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INHIBITION OF RNA-DIRECTED DNA POLYMERASE FROM AVIAN MYELOBLASTOSIS VIRUS BY A 5-BENZYL-6-AMINOURACIL

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5-(p-Chlorobenzyl)-6-aminouracil (5-ClAU) inhibited RNA-dependent DNA polymerase (reverse transcriptase) from avian myeloblastosis virus. Inhibition was expressed only in the presence of a polyribonucleotide template such as mRNA or poly(rA), and kinetic analysis suggested that the action of 5-ClAU is competitive with template:primer. 5-ClAU did not inhibit HeLa DNA polymerase γ, an enzyme that efficiently copies polyribonucleotide templates. A mechanism is proposed in which a 5-ClAU:template complex interferes with enzyme function, based partly on NMR studies indicating that 5-ClAU can form a hydrogen-bonded complex with deoxyadenosine in solution.

N^6-Substituted 6-aminouracils inhibit selected prokaryotic and eukaryotic DNA polymerases by acting as analogs of deoxyguanosine 5'-triphosphate (1,2). This class of compounds acts by hydrogen-bonding with cytosines in the DNA template and by binding, via the phenyl ring, with the enzyme at an inhibitor binding site in the vicinity of the active site region (3). By altering the structure of the N^6-substituent we have been able to tailor componds of this class to selectively inhibit specific polymerases. For example, uracil substituted with the 6-(p-n-butylanilino) group is specific for mammalian DNA polymerase α (2,4), whereas uracil substituted with the 6-(p-methylanilino) group is specific for gram-positive DNA polymerase III (2). We have further manipulated the phenyl substituent in an attempt to develop inhibitors in this class which are active on reverse transcriptases. This paper reports initial results of a study employing reverse transcriptase from avian myeloblastosis virus (AMV). As indicated below, we have identified an active compound which, although structurally related to those active against the conventional DNA-dependent enzymes, apparently acts by a different and novel mechanism.

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MATERIALS AND METHODS

Enzymes. AMV reverse transcriptase was obtained from Dr. Joseph Beard, Life Sciences, Inc. It was diluted 1:5, stored and used in a diluent containing 10% glycerol, 5 mM DTT, 0.5 µg/ml BSA, 50 mM Tris Cl (pH 8.5) and 200 mM KCl. DNA polymerase γ from HeLa cells was a gift from Dr. E. Baril.

Template:primers. Calf thymus DNA was activated by DNAse I treatment according to the method presented in ref. 5. Heterogeneous messenger RNA (mRNA) from Dictyostelium discoideum was a gift from Dr. A. Jacobson; it was mixed with oligo(dT)$_8$ in 5 mM Tris Cl/25 mM NaCl, and incubated for 10 min at 25°C to give a stock solution containing 53 µg/ml mRNA and 13 µg/ml oligo(dT)$_8$. Poly(rA):oligo(dT)$_8$ was prepared by hybridizing poly(rA) and oligo(dT)$_8$ (P.L. Biochemicals) for 10 min at 25°C to give a stock solution containing 125 µg/ml and 25 µg/ml of the respective components.

Enzyme assays. The standard reverse transcriptase assay contained in a volume of 50 µl: 50 mM Tris Cl (pH 8.3), 40 mM KCl, 5 mM NaCl, 8 mM $MgCl_2$, 10% glycerol, 1 mM DTT and 0.1 µg/ml BSA. For assays employing activated DNA, the mixture contained 200 µM dATP and dCTP, 4 µM [^{3}H]TTP (3250 cpm/pmol) and dGTP at either 0 or 200 µM. Assays with mRNA:oligo(dT)$_8$ contained 13.2 µg/ml mRNA, 3.25 µg/ml oligo(dT)$_8$, 250 µM dATP and dCTP, 5 µM [^{3}H]TTP and dGTP at either 0 or 250 µM. Assays with poly(rA):oligo (dT)$_8$ employed 25 µg/ml poly(rA), 6.2 µg/ml oligo(dT)$_8$ and 4 µM [^{3}H]TTP. Reactions were initiated by the addition of 0.01 unit of enzyme and proceeded for 10 min at 37°C. One unit of enzyme activity is that amount of enzyme in 50 µl that incorporates 1 nmol of TMP into acid insoluble product in 10 min at 37°C with poly(rA):oligo(dT)$_8$ as template:primer.

DNA polymerase γ was assayed as described in ref. 6. The work-up and counting of acid insoluble products have been described (7).

Inhibitors. Stock solutions of inhibitors (40 mM) were prepared in dimethylsulfoxide (DMSO) and diluted with 50 mM Tris Cl (pH 8.3). These solutions were diluted into assay media to designated concentrations. Control assays contained an identical amount of dimethylsulfoxide. The syntheses of 5-(p-chlorobenzyl)-6-aminouracil (5-ClAU) and 6-(p-chlorobenzyl-amino)uracil (6-ClAU) have been reported (8).

Spectroscopic experiments. UV absorption data were determined on a Gilford 2400-2 spectrophotometer. All readings were done with polynucleotides (concentrations expressed as M of monomers) in the absence or presence of 5-ClAU (2×10^{-5} M) in 0.02 M potassium phosphate buffer (pH 7) containing 0.1 M NaCl. 5-ClAU was initially dissolved in DMSO and this solution was diluted with buffer; blank solutions contained the same concentration of DMSO.

Nuclear magnetic resonance spectra were obtained at 60MHz on a Perkin Elmer R-12B instrument with a Nicolet TT-7 fourier transform attachment. The ^{1}H spectrum of 5-ClAU has been reported (8).

RESULTS

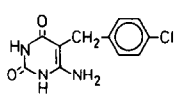
Inhibition of reverse transcriptase by 5-(p-chlorobenzyl)-6-amino-uracil is template specific. Among a series of 6-anilinouracils, 6-(phenyl-alkylamino)uracils and related 6-aminouracils, one compound, 5-(p-chlorobenzyl)-6-aminouracil (5-ClAU), inhibited the reverse transcriptase-catalyzed incorporation of [^{3}H]TMP into mRNA primed with oligo(dT)$_8$. The data of Table 1 show that 1 mM 5-ClAU inhibited 76% of

Table 1: Inhibition of AMV reverse transcriptase by 5-(p-chlorobenzyl)-6-aminouracil with mRNA:oligo(dT)$_8$ as template:primer

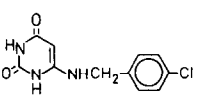
Assay conditions*	Incorporation, pmol [^{3}H]TMP -inhibitor	+1 mM inhibitor	% Inhibition
all dNTPs	2.0	.58	76
-dGTP	.58	.15	72
-dATP	.41	.10	75

*The assay procedure is described in MATERIALS AND METHODS.

enzyme activity in the presence of all four deoxyribonucleoside 5'-triphosphates. Unexpectedly, no increase in inhibition was observed in the truncated assay run in the absence of the putative competitor, dGTP, or in the absence of dATP (Table 1). Furthermore, 5-ClAU did not inhibit reverse transcriptase when activated DNA was used as the template, either in the presence or absence of dGTP (data not shown).



5-ClAU



6-ClAU

The inability of dGTP to attenuate inhibition of reverse transcriptase by 5-ClAU indicated that its mechanism of action differed from that of conventional dGTP-specific polymerase inhibitors such as the analogous 6-(p-chlorobenzylamino)uracil (6-ClAU). 6-ClAU is a potent inhibitor of DNA polymerase III, completely reversible by dGTP (3), and devoid of activity against reverse transcriptase with either activated DNA or mRNA:oligo(dT)$_8$ as template:primers (data not shown). 5-ClAU, in contrast, is a very weak dGTP-reversible inhibitor of the bacterial enzyme, inhibiting only 33% of DNA polymerase III activity in the truncated, dGTP-deficient assay at a concentration of 1.6 mM.

Further evidence of a novel mechanism of 5-ClAU action on reverse transcriptase was the retention of its inhibitory activity in a reaction employing oligo(dT)$_8$-primed poly(rA) (cf. Figure 1), a template that does not contain cytosine.

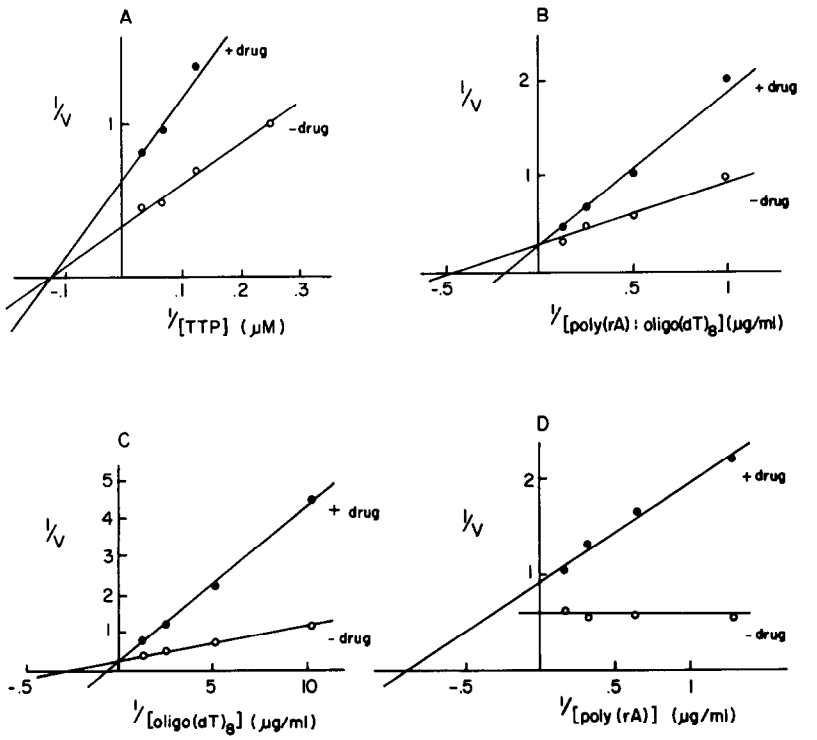


Figure 1: Lineweaver-Burk plots of AMV reverse transcriptase activity inhibited by 5-ClAU as a function of substrate and template concentrations. The panels correspond to experiments in which the concentrations of TTP (A), poly(rA):oligo(dT)$_8$ (B), oligo(dT)$_8$ (C) and poly(rA) (D) were varied. The standard assay procedure is described in MATERIALS AND METHODS. Values of V correspond to pmol of TMP incorporated per 10 min assay. ^{o}, absence of 5-ClAU; $^{\bullet}$, presence of 1.2 mM 5-ClAU.

Inhibition is inversely related to template:primer concentration.

The findings summarized by the Lineweaver-Burk plots in the Figure show that the potency of 5-ClAU on reverse transcriptase varies as a function of template:primer concentration rather than as a function of deoxynucleotide substrate concentration. Part A of the Figure demonstrates the effect of variation of TTP concentration on reverse transcriptase activity in the presence and absence of 5-ClAU at 1.2 mM. The V_{max} decreases in the presence of inhibitor while the K_m for TTP is unchanged. Part B of the Figure shows that variation of poly(rA):oligo(dT)$_8$ in the presence of the drug demonstrates typical competitive kinetics.

The above observations suggested that 5-ClAU interferes with the function of one or both components of the template:primer. We attempted to

distinguish between the latter possibilities by examining independently the effects of poly(rA) and oligo(dT)$_8$ on inhibition by 5-ClAU. The effect of varying the concentration of oligo(dT)$_8$ at constant poly(rA), summarized in part C of the Figure suggests at first glance competitive kinetics. However, this suggestion is ambiguous because the increase in oligo(dT)$_8$ likely increases the number of hybridized primer termini available to the enzyme causing, in effect, a situation comparable to that of increasing total poly(rA):oligo(dT)$_8$. The control curve of part D of the Figure shows that the reaction was unresponsive to additional template. This result was expected because no more primer termini are generated. The inhibited reaction, however, was responsive to added template; increasing template concentration decreased inhibition to a level approaching that of the control uninhibited reaction.

5-ClAU is selective for reverse transcriptase. The apparent capacity of 5-ClAU to compete with template and/or template:primer suggested that other polymerases capable of utilizing poly(rA):oligo(dT)$_8$ might be inhibited by the compound. One such enzyme, DNA polymerase γ, in an assay using poly(rA):oligo(dT)$_8$-directed incorporation of [^{3}H]TMP, was not inhibited by 1.2 mM 5-ClAU at the high template:primer concentration of standard assay conditions or at the sub-optimal ($<K_m$) template:primer concentrations in the range of those for which inhibition of AMV reverse transcriptase is maximized (results not shown).

Analysis of possible inhibitor-template interactions. Spectroscopic methods were employed to detect an interaction between 5-ClAU and poly(rA):oligo(dT)$_8$. The electronic absorption spectrum of 5-ClAU at pH 7 showed λ_{max} 274 nm (ϵ 12,400), unfortunately close to those of poly(rA) (260 nm) and oligo(dT)$_8$ (264.5 nm). The absorbances of a 2×10^{-5} M solution of 5-ClAU at 265, 275 and 285 nm were monitored in the presence of poly(rA) ($1\text{-}15\times10^{-5}$ M) and poly(rA):oligo(dT)$_8$ ($5\text{-}15\times10^{-5}$ M). We observed no changes in absorbance at the indicated wavelengths other than additive increases, nor did we observe a change in λ_{max} of 5-ClAU.

Nuclear magnetic resonance experiments did demonstrate hydrogen bonding interactions between 5-ClAU and deoxyadenosine (dA). Addition of excess dA to a dilute solution of 5-ClAU in DMSO-d_6:$CDCl_3$ (1:1, v/v) caused downfield shifts only in the 3-H resonance of the drug, a result which would occur if it hydrogen bonded in Watson-Crick fashion with dA. The association constant for this interaction, K_a, determined by the method of ref. 9, was 7.8 L/mol. Deoxyguanosine (dG) caused no significant shifts of 5-ClAU resonances when added in a similar excess.

It should be noted that 5-ClAU interacted strongly with deoxycytidine (dC) in similar experiments (K_a 45 L/mol). Both 1-H and 6-NH_2 resonances of 5-ClAU moved downfield in the presence of dC, commensurate with Watson-Crick-like hydrogen bonding as in dC:dG base pairing experiments (10).

DISCUSSION

5-ClAU is a novel and relatively selective inhibitor of AMV reverse transcriptase. Its action is dependent on the use of an RNA template - it is ineffective when enzyme is copying DNA. 5-ClAU also is not a classic competitor of dNTP binding to the enzyme (Fig. part A); rather, it is competitive with template and/or template:primer (Fig. parts B-D), apparently interfering with the binding of template:primer to the reverse transcriptase. Although ultraviolet spectroscopy suggested no intercalative interaction between inhibitor and template or template:primer, the results of NMR study clearly demonstrated the capacity of 5-ClAU to hydrogen bond with adenine residues.

The data presented here support a working hypothesis that the 5-ClAU:adenine interaction is the basis of its mechanism of reverse transcriptase inhibition. We hypothesize that 5-ClAU, with or without the assistance of the transcriptase, binds to adenine residues of template and, thus, interferes with the normal interaction of enzyme and its template:primer. The selectivity of 5-ClAU for reverse transcriptase, suggested by the relative resistance of DNA polymerase γ to the drug (see

Results), is not incompatible with the proposed mechanism. Subtle differences in the structural features of the two enzymes may profoundly affect the way in which each protein perceives, and possibly facilitates, the formation of the inhibitor:template complex. The resistant enzyme may simply fail to sense or facilitate the formation of the complex, whereas the sensitive enzyme does. Experiments exploiting modification of the 5-ClAU prototype inhibitor and "non-complementary", adenine-deficient templates will be done to test the hypothesis of inhibitor mechanism and to determine the basis for its enzyme selectivity.

There are few selective inhibitors of reverse transcriptases. Pyridoxal 5'-phosphate inhibits the enzymes from several RNA tumor viruses (11) and rifamycin derivatives, anthracyclines, certain template:primer analogs and altered nucleotides such as araCTP and dideoxyTTP have been reported to display anti-reverse transcriptase activity (see, for example, refs. 12 and 13 for reviews). Interest in inhibitors of reverse transcriptases has been stimulated recently by observations that 1) a human T-cell leukemia retrovirus, HTLV-III, may be the causative agent of AIDS (14) and 2) the drug suramin, a reverse transcriptase inhibitor, protects T-cells in vitro against infectivity and cytopathic effect of HTLV-III (15). It may be possible, through further studies examining the spectrum of action of 5-ClAU and analogous compounds, to develop selective, clinically useful inhibitors of reverse transcriptases which can be of value in inhibiting the infectivity of medically important RNA viruses such as HTLV-III.

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