Biochemical Pharmacology, Vol. 36, No. 19, pp. 3103–3106, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

RAPID COMMUNICATION

INHIBITION OF HERPESVIRUS AND HUMAN DNA POLYMERASES BY α-HALOGENATED PHOSPHONOACETATES*

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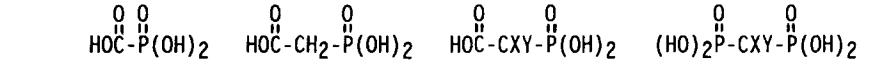
(Received 7 April 1987; accepted 15 July 1987)

Infection of mammalian cells by herpesviruses produces virally coded DNA polymerase, a primary target of several classes of antiviral compounds [1]. The anions of phosphonoformic acid (PFA) and phosphonoacetic acid (PAA) are antiviral agents, related structurally to pyrophosphate, that inhibit a number of viral DNA polymerases [2-4]. Both compounds are believed to interact at the pyrophosphate binding site of the enzyme during polymerization of nucleoside triphosphates and thus interfere with replication of DNA. Modifications in the phosphonate/carboxylate groups of PAA and PFA by esterification or by replacement with other combinations of acidic functional groups have resulted in less active inhibitors [3-6]. PAA inhibits some viral DNA polymerases about as potently as PFA, but with others it is less effective [3,6]. From the perspective of providing access to derivatives useful for probing structure-function relationships, however, the methylene group present in PAA offers a locus for substitution not available in PFA. A number of PAA compounds modified by α-alkyl, α-aryl, and α-hydroxy substituents have been examined as inhibitors of herpesvirus DNA polymerases [3-7], but α-halo substituted PAA analogs have not been evaluated systematically. We recently described a convenient synthesis of fluorophosphonoacetic and difluorophosphonoacetic acids [8,9] and proposed these compounds as possible new inhibitors of viral replication [8].



PFA PAA XYPAA XYMDP

We report here a study of α-monohalo (X = H, Y = F/Cl/Br), α,α-dihalo (X,Y = F/Cl/Br) and α-methyl, α-halo (X = CH_3, Y = F/Cl/Br) XYPAA salts as inhibitors of three viral DNA polymerases (from Herpes simplex type 1 (HSV-1), Herpes simplex type 2 (HSV-2), and Epstein-Barr virus (EBV)) and three human (α, β, and γ) DNA polymerases. For comparison, inhibition results for the corresponding α-monohalo and α,α-dihalomethanediphosphonates (XYMDP; X,Y = H/F/Cl/Br) are also presented.

MATERIALS AND METHODS

General methods and materials. Phosphorus (^{31}P, 109.35 MHz) NMR spectra were obtained on a Bruker WP-270SY spectrometer. Melting points were measured using a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories. [^3H]TTP was purchased from Moravek Biochemicals, Brea, CA. dATP, dCTP, dGTP, TTP, single stranded- and native-DNA cellulose, and calf thymus DNA were obtained from Sigma Biochemicals, St. Louis, MO. DEAE-cellulose and phosphocellulose were products of Whatman Inc., Clifton, NJ. All other chemicals were reagent grade.

Preparation of α-halogenated phosphonoacetate and methanediphosphonate amine salts. We describe elsewhere preparation of FPAA [8,9], F_2PAA [8,9], 2-fluoro-2-phosphonopropionic acid (CH_3FPAA) [9], FMDP [10], F_2MDP [10], and the other α-halo MDP [11] acids. The remaining α-halo PAA triacids were synthesized by refluxing the appropriate triethyl ester [12] in excess HCl for 6 hr. The HCl was then removed at reduced pressure. In some cases the triacid was also made by treatment with bromotrimethylsilane followed by H_2O [13,14]. a. Dicyclohexylamine (DCHA) salts. The product triacid was dissolved in a minimum of absolute ethanol. DCHA was added in excess, precipitating its salt which was recrystallized successively from ethanol/acetone and absolute ethanol. b. Pyridine salts. The product triacid was treated with excess pyridine and let stand overnight at about -5°. The resulting salt was filtered, washed with cold MeOH, air-dried (12 hr), recrystallized from MeOH and then dried for 6 hr at 60°. Both types of derivative were characterized by elemental analysis, melting point, and ^{31}P NMR (Table 1).

*Presented at the Second International Symposium on Phosphorus Chemistry Directed Towards Biology, Lodz, Poland, September 8-12, 1986.

Preparation of DNA polymerases. The activated calf thymus DNA was prepared according to previously published methods [15]. DNA polymerases from HSV-1 and HSV-2 [16], EBV [17], and peripheral blasts from chronic lymphocytic leucophoresed patients undergoing blast crisis [18] were purified by previously published methods. Generally, DNA polymerases were purified by sequential chromatography on DEAE-cellulose, phosphocellulose, and single- or double-stranded-DNA cellulose. The purified enzymes were dialyzed against and stored in 50 mM Tris-Cl (pH 7.5) containing 1 mM each of DTT, EDTA and PMSF, plus 30% glycerol.

DNA polymerase assays. The standard viral DNA polymerase reaction mixture contained the following: 50 mM Tris-Cl, pH 8.0; 4 mM $MgCl_2$; 0.5 mM dithiothreitol (DTT); 0.2 mg/ml bovine serum albumin; 0.15 M KCl; 0.25 mg/ml activated calf thymus DNA; 0.1 mM each of dATP, dCTP and dGTP; and 10 μM [^{3}H]TTP in a final reaction volume of 50 μl. The reaction was started by adding the enzyme to the reaction mixture and allowed to proceed for 20 min at 37°. Samples were spotted onto 2.1 cm GF/A filter discs and processed to determine trichloroacetic acid insoluble, filter-bound radioactivity. When determining the inhibitory action of PAA analogs, the reaction mixture containing the appropriate amount of analog was kept on ice before initiation. Assays with human DNA polymerase α were done similarly except that the pH of the reaction mixture was 7.5 and contained no KCl. For β and γ DNA polymerases the reaction mixture included 100 mM KCl.

TABLE 1. Data for characterization of XYPAA salts

Compound	Formula	Calculated			Found			m.p.	δ ^{31}P (J)[a]
		%C	%H	%N	%C	%H	%N	°C	ppm (Hz)
ClPAA[b]	$C_{26}H_{50}O_5ClN_2P$	58.14	9.38	5.22	58.22	9.41	5.12	196-8[c,d]	12.9 (15)
ClPAA[e]	$C_7H_9O_5ClNP$	33.16	3.58	5.52	33.44	3.63	5.66	167-8	9.9 (17)
BrPAA[b]	$C_{26}H_{50}O_5BrN_2P$	53.70	8.67	4.82	54.06	8.72	4.69	171-3[c,f]	12.8 (14)
BrPAA[e]	$C_7H_9O_5BrNP$	28.21	3.04	4.70	28.05	3.06	4.69	143-4	9.8 (14)
FClPAA[b,g]	$C_{26}H_{49}O_5FClN_2P$	53.65	9.00	4.81	53.48	8.93	4.81	183-4	7.4 (84)
FBrPAA[b]	$C_{26}H_{49}O_5FBrN_2P$	52.09	8.24	4.67	51.66	8.32	4.48	150-2[c,f,h]	7.5 (81)
ClBrPAA[b]	$C_{26}H_{49}O_5ClBrNP$	50.69	8.02	4.55	50.53	7.93	4.47	184-5[i]	9.9
ClBrPAA[e]	$C_7H_8O_5ClBrNP$	25.29	2.43	4.21	25.31	2.40	4.23	137-8[d]	9.7
Cl_2PAA[b]	$C_{26}H_{49}O_5Cl_2N_2P$	54.64	8.64	4.90	54.46	8.42	4.85	224-5[d]	10.3
Cl_2PAA[e]	$C_7H_8O_5Cl_2NP$	29.19	2.80	4.86	29.17	2.87	4.81	166-7[d,f]	10.3
Br_2PAA[b]	$C_{26}H_{49}O_5Br_2N_2P$	47.28	7.48	4.24	47.30	7.08	3.71	221-2[d]	9.6
Br_2PAA[e]	$C_7H_8O_5Br_2NP$	22.31	2.14	3.72	22.43	2.19	3.75	144-5[d,f]	9.7
CH_3ClPAA[b]	$C_{27}H_{52}O_5ClN_2P$	58.84	9.51	5.08	58.46	9.55	5.19	>225	17.4 (13)
CH_3BrPAA[b,j]	$C_{27}H_{52}O_5BrN_2P$	52.85	8.87	4.57	52.71	8.74	4.26	>225	17.5 (14)

a. In D_2O. b. DCHA salt. c. Sensitive to initial temp. d. Melted with dec. (Δ color). e. Pyridine salt. f. Melted with dec. (gas evol.). g. 3/2 H_2O. h. Sintered at 107°. i. Dec. (Δ color) w/o melting. j. 1 H_2O.

RESULTS AND DISCUSSION

Several structure-activity patterns emerge from analysis of the IC_{50} values obtained (Table 2). First, α-monohalo PAA derivatives as a group were much better inhibitors than the corresponding disubstituted compounds. FPAA and ClPAA displayed activity against the HSV DNA polymerases that almost rivaled that of PAA or PFA. BrPAA, previously reported to inhibit HSV-2 DNA polymerase [7], was slightly less active. Second, in the α,α-dihalo (like) disubstituted group, only F_2PAA showed significant inhibition, but with a higher IC_{50} than that found for any of the α-monohalo phosphonoacetates. Third, only the Mendeleevan extremes within our halogen group (F,Br) produced an α,α-dihalo (unlike) disubstituted inhibitor having an IC_{50} < 100 μM. Fourth, combination of methyl and halogen substituents (CH_3YPAA) did not result in effective inhibitors for the viral enzymes tested. Fifth, the potency of the α-halophosphonoacetate inhibitors was dependent on the virus DNA polymerase used; in particular, the α-monohalo PAA IC_{50} values for the HSV-1 and HSV-2 polymerases were 4- to 5-fold smaller than for the EBV enzyme, with lesser differences seen for the active α,α-dihalo inhibitors. In contrast, the IC_{50} values of PFA and PAA were similar for all three viral DNA polymerases. Finally, human α, β and γ DNA polymerase were significantly less sensitive to the active α-halo PAA inhibitors than the viral enzymes. FPAA was at least severalfold more selective than PAA or PFA as an inhibitor of HSV-1 versus human DNA polymerase.

Inhibition data for XYPAA samples tested at a single concentration of 100 μM differentiated some of the compounds having an IC_{50} > 100 μM. The results (not shown) indicated that EBV DNA polymerase, but not the HSV-1 and HSV-2 enzymes, had some sensitivity to the α-methyl α-halo PAA derivatives (40-50% inhibition) and to Cl_2PAA, Br_2PAA and ClBrPAA (30-40% inhibition).

Unlike the nine α-monohalo and α,α-dihalo XYPAA derivatives, among which five showed significant activity, none of the corresponding XYMDP salts had an IC_{50} < 100 μM with the three herpesvirus DNA polymerases tested (Table 1). This apparent difference between the two categories of α-halogenated phosphonates can be compared with the observation that Br_2MDP, Cl_2MDP and ClMDP had IC_{50} values of 10, 75 and 85 μM, respectively, with RNA polymerase from influenza virus A, whereas PAA was a poorer inhibitor [19]. The suggestion of an opposite trend in halogen substitution effects as well as in sensitivity to parent phosphonate structure under specified assay conditions merits further investigation.

TABLE 2. IC_{50} values (μM) for DNA polymerase inhibitors

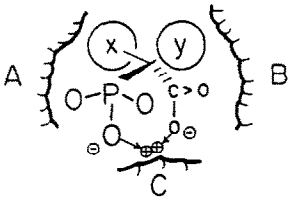
Inhibitor[a]	Synth.	Viral			Human	
		HSV-1	HSV-2	EBV	α	β,γ
PFA	-	1.1	1.1	1.2	15	>300
PAA	-	1.3	1.2	1.6	21	>300
FPAA	b	2.5	3.8	14	>100	>300
ClPAA	c	3.2	5.4	14.5	80	>300
BrPAA	c	6	5.4	25	>100	>300
F_2PAA	b	18	30	65	>100	>300
Cl_2PAA	c	>100	>100	>100	>100	>300
Br_2PAA	c	>100	>100	>100	>100	>300
FClPAA	c	>100	>100	>100	>100	>300
FBrPAA	c	37	70	94	>100	>300
ClBrPAA	c	>100	>100	>100	>100	>300
CH_3FPAA	b	>100	>100	>100	>100	>300
CH_3ClPAA	c	>100	>100	>100	>100	>300
CH_3BrPAA	c	>100	>100	>100	>100	>300
XYMDP[d]	e	>100	>100	>100	>100	>300

a. DCHA salts. b. Ref. 9. c. This work. d. X,Y = H,F; H,Cl; H,Br; F,F; Cl,Cl; Br,Br; F,Cl; F,Br; Cl,Br. e. Ref. 11, except FMDP/F_2MDP (Ref.10).

Frank and Cheng observed that PFA and ACGTP (acyclovir triphosphate) mutually exclude each other by binding at the same site on DNA polymerase [20] and postulated that these two inhibitors, via their negatively charged phosphate and carboxyl moieties, may be binding at a positively charged center in the enzyme [21]. They further predicted that charge alteration in this center might result in reduced affinity for the inhibitors. Reciprocally, any change in the intrinsic ligand properties of the inhibitors should modify their interaction at the putative cationic binding site. Extension of these ideas from PFA to PAA derivatives must take into account the alterations in molecular geometry, chelate ring-size preference [22] and basicity of the phosphonate and carboxylate groups that result from interposition of the methylene 'spacer' in PAA. PFA is the stronger acid and at physiological pH will have a greater average negative charge than PAA [22,23]. We found similar IC_{50} values for PFA and PAA with the HSV-1, HSV-2 and EBV DNA polymerases, in agreement with earlier studies [6]. The inhibition activity of PFA and PAA has been reported to vary significantly with some other viral enzymes [6,24]. Such variations in specificity are not understood at this time but presumably reflect structural differences in the inhibitor binding sites.

The systematic substitution of halogen atoms at the methylene carbon of PAA (and α-methyl PAA) creates a homologous set of compounds presenting a range of acid/base and steric properties. In view of the differing herpesvirus DNA polymerase IC_{50} values observed for these inhibitors, it will be of interest to measure their pK_as and relative affinities for metal dications proposed to be relevant to the binding of pyrophosphate analogues by viral polymerases [19,22]. However, certain relationships between XYPAA structure and activity can be discussed qualitatively without the benefit of additional data. It is clear that the increasing van der Waal's radius of the halogen substituent going from F to Cl to Br in α-monohaloPAA should weaken the inhibitor-polymerase binding interaction if a steric effect is operative, unless the accompanying decrease in inhibitor basicity expected to occur has a countervailing effect. In the series PAA, FPAA, ClPAA and BrPAA a small, continuous increase in IC_{50} was observed for the HSV and EBV polymerases, consistent with an inverse relationship between activity and substituent size, but not correlating in a simple way with substituent electronegativity. A second halogen atom always substantially diminished activity, and CH_3FPAA, CH_3ClPAA and CH_3BrPAA bound poorly regardless of the α-halogen atom present, again demonstrating the importance of steric effects on binding. The existence of non-steric factors affecting inhibitor binding is indicated by the observation that FBrPAA was a better inhibitor of the viral polymerases than FClPAA. An alternate explanation for this result would be that FBrPAA was transformed during the inhibition assay into a more active compound, e.g. FPAA if debromination by DTT were to occur. ^{31}P NMR analysis of FBrPAA incubated in a polymerase assay mixture provided no evidence to support this hypothesis.

One other interesting question concerning structure-activity relationships in these compounds is whether stereoisomerism, when present, is important. PFA and PAA are achiral molecules, but XYPAA derivatives that are α-monosubstituted, or α-disubstituted with unlike substituents, possess a chiral center. Since the inhibition data reported here are for the racemic modifications of such compounds, it is possible that, in a given racemate, one component enantiomer is a better inhibitor than the other, or indeed may be responsible for all the observed activity. Thus, the phosphonate and carboxylate groups of an inhibitor XYPAA where X ≠ Y might interact non-equivalently with a cationic polymerase site, represented as "C" in the accompanying scheme, depending on whether they are oriented as shown or interchanged by a 180° rotation. If the molecule encountered significantly different steric hindrance at regions "A" and "B" in the two orientations, enantiospecific binding could result. Work is in progress on the problem of isolating individual enantiomers of chiral α-substituted phosphonoacetates to determine whether their stereochemistry affects their interaction with DNA polymerases.



The varying effectiveness observed for particular analogs as inhibitors of different viral and mammalian DNA polymerases demonstrates differences in the pyrophosphate binding sites of these enzymes. Mammalian DNA polymerase α and virally coded DNA polymerase are assumed to be the key DNA synthesizing enzymes during host cell replication and viral biosynthesis. They are processive enzymes in which displacement of the pyrophosphate moiety appears to be rate-limiting. DNA polymerase β, on the other hand, shows no sensitivity to inhibitor structure. This enzyme is distributive, and displacement of pyrophosphate may not be a rate-limiting step. Its primary function appears to be strand displacement in the post-replicative repair function, with polymerization of deoxynucleoside triphosphates as its secondary function. DNA polymerase γ is also processive but differs from DNA polymerase α in that it is a mitochondrial genomic replicative enzyme. The origin of its insensitivity to inhibition by PFA, PAA and active XYPAA analogs is unclear. However, the reverse transcriptase-like activity of this enzyme may indicate that the sugar moiety of the substrates, rather than pyrophosphate binding, plays an important role in the polymerization process. Differences between mammalian and viral enzymes such as thymidine kinase and DNA polymerase have been exploited for the development of new antiviral drugs, and a clear understanding of their reaction mechanisms may prove useful in the development of more effective antiviral drugs with less cytotoxic effects.

Acknowledgements - This research was supported by grants from the National Institutes of Health (AI-21871) and the University of Southern California (Faculty Research and Innovation Fund award) to C.E. McKenna and by a grant from the American Cancer Society (CH-29) to Y.-C. Cheng.

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