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► To cite this version:

Carlos Cruchaga, Elena Anso, Maria Font, Virginia S. Martino, Ana Rouzaut, et al.. A new strategy to inhibit the excision reaction catalyzed by HIV-1 reverse transcriptase: compounds that compete with the template-primer. *Biochemical Journal*, 2007, 405 (1), pp.165-171. 10.1042/BJ20061831 . hal-00478714

HAL Id: hal-00478714

<https://hal.science/hal-00478714>

Submitted on 30 Apr 2010

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A NEW STRATEGY TO INHIBIT THE EXCISION REACTION CATALYZED BY HIV-1 REVERSE TRANSCRIPTASE: COMPOUNDS THAT COMPETE WITH THE TEMPLATE/PRIMER

Carlos CRUCHAGA*, Elena ANSO*, María FONT[†], Virginia S. MARTINO[‡], Ana ROUZAUT* and Juan J. MARTINEZ-IRUJO*

*Departamento de Bioquímica y Biología Molecular, Facultad de Medicina and

[†]Departamento de Química Orgánica y Farmacéutica, Facultad de Farmacia, Universidad de Navarra, 31008 Pamplona, Spain; [‡]Cátedra de Farmacognosia, IQIMEFA (UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, 1113 Buenos Aires, República Argentina.

Running title: New inhibitors of HIV-1 RT-catalyzed phosphorolysis

Keywords: HIV, reverse transcriptase, phosphorolysis, combined chemotherapy, nucleoside excision, AZT

Address correspondence to: Juan J. Martínez-Irujo. Departamento de Bioquímica y Biología Molecular, Edificio de Investigación, Universidad de Navarra, 31008, Pamplona, Spain, Tel.: +34 048 425 649, Fax: +34 948 425 649, E-Mail: jjmirujo@unav.es

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

ABSTRACT

Inhibitors of the excision reaction catalyzed by HIV-1 reverse transcriptase (RT) represent a promising approach in the fight against HIV, because these molecules would interfere with the main mechanism of resistance of this enzyme toward chain-terminating nucleotides. Only a limited number of compounds have demonstrated to inhibit this reaction to date, including nonnucleoside RT inhibitors (NNRTIs) and certain pyrophosphate analogs. We have previously found that 2-*O*-galloylpunicalin (2GP), an antiviral compound extracted from the leaves of *Terminalia triflora*, was able to inhibit both the reverse transcriptase and the ribonuclease H activities of HIV-1 RT without affecting cell proliferation or viability. Herein we show that 2GP also inhibited the ATP- and PP_i-dependent phosphorolysis catalyzed by wild-type and AZT-resistant enzymes at submicromolar concentrations. Kinetic and direct binding analysis showed that 2GP was a noncompetitive inhibitor against the nucleotide substrate, while it competed with the binding of RT to the template/primer ($K_d = 85$ nM). As expected from their 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-catalyzed phosphorolysis was responsible for the synergy found. While 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 analogs.

INTRODUCTION

Combined chemotherapy has dramatically reduced morbidity and mortality in patients with AIDS [1]. Nowadays the main target for the treatment of the human immunodeficiency virus type 1 (HIV-1) is the reverse transcriptase (RT). In fact, 14 of the 24 approved drugs for the treatment of HIV infection are RT inhibitors [2]. They can be divided in two broad groups: nucleoside analogs and non-nucleoside reverse transcriptase inhibitors (NNRTIs) [3, 4]. Nucleoside analogs include compounds such as AZT, 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 the susceptibility to antiretroviral drugs. This problem is more pronounced with NNRTIs, because a single mutation in the RT gene can cause cross-resistance to 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 analogs arises in two different ways [8]. Some mutations allow the enzyme to discriminate between the natural deoxynucleotide and the analog 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 analog once incorporated into the primer. This is the case for AZT, in which the resistant enzyme (RT^{AZT}) efficiently catalyzes the ATP-mediated excision of AZTMP from the terminated primer [8-10]. Currently the excision mechanism has become the dominant mechanism of nucleoside analog resistance, and it seems that some mutants of RT are able to excise most NRTIs with varying efficiencies.

Several strategies to block the excision reaction catalyzed 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 block DNA polymerization, inhibit RT-catalyzed phosphorolysis [11-13]. Recently, we have also found that some pyrophosphate analogs inhibit the excision of chain-terminating nucleotides by competing with ATP or PP_i, while other analogs are used by RT as substrates for this reaction [14]. It has been suggested that compounds that interfere with the productive binding of ATP and analogs of the dinucleotide tetraphosphate product of the excision

reaction, may also be suitable for this purpose [8]. While 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.

Previously we have reported that aqueous leaf extracts of *Terminalia triflora* were active in inhibiting the DNA polymerase and RNase H activities of HIV-1 RT at not cytotoxic concentrations [15]. The bioassay-guided fractionation of these extracts lead us to the purification and characterization of 2-*O*-galloylpunicalin (2GP) [16]. This compound inhibited HIV-1 reverse transcriptase activity and viral replication at submicromolar concentrations. In this paper we go into the mechanism of action of this compound demonstrating that 2GP also inhibited the excision reaction catalyzed 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 analogs with chain-terminating nucleotides.

EXPERIMENTAL

Enzymes and nucleic acids. Recombinant p66/p51 wild-type (RT) and AZT resistant HIV-1 RT (RT^{AZT}) were expressed and purified as described [12]. RT carrying mutations D67N, K70R, T215Y, K219Q and Y181C (RT^{5mut}) was obtained from the plasmid coding for p66-RT^{AZT} (D67N, K70R, T215Y, K219Q), by changing the TAT 181 codon for 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 [12]. The presence of the mutations was verified by analyzing 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'-AAAAAAAAUAAAAGAACAGGUCGACUCUAGAGGAUCCCC-3'); r36 template, (5'-AAAAAAAAAAAAAAAAAGGUCGACUCUAGAGGAUCCCC-3'); d21 primer, (5'-GGGGATCCTCTAGAGTCGACC-3'). AZTMP-terminated d21 primer was obtained from d21 and purified by PAGE as described [14].

Reverse transcriptase activity. Enzyme activity was measured as described [19]. Briefly, RT was incubated in a total volume of 50 µl for 10 min with the indicated

amounts of enzyme, poly(rA)/dT₂₀ and labeled dTTP in a buffer containing 50 mM Tris/HCl (pH 8.3), 100 mM KCl, 0.05% Nonidet P-40, 7 mM MgCl₂, 2 mM EGTA, and 2 mM DTT. 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, 1.25 mM EGTA, 0.5 mM EDTA, 0.05% NP40, and 10 mM MgCl₂ at pH 8) containing 100 mM NaCl with 3 nM of the 5'-³²P-labeled d21 primer annealed to r36 template and 20 μM dTTP in a final volume of 50 μl. After 60 min 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 analyzed by denaturing PAGE using 12% polyacrylamide gels containing 7 M urea.

Phosphorolysis assay. 1 nM of 5'-³²P-labeled d21-AZT annealed to r39 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 this period of time, reactions were quenched by addition of the same volume of loading buffer and samples were analyzed 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-catalyzed phosphorolysis by 2GP, the inhibitor was added after the formation of the complex between RT and r39/d21-AZT. In these experiments RT was preincubated 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'-³²P- 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 dT₂₀ was labeled with polynucleotide kinase using [γ -³⁵S]ATP as substrate and hybridized with poly(rA) [14]. RT (60 nM) was incubated with 0.5 nM labeled-poly(rA)/dT₂₀ and the indicated amounts of RT inhibitors in binding buffer (20 mM Tris-HCl, 50 mM NaCl, 2 mM MgCl₂, and 2 mM DTT at pH 8.0). After 30 min of incubation at 37 °C, reactions were filtered under suction through a

Multiscreen nitrocellulose filter plate (MAHA N45, Millipore) and rinsed with 50 μ l 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'-³²P-labeled d21-AZTMP was annealed to 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 μ M poly(rA)/dT₂₀. After 2 min of incubation, 5 μ l of loading buffer (30% glycerol and 0.025% bromophenol blue) was added and the complexes resistant to this trap were resolved in a nondenaturing 5% polyacrylamide gel. Apparent K_d for 2GP was estimated from the fraction of template/primer detected in complex in the mobility shift assay as a function of 2GP concentration. Quantification of the radioactivity and curve fitting were performed with 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)/dT₂₀, 20 μ M [α -³³P]dTTP, and the indicated amounts of the inhibitors with or without 250 μ M PP_i. Reactions were incubated at 37 °C for the indicated periods of time and quenched by adding 5 μ l of 0.5 M EDTA. Aliquots of 15 μ l were spotted onto a DE81 paper (Whatman), washed three times with 0.5 M phosphate buffer (pH 7.5), dried and counted. Synergy analysis was done both by the Yonetani-Theorell plot and by calculating the interaction indexes for each combination as previously described [18, 20].

RESULTS

Inhibition by 2GP of RT-catalyzed phosphorolysis. We tested in the first place whether 2GP inhibited the ATP- and PP_i-dependent phosphorolysis catalyzed by wild-type (RT) and AZT-resistant RTs (RT^{AZT}). Fig. 1 shows that 2GP effectively inhibited the

pyrophosphorosis catalyzed by both enzymes with similar potency. The amount of 2GP needed for half-inhibit these reactions were $0.06 \pm 0.01 \mu\text{M}$ and $0.088 \pm 0.01 \mu\text{M}$ for RT and RT^{AZT}, respectively, showing that the presence of AZT-related mutations did not affect to the activity of this compound. Interestingly, 2GP inhibited the ATP-mediated excision catalyzed by RT^{AZT} phosphorolysis 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 previously reported that some NNRTIs effectively inhibited the phosphorolysis catalyzed 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 reverse transcriptase activity at several concentrations of each substrate (dNTP or template/primer). Fig. 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 which fitted the experimental data best was plotted. Fig. 2A shows that 2GP was a linear noncompetitive 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)/dT₂₀ while maintaining dTTP at a fixed concentration of 10 μM . As shown in Fig. 2B, 2GP displayed a competitive inhibition pattern with respect to poly(rA)/dT₂₀. The calculated competitive constant was $0.031 \pm 0.007 \mu\text{M}$.

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 Fig. 3A, 2GP inhibited the binding of poly(rA)/dT₂₀ to RT in a dose-response form. The apparent K_d value for the binding of RT to poly(rA)/dT₂₀ determined from an independent experiment was $0.1 \pm 0.02 \text{ nM}$ (data not shown). Experimental data were fitted to the quadratic equation describing the competitive binding of two ligands, 2GP and poly(rA)/dT₂₀, to a single site [17], resulting in an apparent K_d for 2GP of $80 \pm 6 \text{ nM}$. As a control we measured the effect

of delarvidine (U-90152s) in this assay. This NNRTI did not affect to the binding of template/primer to HIV-1 RT (Fig 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 (Fig. 3B). These data directly demonstrated that 2GP competed with template/primer on the binding to RT. Quantification of the intensity of the band gave an apparent K_d for 2GP of 89 ± 4 nM.

2GP abolishes HIV-1 reverse transcriptase activity on wild-type and mutant RTs 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 were 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 reverse transcriptase activity using an excess of enzyme over template/primer. Reactions were incubated for 60 min and polymerization products were separated in a polyacrilamide 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 in inhibiting HIV-1 RT, while 2GP retained its activity at submicromolar concentrations. Neither the presence of AZT-related mutations nor the presence of a fifth mutation (Y181C) affected to the activity or 2GP. However, the presence of the Y181C mutation conferred high resistance to all NNRTIs tested to RT^{5mut}. The IC₅₀ 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 quantified because the low activity of these compounds on RT^{5mut} under these conditions.

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. While 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 testing the phosphorolysis. Under these conditions, some phosphorolytic activity, corresponding to the preformed 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 preincubated 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 dissociates from the template/primer. As control, we studied the effect of an excess of unlabeled poly(rA)/dT₂₀ or efavirenz under the same conditions.

Phosphorolytic activity was detected when RT was preincubated with the template/primer before adding an excess of 2GP or cold template/primer (Fig. 4). This activity was absent if RT was preincubated 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 clearly demonstrate that 2GP inhibited phosphorolysis by competing with the template/primer.

Effect of the combination of 2GP and AZTTP on RT activity. We have previously reported that the synergy found in combinations of AZT with NNRTIs or certain PP_i analogs was due to the inhibition by these compounds of the nucleotide excision reaction catalyzed by HIV-1 RT [12, 18]. For this reason we analyzed 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 (Fig. 4A). 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 μ M PP_i the combination of 2GP with AZTTP was highly synergistic as judged by the Yonetani-Theorell plot, with a $-intercept/IC_{50}$ value of 0.24 ± 0.04 (Fig. 4B). 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

(-intercept/ $IC_{50} = 0.15 \pm 0.02$), nevirapine (0.17 ± 0.2) or pamidronate (0.23 ± 0.13) (data not shown). In addition, synergy increased by extending the time of incubation. For example, interaction 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 (Fig. 4C). Under these conditions RT-catalyzed phosphorolysis severely reduced the potency of AZTTP as a chain-terminating agent.

DISCUSSION

Inhibition of the ATP- or PP_i - dependent phosphorolysis catalyzed by HIV-1 RT is a novel approach to fight against viral resistance to chain-terminating nucleotides. However, only a few compounds have been demonstrated to inhibit this reaction [12-14]. Previously we have found that 2GP, a natural compound isolated from *Terminalia triflora*, inhibited both the DNA polymerase and RNase H activities of HIV-1 RT. In this work we extend our previous observations showing that 2GP also inhibited the ATP- and PP_i -dependent excision of chain-terminated primers catalyzed by HIV-1 RT. 2GP was as effective as NNRTIs in inhibiting this activity and, unlike some PP_i analogs such as phosphonoformate [14], 2GP was able to fully block the RT-catalyzed phosphorolysis. 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 directly demonstrated that 2GP binds to the free enzyme with a K_d of about 85 nM.

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 reverse transcriptase 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 [23], KM043 [24], avarol and avarone derivatives [24] and toxiuxol [25]. Some of them, such as the KM-1, avarol or the avarone derivatives have also been found to inhibit the RNase H activities of HIV-1 RT [17, 24]. However, it seems that the potential of these compounds as inhibitors of the phosphorolytic reaction catalyzed 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. Firstly, 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 catalyzed by HIV-1 RT has not been analyzed, although, given their mechanism of action, we anticipate that molecules such as KM-1 [17] will be effective in blocking RT-catalyzed 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]. And 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 inhibit viral replication with an EC_{50} of 0.21 μ M, but does not affect cell proliferation or viability below 40 μ M [17]. 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 in cell-based assays, such as in the inhibition of the viral binding to the cell surface [26]. Moreover, several analogs of the template/primer are either charged or have hydrophilic nature and probably their bioavailability should 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 analogs, and their potential for combined chemotherapy merits further development..

The results obtained in this work provide a rationale for combining molecules that block the binding of the template/primer to RT with nucleoside analogs. 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 previously shown that the inhibition of RT-catalyzed phosphorolysis seems to be related to the superior long term efficacy of combinations containing a NNRTI over combinations containing only nucleoside analogs [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 analogs by interfering with the main mechanism of resistance of this enzyme to chain-terminating nucleotides.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Departamento de Educación y Cultura from the Gobierno de Navarra and by a joint grant from the Gobierno de Navarra and the Conseil Regional d'Aquitaine (Fondo Común de Cooperación Navarra-Aquitania). The following reagent was obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH: Efavirenz. We also thank Drs. V. Dollé and C. H. Nguyen (UMR 176 CNRS-Institut Curie, Orsay cedex, France) for supplying us with 4-arylmethylpyridinone.

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TABLES

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

	IC ₅₀ (μM) ^a		
	RT	RT ^{AZT}	
	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

^a The experiments were carried out as described in the caption to Fig.1.

Table 2: Inhibition of DNA polymerization catalyzed by wild-type and resistant RTs using a large excess of enzyme over template/primer.

	IC ₅₀ (μM) ^a		
	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

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

FIGURE LEGENDS

Fig 1. Inhibition of RT-catalyzed phosphorolysis by 2GP. 1 nM of 5'-³²P-labeled primer (d21-AZTMP) annealed to the r39 template was incubated in buffer A containing 100 mM NaCl with 25 nM RT, the indicated amount of 2GP and 150 μ M 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 products analyzed by denaturing PAGE. Under these conditions, no ATP-dependent phosphorolysis was detected for wild-type RT.

Fig 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 (\circ), 0.2 (\bullet), 0.5 (\square), 0.8 (\blacksquare), and 2 μ M (\triangle). In the direct plot all the experimental data were simultaneously fitted by nonlinear regression to the three dimensional equation defining non-competitive inhibition, which gave a K_i value of $0.41 \pm 0.02 \mu$ M. (B) Michaelis-Menten and Lineweaver-Burk plots for the inhibition of RT activity by 2GP with poly(rA)/dT₂₀ as the variable substrate. Concentrations of 2GP were 0 (\circ), 0.05 (\bullet), 0.15 (\square), and 0.5 μ M (\blacksquare). In this case the best fit corresponds to a competitive inhibition with $K_{ic} = 0.031 \pm 0.007 \mu$ M.

Fig 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 labeled poly(rA)/dT₂₀ and 2GP (\bullet) or U-90152s (\blacksquare). Reactions were filtered through a Multiscreen nitrocellulose filter plate, rinsed twice with 50 μ l of binding buffer, and 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. 200 nM RT was added to a mixture containing 4 nM of 5'-³²P-labeled d21-AZTMP annealed with r39 primer and 2GP. The putative complexes formed were challenged by the addition of 1.5 μ M poly(rA)/dT₂₀ and the complexes resistant to this trap were resolved in a non-denaturing 5% polyacrilamide gel.

Fig 4. Mechanism of inhibition of phosphorolysis by 2GP. 1 nM of 5'-³²P-labeled primer (d21-AZTMP) annealed to the r39 template was preincubated 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 MgCl₂ along with the indicated amounts of 2GP, efavirenz or unlabeled poly(rA)/dT₂₀ (cold T/P). After 2 min, reactions were stopped and products analyzed by denaturing PAGE. In other tubes, RT was first preincubated with each inhibitor and reactions started by adding 10 mM MgCl₂ along with the labeled r39/d21-AZT. The addition of 2GP or poly(rA)/dT₂₀ did not completely inhibit phosphorolysis if RT was preincubated with the substrate, due to the activity of preformed RT·template/primer complexes. Lane C represents a control reaction carried out in the absence of RT.

Fig 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)/dT₂₀ and 20 μM [α-³³P]dTTP. The concentrations of AZTTP were 0 (O), 0.0125 (●), 0.025 (□), 0.05 (■), and 0.1 μM (△). 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 (O), 0.025 (●), 0.05 (□), 0.1 (■), and 0.2 μM (△). Lines converging at the left 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.

Fig 1.

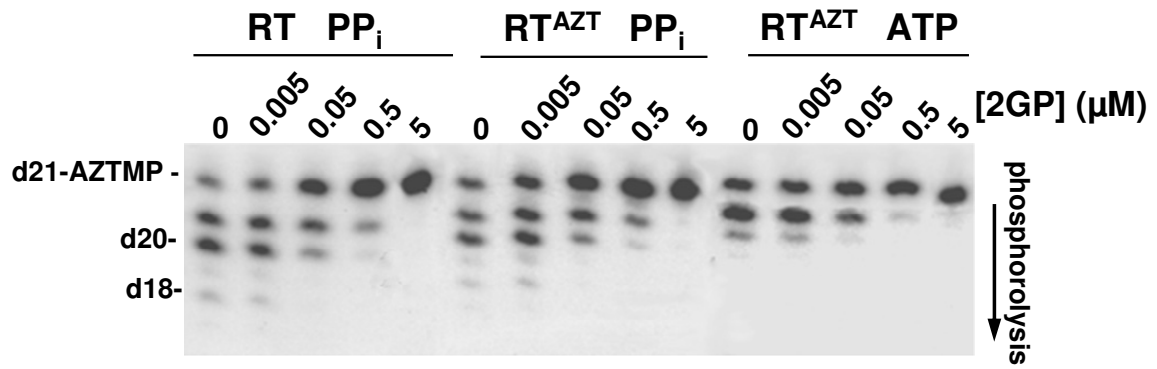
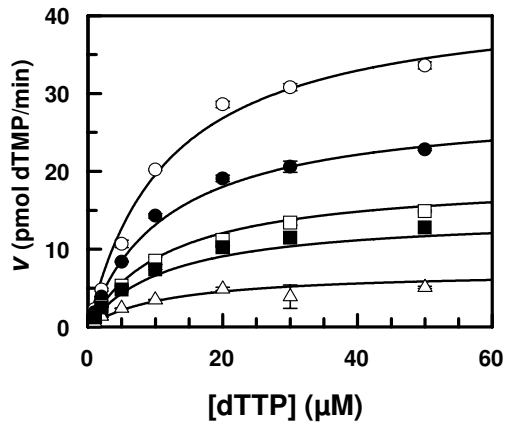
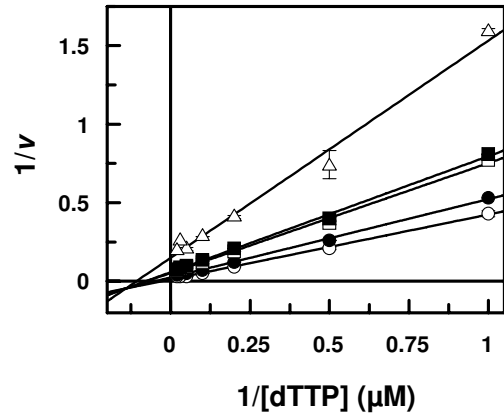


Fig 2.

A Michaelis-Menten plot



Lineweaver-Burk plot



B

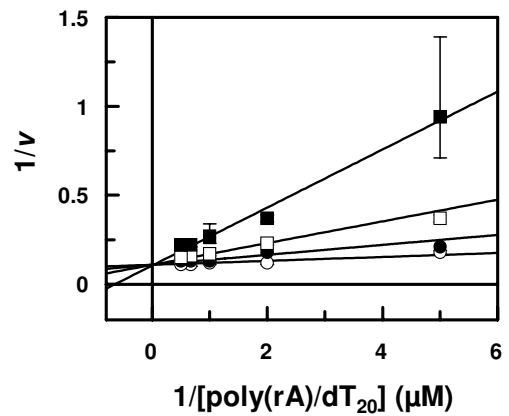
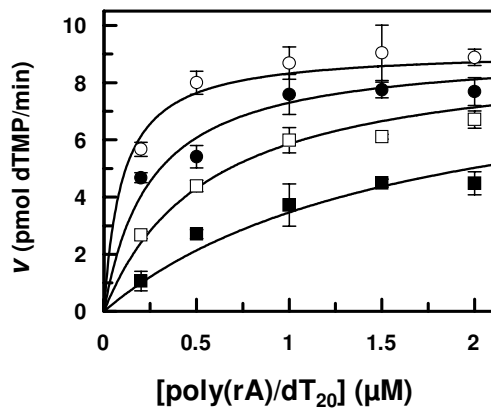


Fig 3.

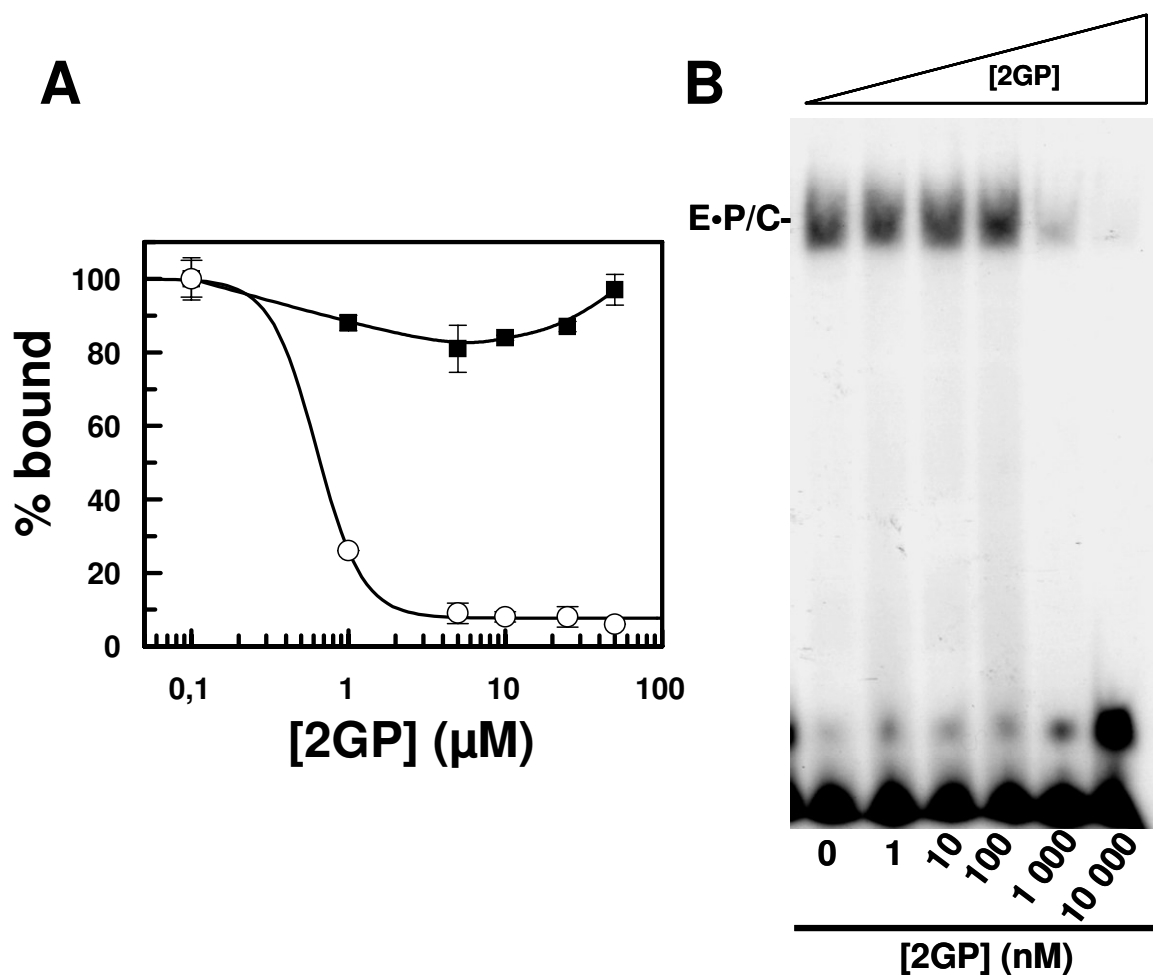


Fig 4.

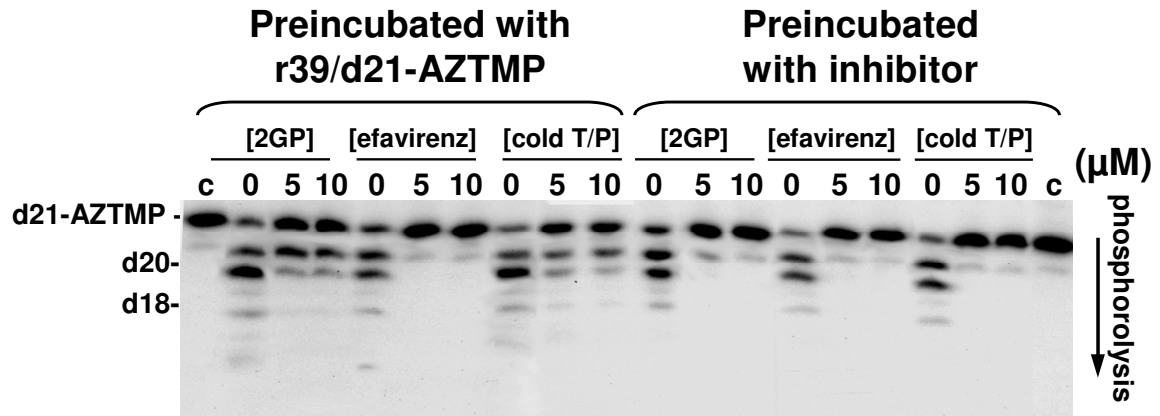


Fig 5.

