A), 2GP inhibited the binding of poly(rA)–dT_{20} to RT in a dose-responsive manner. The apparent K_d value for the binding of RT to poly(rA)–dT_{20} determined from an independent experiment was 0.1 ± 0.02 nM (results not shown). Experimental data were fitted to the quadratic equation describing the competitive binding of two ligands, 2GP and poly(rA)–dT_{20}, to a single site [17], resulting in a K_d (app) for 2GP of 80 ± 6 nM. As a control we measured the effect of delarvidine (U-90152s) in this assay. This NNRTI did not affect the binding of template–primer to HIV-1 RT (Figure 3A).

The displacement of the template–primer by 2GP was also confirmed in a mobility shift assay. This assay detected the presence of a stable complex between HIV-1 RT and the AZT-terminated template–primer. The apparent dissociation constant for the RT–r39–d21-AZT complex under these conditions was 1.1 ± 0.1 nM. In the presence of increasing concentrations of 2GP the amount of template–primer bound to RT was reduced, and eventually the band corresponding to this complex disappeared (Figure 3B). These results demonstrate directly that 2GP competes with template–primer for binding to RT. Quantitation of the intensity of the band gave a K_d (app) for 2GP of 89 ± 4 nM.

2GP abolishes HIV-1 wild-type and mutant RT activity

It has been demonstrated that a substantial excess of RT over viral RNA exists *in vivo*. Therefore complete inhibition of viral replication would require that RT activity be fully abolished by the inhibitor, because residual activity of RT would lead to sustained viral replication and to the rapid emergence of viral resistance. For this reason, we have measured the effect of several inhibitors on RT activity using an excess of enzyme over template–primer. Reactions were incubated for 60 min and the polymerization products were separated in a polyacrylamide gel. As a control, we also measured the effect of three structurally unrelated NNRTIs that inhibited the excision reaction [12]. Table 2 shows that under these stringent conditions 9-Cl-TIBO and nevirapine were far less effective than 4-arylmethylpyridinone [3-dimethylamino-4-(3,5-dimethylbenzyl)-5-ethyl-6-methylpyridin-2(1*H*)-one] in inhibiting HIV-1 RT, whereas 2GP retained its activity at submicromolar concentrations. Neither the presence of AZT-related mutations nor the presence of a fifth mutation (Y181C) affected the activity of 2GP. However, the presence of the Y181C mutation conferred high resistance to all NNRTIs tested to RT^{5mut}. The IC_{50} for 4-arylmethylpyridinone increased more than 10-fold with respect to wild-type RT, and the resistance toward nevirapine and to 9-Cl-TIBO could not be quantitated due to the low activity of these compounds on RT^{5mut} under these conditions.

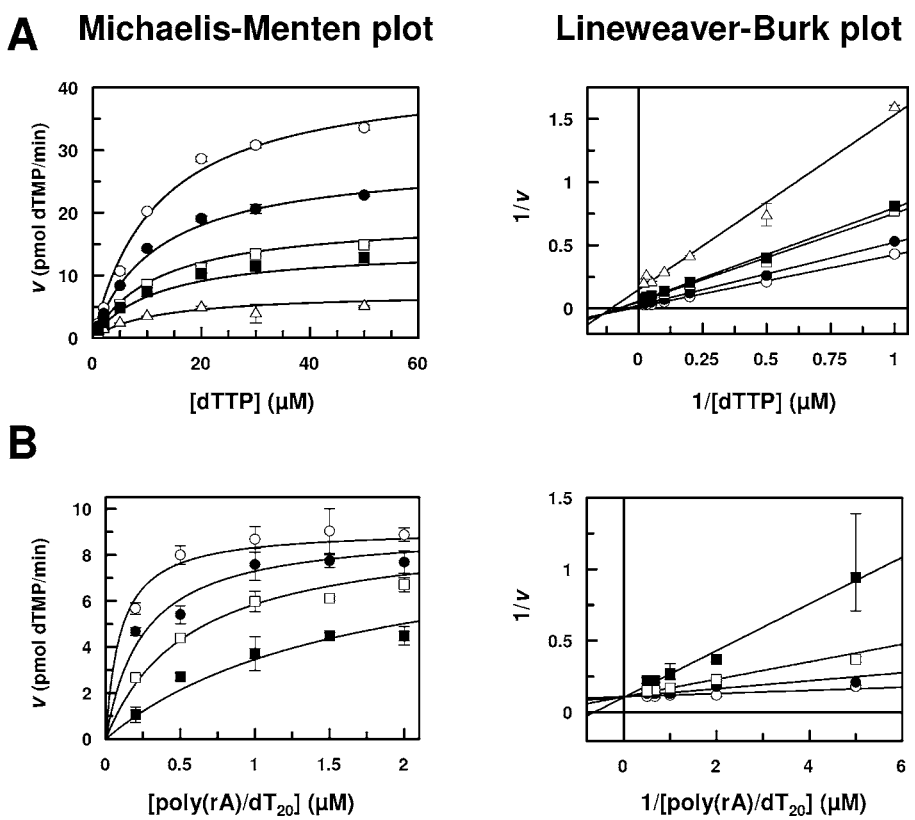

Figure 2 Inhibition of HIV-1 RT-associated DNA polymerase by 2GP

(**A**) Michaelis–Menten and Lineweaver–Burk plots for the inhibition of RT activity by 2GP with dTTP as the variable substrate. The concentrations of 2GP were 0 (○), 0.2 (●), 0.5 (□), 0.8 (■) and 2 μM (△). In the direct plot, all the experimental data were fitted simultaneously by nonlinear regression to the three-dimensional equation defining non-competitive inhibition, which gave a K_i value of 0.41 ± 0.02 μM. (**B**) Michaelis–Menten and Lineweaver–Burk plots for the inhibition of RT activity by 2GP with poly(rA)–dT_{20} as the variable substrate. Concentrations of 2GP were 0 (○), 0.05 (●), 0.15 (□) and 0.5 μM (■). In this case the best fit corresponds to a competitive inhibition with $K_{ic} = 0.031 \pm 0.007$ μM.

Mechanism of inhibition of phosphorolysis by 2GP

We have found that 2GP showed a competitive pattern against template–primer in DNA polymerase assays and that this compound interfered with the binding of RT to the template–primer in direct-binding assays. Although this mechanism may explain the inhibition of the phosphorolytic activity by 2GP, a direct relationship between the inhibition of DNA binding and the inhibition of phosphorolysis was not formally demonstrated. For this reason, we performed an experiment adding the inhibitor after the formation of the complex between RT and AZT–DNA and then examined the phosphorolysis. Under these conditions, some phosphorolytic activity, corresponding to the pre-formed RT–template–primer complexes, must be detected before RT dissociates and binds to an excess of 2GP. This activity must be absent if the enzyme is pre-incubated in the presence of 2GP. In these experiments we added millimolar concentrations of PP_i in order to obtain a substantial amount of phosphorolysis before RT dissociated from the template–primer. As control, we studied the effect of an excess of unlabelled poly(rA)–dT_{20} or efavirenz under the same conditions.

Phosphorolytic activity was detected when RT was pre-incubated with the template–primer before adding an excess of 2GP or unlabelled template–primer (Figure 4). This activity was absent if RT was pre-incubated with the inhibitors prior to incubation with the template–primer. Interestingly, no activity was detected with efavirenz irrespective of the order of addition, as expected for a compound that binds to another site on the enzyme. These results demonstrate clearly that 2GP inhibited phosphorolysis by competing with the template–primer.

Effect of the combination of 2GP and AZTTP on RT activity

We have reported previously that the synergy found in combinations of AZT with NNRTIs or certain PP_i analogues was due to the inhibition by these compounds of the nucleotide excision reaction catalysed by HIV-1 RT [12,18]. For this reason, we analysed the effect of the combination of 2GP with AZTTP on HIV-1 RT. In the absence of PP_i, the combination of 2GP and AZTTP resulted in parallel lines in a Yonetani–Theorell plot, indicating that this combination was merely additive (Figure 5A). Interaction indexes for this combination were close to 1 (1.06, 1.05 and 1.03 at 50, 70 and 95 % of inhibition respectively), as expected for an additive combination.

We also tested the same combination under conditions in which phosphorolysis can take place. In the presence of 250 μM PP_i the combination of 2GP with AZTTP was highly synergistic as judged by the Yonetani–Theorell plot, with a $-\text{intercept}/IC_{50}$ value of 0.24 ± 0.04 (Figure 5B). For synergistic combinations this parameter takes positive values that decrease as synergy increases [18,19]. This amount of synergy was similar to that found under the same conditions for the combinations of AZTTP with efavirenz ($-\text{intercept}/IC_{50} = 0.15 \pm 0.02$), nevirapine ($-\text{intercept}/IC_{50} = 0.17 \pm 0.2$) or pamidronate ($-\text{intercept}/IC_{50} = 0.23 \pm 0.13$) (results not shown). In addition, synergy increased by extending the time of incubation. For example, interaction

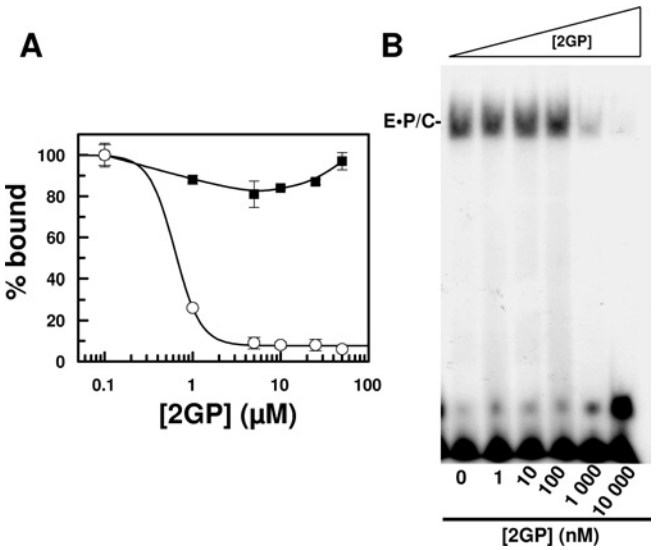

Figure 3 Effect of 2GP on the binding of RT to template–primer

(**A**) Filter binding assay. RT (60 nM) was incubated with 0.5 nM labelled poly(rA)–dT_{20} and 2GP (○) or U-90152s (■). Reactions were filtered through a Multiscreen nitrocellulose filter plate, rinsed twice with 50 μl of binding buffer and the radioactivity remaining on the filters was measured by scintillation counting. (**B**) Electrophoretic mobility shift assays were carried out to confirm the competition between 2GP and the template–primer. RT (200 nM) was added to a mixture containing 4 nM of 5′-^{32}P-labelled d21-AZTMP annealed to the r39 primer and 2GP. The putative complexes formed were challenged by the addition of 1.5 μM poly(rA)–dT_{20} and the complexes resistant to this trap were resolved in a non-denaturing 5 % polyacrilamide gel.

Table 2 Inhibition of DNA polymerization catalysed by wild-type and resistant RTs using a large excess of enzyme over template–primer

RT (10 nM) was incubated with 3 nM (^{32}P labelled) d21 primer annealed to r36 template, 20 μM dTTP and the indicated inhibitors. The reactions where stopped after 1 h, and the products were analysed by denaturing PAGE. Inhibition of RT activity was obtained by densitometric quantitation in each reaction of all oligonucleotides larger than 35 nucleotides [12].

	IC_{50} (μM)		
Inhibitor	RT	RT^{AZT}	RT^{5mut}
4-Arylmethylpyridinone	0.75 ± 0.15	0.50 ± 0.01	6.0 ± 2.4
Nevirapine	30 ± 8	32 ± 8	>50
9-Cl-TIBO	>50	>50	>50
2GP	0.20 ± 0.01	0.40 ± 0.05	0.38 ± 0.05

indexes calculated at 50 % of inhibition for the combination of 2GP with AZTTP in presence of PP_i decreased from 0.62 to 0.42 when the reaction time increased from 20 to 60 min (Figure 5C). Under these conditions RT-catalysed phosphorolysis severely reduced the potency of AZTTP as a chain-terminating agent.

DISCUSSION

Inhibition of the ATP- or PP_i-dependent phosphorolysis catalysed by HIV-1 RT is a novel approach to fight against viral resistance to chain-terminating nucleotides. However, only a few compounds that are able to inhibit this reaction [12–14]. We have found previously that 2GP, a natural compound isolated from *T. triflora*, inhibited both the DNA polymerase and RNase H activities of HIV-1 RT. In the present study, we extend our previous observations by showing that 2GP also inhibits the ATP- and PP_i-dependent excision of chain-terminated primers catalysed by HIV-1 RT. 2GP was as effective as NNRTIs in inhibiting this activity and, unlike some PP_i analogues, such as phosphonoformate [14], 2GP was able to block the RT-catalysed phosphorolysis fully. Kinetic studies indicated that 2GP acts by interfering with the binding of HIV-1 RT to the template–primer. Both the filter binding and the mobility shift assays demonstrated directly that 2GP binds to the free enzyme with a K_d of approx. 85 nM.

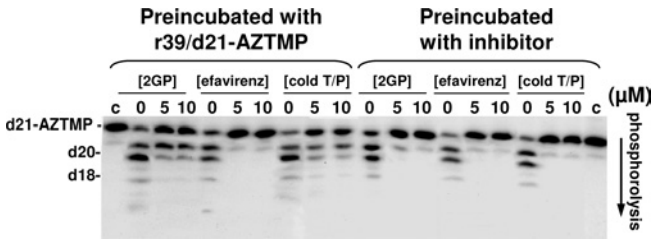

Figure 4 Mechanism of inhibition of phosphorolysis by 2GP

5′-^{32}P-labelled primer (d21-AZTMP; 1 nM) annealed to the r39 template was pre-incubated in the absence of Mg^{2+} with 25 nM RT in buffer A containing 20 mM NaCl and 2.5 mM PP_i. Reactions were then started by adding 10 mM $MgCl_2$ along with the indicated amounts of 2GP, efavirenz or unlabelled poly(rA)–dT_{20} (cold T/P). After 2 min, the reactions were stopped and products analysed by denaturing PAGE. In other tubes, RT was first pre-incubated with each inhibitor and the reactions were started by adding 10 mM $MgCl_2$ along with the labelled r39–d21-AZT. The addition of 2GP or poly(rA)–dT_{20} did not completely inhibit phosphorolysis if RT was pre-incubated with the substrate, due to the activity of preformed RT template–primer complexes. c, control reaction carried out in the absence of RT.

As far as we know, this is the first demonstration that a compound that competes with the template–primer is able to inhibit phosphorolysis. In fact, there are a number of natural and synthetic compounds that have been shown to inhibit RT activity either by competing with the template–primer or by selective binding to the free enzyme. These include, at least, the carboxanilide derivative UC-84 [21], indolyl aryl sulfone derivatives [22], KM-1 [17], KM043 [23], avarol and avarone derivates [24] and toxiuxol [25]. Some of them, such as the KM-1, avarol or the avarone derivates have also been shown to inhibit the RNase H activity of HIV-1 RT [17,24]. However, it seems that the potential of these compounds as inhibitors of the phosphorolytic reaction catalysed by HIV-1 RT has not been recognized.

Compounds that interfere with the binding of template–primer to the enzyme may represent an interesting alternative to the NNRTIs for combined chemotherapy. First, because some of them, such as 2GP, inhibit polymerization and phosphorolysis in the nanomolar range. The potential of other members of this family as inhibitors of the nucleotide excision catalysed by HIV-1 RT has not been analysed, although, given their mechanism of action, we anticipate that molecules such as KM-1 [17] will be effective in blocking RT-catalysed phosphorolysis. Secondly, because the activity of compounds that interfere with the binding of RT to the template–primer is not affected by mutations that confer severe resistance to NNRTIs. Often a single mutation in the RT may cause resistance to a variety of NNRTIs, precluding the inhibition of both the polymerase and the phosphorolytic activities. As expected, 2GP or KM-1 activity is neither affected by TAM or Y181C mutations [17]. Lastly, because some of these compounds inhibit HIV-1 RT and viral replication at concentrations that do not affect cell proliferation or viability. For example, 2GP inhibits viral replication with an EC_{50} of 0.21 μM, but does not affect cell proliferation of viability below 40 μM [16]. This profile it is not general to other members of this family, since some of them exhibit high cytotoxicity *in vitro* precluding their therapeutic application [23,25]. It cannot be discarded that some of these compounds may have alternative modes of action

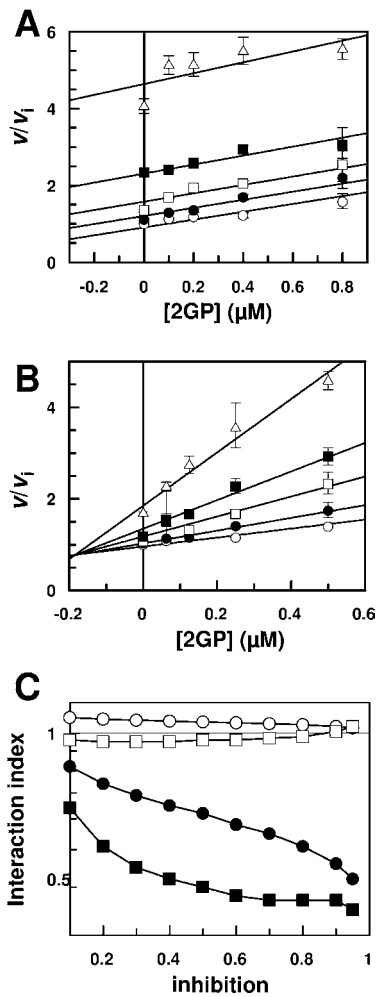

Figure 5 Effect of the combination of 2GP with AZTTP on HIV-1 RT activity

(**A**) Effect of the combination in the absence of PP_i or ATP. RT (25 nM) was incubated with 2GP and AZTTP in a buffer containing 3 nM poly(rA)–dT_{20} and 20 μM [α-^{33}P]dTTP. The concentrations of AZTTP were 0 (○), 0.0125 (●), 0.025 (□), 0.05 (■) and 0.1 μM (Δ). Parallel lines in the Yonetani–Theorell plot indicate that this combination showed mutually exclusive effects. (**B**) Effect of the same combination in the presence of 250 μM PP_i. Concentrations of AZTTP were 0 (○), 0.025 (●), 0.05 (□), 0.1 (■) and 0.2 μM (Δ). Lines converging at the left-hand side of the y-axis show that this combination synergistically inhibited HIV-1 RT. (**C**) Interaction indexes for the combination of AZT with 2GP. AZTTP and 2GP were combined at fixed molar ratios and assayed on RT as before without (open symbols) or with 250 μM PP_i (closed symbols). Reactions were incubated for 20 min (circles) or 60 min (squares) at 30 °C.

in cell-based assays, such as in the inhibition of the viral binding to the cell surface [26]. Moreover, several analogues of the template–primer are either charged or have hydrophilic nature and their bioavailability should probably be improved before their therapeutic potential can be fully exploited. However, the high potency of these compounds as RT inhibitors, some of them in the nanomolar range, their lack of cross-resistance with NNRTIs or nucleoside analogues and their potential for combined chemotherapy merits further development.

The results obtained in the present study provide a rationale for combining molecules that block the binding of the template–primer to RT with nucleoside analogues. Combinations of 2GP with AZTTP were highly synergistic on HIV-1 RT when tested in the presence of ATP or PP_i. In fact, the amount of synergy obtained for the combination of 2GP and AZTTP was similar to those found for combinations of AZTTP with NNRTIs. We have shown previously that the inhibition of RT-catalysed phosphorolysis seems to be related to the superior long-term efficacy of combinations containing an NNRTI over combinations containing only nucleoside analogues [12]. Taken together, these results support the notion that compounds that inhibit the formation of the RT–DNA complex may be useful in combined chemotherapy with nucleoside analogues by interfering with the main mechanism of resistance of this enzyme to chain-terminating nucleotides.

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