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INHIBITION OF HUMAN PURINE NUCLEOSIDE PHOSPHORYLASE BY ACYCLIC NUCLEOSIDES AND NUCLEOTIDES

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Abstract—In an effort to develop more potent inhibitors of human purine nucleoside phosphorylase (PNP) as immunosuppressive and cancer chemotherapeutic agents, the affinity of the erythrocytic enzyme for 30 acyclic nucleosides, nucleotides and related compounds was determined. Among the acyclonucleosides, 2′-nordeoxyguanosine [2′NDG, 9-(1,3-dihydroxy-2-propoxymethyl)guanine] had a 3-fold greater affinity than acyclovir, and 8-amino-2′NDG was the best inhibitor with $K_i = 2.6 \times 10^{-7}$ M. The ether moiety of the acyclovir and 2′NDG side-chains was not important for binding. Phosphorylated 2′NDG analogs appeared to act as multisubstrate analogs with optimal binding at low (1 mM) phosphate concentration. The 2′NDG mono- and triphosphates had higher affinities than those reported for the phosphorylated acyclovir derivatives but the diphosphate had a similar K_i value of 9×10^{-9} M. Poor affinity, independent of phosphate concentration, was found for 9-(2-phosphonoethyl)guanine. The 3′-phosphate derivative of 8-(3-hydroxypropyl)-9-methylguanine inhibited with a $K_i = 2 \times 10^{-5}$ M in 1 mM phosphate. The chemical syntheses of new analogs are described.

Appreciation of the importance of purine nucleoside phosphorylase (PNP‖, EC 2.4.2.1) in immunodevelopment and purine nucleoside analog metabolism has inspired detailed structural and kinetic studies and has stimulated efforts to discover PNP inhibitors. Since PNP deficiency results in selective cellular immunodeficiency [1, 2], inhibition of this enzyme has been proposed for the chemotherapy of T-cell leukemias and certain autoimmune diseases, for the suppression of host-versus-graft reaction without destruction of the patient's B-cell response, and in the treatment of metabolic disorders such as secondary or xanthine gout [3–5].

Human erythrocytes, unlike those of certain laboratory animals, have very high PNP activity [6]. Furthermore, partially deficient patients, with as little as 0.5% of normal PNP activity, exhibit diminished symptoms of PNP-related cellular immunodeficiency [7]. Therefore, a clinically useful inhibitor must inactivate the huge reservoir of PNP in human blood as well as in lymphoid and other tissues [8] and should itself be resistant to metabolic activation resulting in non-specific toxicities. These and other considerations have been discussed in several recent reviews [9–11].

Studies of purine base and nucleoside analogs have identified several moderately potent inhibitors of PNP. The C-nucleosides, formycin B (8-aza-9-deaza) and 9-deaza type compounds, are not cleaved by PNP and illustrate that C(5′) modifications can increase the affinity for PNP as well as prevent further metabolism. 5′-Iodoformycin B ($K_i = 7 \times 10^{-6}$ M [12], compared with 1×10^{-4} M for formycin B [13]) and 5′-iodo-9-deazainosine ($K_i = 1.8 \times 10^{-7}$ M) [14] are the best inhibitors from this group. Affinity for PNP is also greatly increased by an amino group at C(8), as in the alternative substrate, 8-aminoguanine ($K_i = 2 \times 10^{-7}$ M, compared with 5×10^{-6} M for guanine [12]). 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamidine resembles 5-amino-4-imidazolecarboxamide ribonucleotide, an intermediate of purine biosynthesis *de novo*, and is a noncleavable inhibitor ($K_i = 5 \times 10^{-6}$ M [15]) in which a complete ring structure in the base moiety is not required for effective binding.

Recently, nucleoside-like analogs with open or "acyclo" structures replacing the sugar moiety were shown to inhibit PNP. The diphosphate derivative of acyclovir is the best PNP inhibitor currently available, with $K_i = 8.7 \times 10^{-9}$ M in the presence of 1 mM phosphate [16]. In contrast, acyclovir itself has relatively weak affinity for PNP ($K_i = 10^{-4}$ M). Therefore, it was postulated that this compound functions as a multisubstrate analog that can interact with both the nucleoside and phosphate binding sites. The structure and positioning of the phosphate group seem crucial, since the diphosphate has a much higher affinity than the mono- or triphosphate. Unfortunately, the diphosphate group also poses problems for metabolic stability and transport across cell membranes which preclude the use of acyclovir diphosphate as a PNP inhibitor *in vivo*. The 9-phosphonoalkyl derivatives of hypoxanthine with 5, 6, and 7 carbons between the base and the phos-

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‖ Abbreviations: 2′NDG, 2′-nordeoxyguanosine or 9-(1,3-dihydroxy-2-propoxymethyl)guanine; carba-acyclovir, 9-(4-hydroxybutyl)guanine; carba-2′NDG, 9-(4-hydroxy-3-hydroxymethylbutyl)guanine; and PNP, purine nucleoside phosphorylase.

phorus atom inhibit PNP with K_i values of 0.9–2×10^{-6} M [17]. They are somewhat better inhibitors than acyclovir monophosphate, showing that neither the ether nor the ester oxygen is required for activity. Since phosphonates are resistant to esterases and less charged than the phosphate esters, they have potential for biological activity.

In the present study, structure–activity correlations were established for a large number of acyclic analogs, both uncharged and phosphorylated, as part of the ongoing efforts to design better PNP inhibitors.

MATERIALS AND METHODS

Materials

Human erythrocytic PNP (sp. act., 1 unit/mg protein) was purified at the New England Enzyme Center, Boston, MA, as described previously [18]. Xanthine oxidase (grade III) and 8-bromoguanosine were purchased from the Sigma Chemical Co., St. Louis, MO. Ultraviolet spectra were taken on a Perkin–Elmer model 552A spectrophotometer, NMR spectra on a Varian XL-200 spectrometer, ^{13}C-NMR spectra on a Varian XL-400 spectrometer, and mass spectra on a Varian MAT-731 instrument using FAB. Thin-layer chromatography was carried out on Analtech Uniplate silica gel GF plates, and silica gel column chromatography utilized E. M. Merck silica gel 60 (70–230 mesh) or Baker 3405 silica gel. Analytical HPLC analyses of phosphorylated acyclonucleosides employed an AX-10 anion exchange column and a linear gradient of 0–100% 1 M monopotassium phosphate in 10 min, flow rate 3 ml/min, as described earlier [19].

Syntheses of the following compounds have been reported elsewhere: 8-aminoacyclovir [20]; 8-azaacyclovir [21]; 9-(4-hydroxybutyl)guanine [22]; 2′NDG [23]; 8-amino-2′NDG and 8-bromo-2′NDG [24]; *S*-iso-2′NDG and *R*-iso-2′NDG [25]; 9-(4-hydroxy-3-hydroxymethylbutyl)guanine [26–28]; *S*-2′NDG mono-, di- and triphosphates and 2′NDG 3′-diphosphate (*R*-2′NDG diphosphate) [29]; and *seco*-guanosine diphosphate [30] with modifications [the reaction solvent was water and product isolation employed DEAE-Sephadex column chromatography (formate form) with gradient elution by 0 to 1.75 M ammonium formate].

8-Thio-2′NDG (**11**) was prepared by nucleophilic displacement of the 8-bromo derivative. The corresponding 8-aza analog (**13**) was prepared by direct alkylation of silylated 8-azaguanine analogous to the preparation [31] of glycosylated 8-azapurines.

The synthesis of 9-(phosphonoethyl)guanine (**22**) was accomplished by a variation of a previously published procedure [17]. Thus, 2-amino-6-benzyloxypurine was used as a pro-guanine derivative [32] and was alkylated regioselectively using diethyl 2-bromoethylphosphonate. An unusual by-product, 2-amino-6-benzyloxy-9-ethylpurine (3% yield), was produced in the reaction, presumably by ethylation with the diethylphosphonate. Deprotection of the 6-*O*-benzylphosphonate ester of **22** resulted in concomitant debenzylation of the protected guanine to afford the desired product. Sites of attachment of alkyl side-chains were determined unequivocally by ^{13}C-NMR studies.

Treatment [33] of 2,5-diamino-6-methylamino-4-benzyloxypyrimidine with butyrolactone gave a 5-acylamino derivative which could be ring-closed [34] to the 8-substituted purine derivative (**23**) in good yield. The corresponding phosphate derivative (**24**) could not be prepared by the conventional phosphorous oxychloride–triethylphosphate method [35], but the procedure of Tener [36] produced **24** efficiently.

9-(1,3-Dihydroxy-2-propoxymethyl)-8-mercaptoguanine (**11**). A mixture of 200 mg (0.63 mmole) of 9-(1,3-dihydroxy-2-propoxymethyl)-8-bromoguanine and thiourea (160 mg; 2.10 mmoles) in methoxyethanol (10 ml) was heated under reflux for 18 hr. The solution was allowed to cool to room temperature, and the precipitated product was filtered and recrystallized from water to give 70 mg (0.24 mmole, 38%) of the title compound. An analytical sample was obtained by recrystallization from H_2O. UV: (pH 7.5), λ_{max} 282 (17,300), sh 300 (16,000); (0.01 M HCl), λ_{max} 281 (17,800), 302 (17,000); (0.01 M NaOH), λ_{max} 292 (18,300); NMR: (DMSO-d_6, δ from TMS 3.55–3.24 (m, $CH_2OH + H_2O$), 3.83 (quintet, 1, $OCH(CH_2OH)_2$, J = 5.0 Hz), 4.46 (t, 2, OH, J = 5.0 Hz), 5.48 (s, NCH_2O), 6.68 (s, NH_2). Anal. Calc. for $C_9H_{13}N_5O_4S \cdot 0.5H_2O$ (296.30): C 36.48, H 4.76, N 23.64, S 10.89; Found: C 36.49, H 4.38, N 23.75, S 10.90.

3-(1,3-Dihydroxy-2-propoxymethyl)-5-amino-β-triazolo[4,5-d]pyrimidin-7-one (**13**). To per-trimethylsilyl-8-azaguanine [31] (1.64 mmoles in 3 ml toluene) was added 1,3-diacetoxy-2-acetoxymethoxypropane [24] (500 mg, 2 mmoles). The toluene was removed by distillation, and the residue was fused at 150° for 1 hr, when starting heterocycle was shown to be gone by thin-layer chromatography ($CHCl_2$/MeOH/H_2O, 80:20:2). The cooled syrup was dissolved in ethyl acetate (3 ml), and 1 ml of 20% aqueous ethanol was added. After stirring for 2 hr, the mixture was evaporated to dryness under reduced pressure, and the residue was acetylated by heating at 80° for 1 hr with acetic anhydride (5 ml). The remaining anhydride was removed by evaporation under reduced pressure, and the residue was dissolved in CH_2Cl_2, filtered to remove insolubles (discarded), and chromatographed with CH_2Cl_2, 2% methanolic and then 3% methanolic CH_2Cl_2 as eluants in turn. The 9-isomer (by UV) was collected (140 mg, 23%). Treatment of this material with 40% aqueous methylamine (1.4 ml) at reflux for 1 hr gave the title compound after evaporation of the solvent under a stream of nitrogen and recrystallization from water (40 mg, 41%), m.p. 196–199°, single spot on TLC *vide supra*. UV: (pH 7), λ_{max} 251 (13,000), sh 267 (7,300), (pH 11), λ_{max} 279 (12,000); NMR: (DMSO, δ from TMS) 3.17–3.51 (4, m, CH_2 + HDO), 2.65 (1, m, CH), 5.73 (2H, s, NCH_2O). Anal. Calc. for $C_8H_{12}O_6N_4 \cdot 0.75H_2O$: C 35.61, H 5.04, N 31.16; Found: C 35.61, H 4.78, N 30.93.

9-(2-Phosphonoethyl)guanine (**22**). 2-Amino-6-benzyloxypurine (2.41 g, 10 mmoles) was dissolved

in sieve-dried dimethyl sulfoxide (DMSO) (50 ml), and $KHCO_3$ (1.52 g, 15.2 mmoles) was added followed by diethyl 2-bromoethylphosphonate (1.92 ml, 10 mmoles). This mixture was stirred at room temperature for 5 days under N_2 and then was heated at 100° for 4 hr. Upon cooling to room temperature, the mixture was diluted to 500 ml with H_2O and extracted with CH_2Cl_2 (3 × 300 ml). The pooled organic phases were dried ($MgSO_4$), filtered, and evaporated to dryness. This residue was dissolved in a minimum volume of CH_2Cl_2 and adsorbed onto silica gel 60 before being placed on a dry-packed silica gel 60 column (3 × 49 cm). The column was developed successively with CH_2Cl_2 (500 ml), CH_2Cl_2-EtOH (49:1, 500 ml), and then CH_2Cl_2-EtOH (97:3) until completion. A by-product, 2-amino-6-benzyloxy-9-ethylpurine, was eluted first, and appropriate fractions were evaporated to a syrup, which upon trituration under Et_2O gave 86 mg (0.32 mmole) of a white powder. NMR: ($CDCl_3$, δ ppm from TMS) 1.47 (3, t, CH_3, J = 7.5 Hz), 4.10 (2, q, $\underline{CH_2}CH_3$, J = 7.5 Hz), 4.84 (2, br s, NH_2), 5.57 (2, s, $\underline{CH_2}O$), 7.26–7.56 (5, aromatics), 7.60 (1, s, H8); CMR: (d_6-DMSO), verifies N^9-isomer. Mass spec.: M^+ at *m/e* 269. Anal. Calc. for $C_{14}H_{15}N_5O \cdot 0.5H_2O$ (278.318): C 60.42, H 5.80, N 25.17; Found: C 60.77, H 5.55, N 24.93. The required product, 2-amino-6-benzyloxy-9-[2-(diethoxyphosphinyl)ethyl]purine, was obtained in later fractions as a syrup, which upon standing gave a semi-solid. Trituration under Et_2O gave 813 mg (2 mmoles) of a pure white powder. NMR: ($CDCl_3$, δ ppm from TMS) 1.26 (6, t, $\underline{CH_3}CH_2$, J = 7.0 Hz), 2.36 (2, d of t's, CH_2P, $^3J_{HH}$ = 7.5 Hz, $^2J_{HP}$ = 18.25 Hz), 4.05 (4, d of q's, $\underline{CH_2}CH_3$, $^3J_{HH}$ = 7.0 Hz, $^3J_{HP}$ = 8.25 Hz), 4.32 (2, d of t's, N-CH_2, $^3J_{HH}$ = 7.5 Hz, $^3J_{HP}$ = 13.5 Hz), 4.88 (2, br s, NH_2), 5.56 (2, s, CH_2O), 7.26–7.54 (5, aromatics), 7.62 (1, s, H8); CMR: (d_6-DMSO), verifies N^9-isomer. Mass spec.: M^+ at *m/e* 405. Anal. Calc. for $C_{18}H_{24}N_5O_4P_1$ (405.40): C 53.33, H 5.97, N 17.28; Found: C 53.24, H 5.96, N 17.44. Overlapping fractions (2.19 g) could be rechromatographed to furnish additional product.

The foregoing product (121.5 mg, 0.30 mmole) was suspended in bromotrimethylsilane (0.5 ml) and stirred at room temperature under N_2 for 3 hr. Dissolution did not occur, and CH_2Cl_2 (2 ml) was added. After an additional 4.5 hr at room temperature, TLC ($MeOH/CH_2Cl_2$, 5:95) showed no starting material remaining, and the reaction mixture was evaporated to dryness under a stream of N_2. This residue was stirred under CH_3CN–H_2O (1:1, 4 ml total) for 30 min at room temperature, and the solid was filtered off and washed with 1 ml of CH_3CN–H_2O (1:1). After drying *in vacuo*, 70 mg (0.26 mmole, 86% yield) was obtained. UV: (pH 7.5, buffer), λ_{max} 252 (10,900), sh 270 (8,100), λ_{min} 224 (3,900); (0.01 M NaOH), λ_{max} 252 (13,300), sh 270 (10,200), λ_{min} 225 (9,100); (0.01 M HCl), λ_{max} 252 (8,800), sh 276 (6,000), λ_{min} 222 (1,800); NMR: (dilute NaOD, δ from TSP) 1.97 (2, m, CH_2-P), 4.20 (2, d of t's, J_{HH} = 5.0 Hz, $^3J_{HP}$ = 12.5 Hz), 7.79 (1, s, H8). Anal. Calc. for $C_7H_{10}N_5O_4P \cdot 0.7H_2O$ (271.77): C 30.93, H 4.22, N 25.77; Found: C 31.23, H 4.08, N 25.38.

8-(3-*Hydroxypropyl*)-9-*methylguanine* (**23**). 2,5-Diamino-6-methylamino-4-benzyloxypyridimine (5.64 g, 23 mmoles, prepared in a manner analogous to 2,4,5-triamino-6-benzyloxypyrimidine [37]) was dissolved in γ-butyrolactone (50 ml, 56 g, 0.96 mole) and heated at 130–140° under house vacuum for 5 hr, and then was held at ambient temperature overnight. The mixture was evaporated to dryness *in vacuo* (bath temp. 60°), and the dark residue was suspended in a minimum volume of CH_2Cl_2 and applied to a Baker 3405 silica gel column; elution was with CH_2Cl_2 and then with a stepwise gradient of MeOH in CH_2Cl_2 adding MeOH in 1% increments up to 5%. Fractions containing chromatographically pure 2-amino-6-methylamino-4-benzyloxy-5(4-hydroxybutyrylamino)pyrimidine were pooled and evaporated to dryness (1.46 g, 4.41 mmoles, 20% yield). Without further purification this intermediate was suspended in 1.5 N NaOH (105 ml) and heated under reflux for 1 hr. The mixture was cooled and the pH adjusted to 4.0 before being evaporated to dryness. The dark residue was triturated with MeOH, and insoluble material was filtered. The filtrate was evaporated to dryness (4.04 g), and the residue was treated with CF_3COOH (50 ml) for 15 min and then was evaporated to dryness, followed by several successive additions and evaporations of water. The residue was purified on a Partisil M20 10/50 ODS-3 preparative HPLC column using CH_3CN–H_2O (4:96) as eluent. Evaporation to dryness of the pure fractions and crystallization of the residue from H_2O gave 267 mg (1.2 mmoles, 27% yield) of product. UV: (pH 7.5 buffer), λ_{max} 251 (13,400), sh 268 (9,900), λ_{min} 224 (4,600); (0.01 M NaOH), λ_{max} 268 (11,100), sh 259 (10,700), λ_{min} 231 (5,300); (0.01 M HCl), λ_{max} 251 (12,400), sh 275 (7,900), λ_{min} 224 (3,600). NMR: (d_6-DMSO, ppm δ from TMS) 1.85 (2, q, $CH_2\underline{CH_2}CH_2$, J = 6.8 Hz), 2.71 (2, t, $\underline{CH_2}$–C8), J = 7 Hz), 3.43–3.59 (5 mm's, $HO\underline{CH_2}$, $\underline{CH_2}$), 4.61 (1, t, HO, J = 5 Hz), 6.40 (2, s, NH_2). Anal. Calc. for $C_9H_{13}N_5O_2 \cdot 0.5H_2O$: C 46.54, H 6.08, N 30.16; Found: C 46.31, H 5.79, N 30.26.

8-(3-*Hydroxypropyl*)-9-*methylguanine* 3′-*monophosphate* (**24**). A suspension of 217 mg (0.67 mmole) of barium cyanoethylphosphate in 50% aqueous pyridine was treated with AG 50WX8 (Py^+) resin to convert the barium salt to the pyridinium salt, and then 8-(3-hydroxypropyl)-9-methylguanine (50 mg, 0.22 mmole) was added. This mixture was dried by repeated addition and evaporation of dry pyridine *in vacuo*, finally concentrating to about 1 ml. Dicyclohexylcarbodiimide (DCC, 460 mg, 2.23 mmoles) was then added, and the mixture was stirred at ambient temperature under N_2. After 16 hr, more DCC (300 mg, 1.46 mmoles) was added, followed by 1 ml of dry *N,N*-dimethylformamide (DMF). After an additional 24 hr, TLC [CH_2Cl_2/MeOH/H_2O (90:10:1)] indicated almost complete reaction, and H_2O (10 ml) was added. The dicyclohexylurea was filtered, the filtrate was evaporated to dryness, and the residue was partitioned between H_2O (12 ml) and $CHCl_3$ (20 ml). The aqueous layer was further extracted (3× with $CHCl_3$), and then 2 N LiOH was added until the solution became 9.5 N with respect to LiOH. The mixture was heated at 100° for 30 min, cooled, and the pH was adjusted to 7 with 2 N HOAc. This solution was applied to a

column of AG 1 × 8 (HCO_3^-, 100 ml) and eluted first with H_2O (300 ml) and then with a linear gradient of H_2O (500 ml) to 6 N HCO_2H (500 ml). Fractions containing the desired product (by UV) were pooled and evaporated to dryness to give 27 mg of a pale yellow solid (0.08 mmole as dihydrate; 36% yield). NMR: (as $diNH_4^+$ salt, D_2O, δ from TSP) 2.07 (2, q, $CH_2\underline{CH_2}CH_2$, J = 7 Hz), 2.92 (2, t, C8-$\underline{CH_2}CH_2CH_2$, J = 7 Hz), 3.58 (3, s, CH_3), 3.94 (2, q, $CH_2CH_2\underline{CH_2}OP$, J_{HH} = 7 Hz, J_{HP} = 6 Hz). Anal. Calc. for $C_9H_{14}N_5PO_5 \cdot 2H_2O$ (339.24): C 31.86, H 5.35, N 20.65; Found: C 31.97, H 5.17, N 20.61.

Methods

Compounds were dissolved in water or *N,N*-dimethylformamide and water, and tested as inhibitors of PNP by observing their effects on inosine phosphorolysis. Reactions at 30° were monitored spectrophotometrically by the coupled xanthine oxidase assay [11, 38].

Nonphosphorylated compounds were tested in 50 mM potassium phosphate (pH 7.4), and reactions were started by addition of enzyme. Apparent K_i values were estimated by linear regression analysis from Dixon plots [39] of 1/reaction velocity versus inhibitor concentration. K_i values were calculated using the K_m value for inosine determined on the same day, from the equation $K_{i(app)} = K_{is}(1 + [S]/K_m)$. The enzyme was diluted with 50 mM phosphate buffer and preincubated with 1 mM dithiothreitol before use. The average K_m value for inosine was 30×10^{-6} M.

The phosphorylated compounds were preincubated with enzyme in 1.0 mM potassium phosphate supplemented with 100 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (pH 7.4). Reactions were started by addition of inosine. The enzyme was diluted with the above buffer and preincubated with 1 mM dithiothreitol prior to use. Apparent K_i values were determined with a weighted regression analysis program adapted by S. Cha from Cleland [40] to a Wang computer and extended to compute both K_m and K_i values from plots of $1/v$ versus $1/S$ at multiple inhibitor concentrations. Inosine concentrations were varied from 0.010 to 0.100 mM.

Because concentrations of DMF as high as 5% were present in some reaction mixtures, its effect on enzymatic activity was determined. Each 1% increment in solvent concentration caused a 4% decrease in reaction velocity and proportional corrections were made in the data.

RESULTS

The structures of the 8- and 9-substituted guanine analogs that were synthesized and evaluated here are presented in Fig. 1, in comparison with the acyclovir derivatives reported by Tuttle and Krenitsky [16]. The analogs with inhibition constants of approximately 100 μM or less are listed in Table 1. They fall into three general categories on the basis of their acyclic substituents. The first group includes acyclovir derivatives (**3–5**) which may be compared to the previously studied acyclovir (**1**) and 8-hydroxyacyclovir (**2**) [16]. 8-Hydroxy- and 8-amino-acyclovir were much better inhibitors than acyclovir itself, but much less effective than the phosphorylated acyclovirs (**6–8**) described by Tuttle and Krenitsky. 8-Azaacyclovir offered 3-fold improvement over the parent compound. 9-(4-Hydroxybutyl)guanine, or carba-acyclovir (**5**), has the same chain length as acyclovir but lacks the ether oxygen and showed a 2-fold greater affinity.

The second group of analogs (**9–21**) includes 2'-nordeoxyguanosine (2'NDG) and its derivatives, which differ from the acyclovir class of compounds by virtue of an additional hydroxymethyl branch in the acyclic substituent. They were generally better inhibitors of PNP than the acyclovirs, or at least comparably effective. Within this group, 8-amino-2'NDG was the best uncharged inhibitor tested ($K_i = 2.6 \times 10^{-7}$ M represents a 100-fold improvement over the parent compound, 2'NDG), whereas other C(8) alterations (thio- and bromo-substituents or 8-aza modification of **11–13**) gave K_i values comparable to that of 2'NDG itself. The ribonucleoside, 8-bromoguanosine (not shown in Fig. 1), caused only 23% inhibition of 30 μM inosine phosphorolysis when present at a 200 μM concentration. Compounds **14** and **15**, straight-chain chiral isomers of 2'NDG in which the hydroxymethyl group of the side-chain is one carbon further removed from the purine base, displayed poorer affinities than 2'NDG, and were comparable to acyclovir. Compound **16** (carba-2'NDG) had an affinity similar to that of 2'NGD. No substrate activity was detected for 2'NDG, although it resembles 2'-deoxyguanosine more closely than does acyclovir.

Phosphorylation improved the affinity of 2'NDG dramatically: 14-fold in the monophosphate, 166-fold in the triphosphate, and 1500- to 3000-fold in the diphosphates. The K_i value of the diphosphate (**18**) was close to that of acyclovir diphosphate, whereas those of the mono- and triphosphates were 2- to 3-fold lower than those of the corresponding phosphorylated acyclovirs. However, the presence of an additional hydroxymethyl group at C(1') in *seco*-guanosine diphosphate (**20**) made it a much poorer inhibitor than the *S*- and *R*-2'NDG diphosphates (**18** and **19** respectively). 2'NDG 3'-diphosphate (*R*-2'NDG diphosphate, **19**) had slightly lower affinity than its chiral isomer (**18**).

The last three compounds in Table 1 differ most from the acyclovir and 2'NDG prototypes. The affinity of 8-(3-hydroxypropyl)-9-methylguanine (**23**) for PNP was comparable to that of acyclovir. Its phosphorylated derivative (**24**) is unique in that the phosphate group extends from C(8) rather than from the normal glycosidic position. This analog had a K_i value of 20 μM in the presence of 1 mM phosphate but it afforded only 15% inhibition when tested against 30 μM inosine in the presence of 50 mM phosphate. Finally, 9-(2-phosphonoethyl)guanine (**22**) was a very weak ligand for PNP and at 500 μM concentration caused only 30% inhibition of 30 μM inosine phosphorolysis. Phosphate concentration had no significant effect on its potency.

In addition, several acyclic adenosines not shown in Fig. 1 were tested as inhibitors of PNP. Acycloadenine, 2'-nordeoxyadenosine, and 9-(2,3-dihydroxypropyl)adenine gave less than 5% inhibition when assayed at 100 μM concentration with

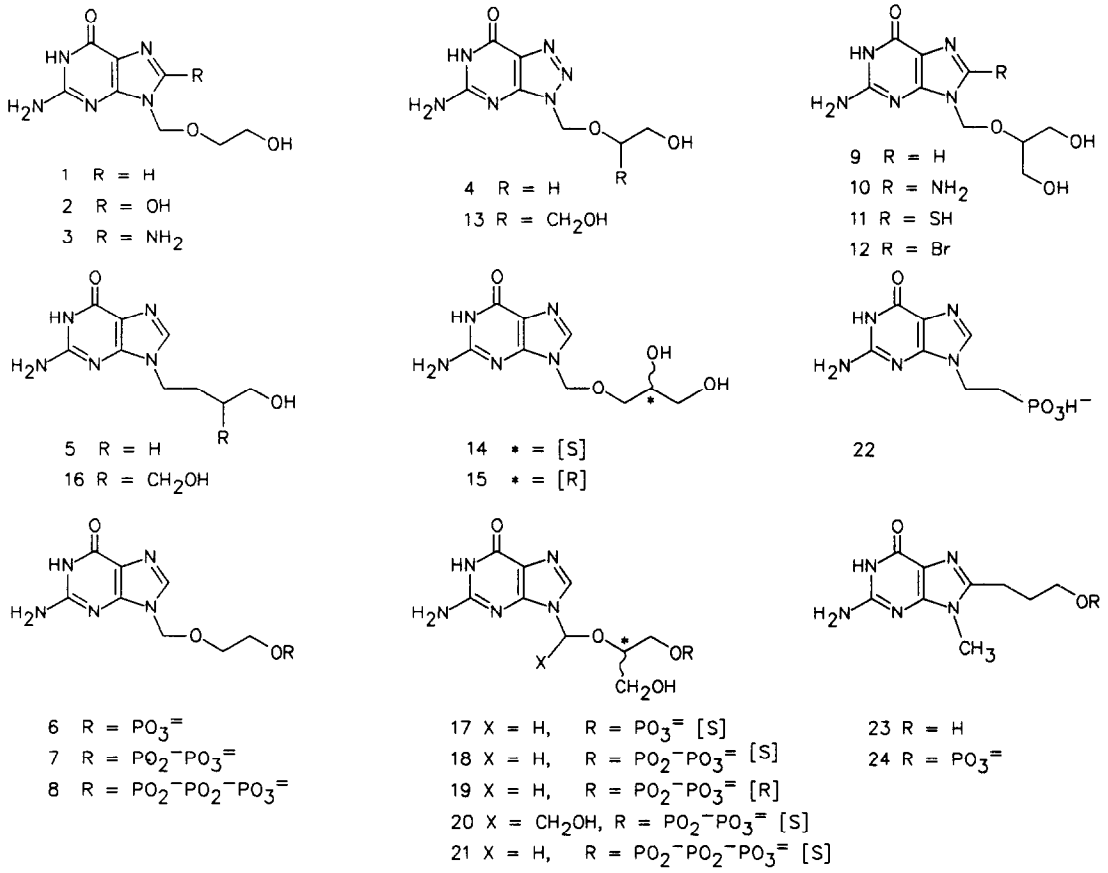

Fig. 1. Structures of the acyclonucleosides and acyclonucleotides presented in Table 1.

30 μM inosine and 50 mM phosphate. These results reflect the low affinities of adenine and adenosine with the enzyme [41]. In contrast, weak but significant inhibition occurred with 6-amino-2′NDG and 6-hydrazino-2′NDG, which appear to benefit from the 2-amino functionality on purine and gave 18 and 14% inhibition, respectively, under the same conditions.

DISCUSSION

The results presented in Table 1 offer several important clues for understanding the interaction of acyclic nucleosides and nucleotides with the pentose binding site of PNP. These relate to the composition and length of the C(9) substituent as well as the effects of substitutions on the tail or on the purine base.

The ether oxygen appears to be unnecessary for inhibition, as was found with the 9-(phosphonoalkyl)hypoxanthines [17], since carba-acyclovir (**5**) and carba-2′NDG (**16**) were not markedly different from their parent compounds. It is interesting that the affinity of acyclovir was actually improved 2-fold, whereas that of 2′NDG was not affected appreciably.

2′-Nordeoxyguanosine and its derivatives were generally more effective PNP inhibitors than analogous acyclovir compounds, with the exception of the diphosphates and 8-aza-2′NDG, in which the beneficial effect of the extra hydroxymethyl group was lost or cancelled. The location of the additional hydroxymethyl group on the side-chain was important. In the linear isomers of 2′NDG (**14** and **15**), the extra group is inserted within the chain, and there was no significant improvement in affinity with respect to acyclovir. In 2′NDG, the location of this group in the acyclic side-chains may be comparable to that of the C(3′)-OH of guanosine. Therefore, their C(9) substituents bear greater resemblance to the pentose moiety of the natural nucleoside substrates of PNP. This specificity at the 3′ position has been demonstrated previously with analogs in which a 3′-deoxy or 3′-*xylo* modification caused a great loss in affinity as well as reactivity [42].

Phosphorylation of either acyclovir or 2′NDG markedly increased affinity for the enzyme, and this may be due to occupation of the specific binding site of the enzyme for the phosphate substrate as suggested by Tuttle and Krenitsky [16]. Two lines of evidence support this proposal. First, the affinities of the phosphorylated compounds were strongly dependent on phosphate concentration. Second, preliminary X-ray analysis of the PNP-acyclovir diphosphate complex crystallized from ammonium sulfate suggests that the distal phosphate displaced sulfate from the putative phosphate binding site of the enzyme in this complex.*

* S. E. Ealick, personal communication, cited with permission.

Table 1. Inhibition constants of some acyclic nucleosides and nucleotides with human PNP

Compound	K_i (μM)
1. Acyclovir	91*
2. 8-Hydroxyacyclovir	4.6*
3. 8-Aminoacyclovir	3.8
4. 8-Azaacyclovir	28
5. 9-(4-Hydroxybutyl)guanine (carba-acyclovir)	42
6. Acyclovir monophosphate	6.6*
7. Acyclovir diphosphate	0.0087*
8. Acyclovir triphosphate	0.3*
9. 2'Nordeoxyguanosine (2'NDG)	30
10. 8-Amino-2'NDG	0.26
11. 8-Thio-2'NDG	13
12. 8-Bromo-2'NDG	34
13. 8-Aza-2'NDG	32
14. *S*-iso-2'NDG	110
15. *R*-iso-2'NDG	80
16. 9-(4-Hydroxy-3-hydroxymethylbutyl)guanine (carba-2'NDG)	38
17. 2'NDG monophosphate (*S*-2'NDG monophosphate)	2.2†
18. 2'NDG diphosphate (*S*-2'NDG diphosphate)	0.009†
19. 2'NDG 3'-diphosphate (*R*-2'NDG diphosphate)	0.018†
20. *Seco*-guanosine diphosphate	14†
21. 2'NDG triphosphate (*S*-2'NDG triphosphate)	0.16†
22. 9-(2-Phosphonoethyl)guanine	>500
23. 8-(3-Hydroxypropyl)-9-methylguanine	92
24. 8-(3-Hydroxypropyl)-9-methylguanine 3'-monophosphate	20†

Values were determined from Dixon plots obtained at 50 mM phosphate, except where noted, and represent the average of two experiments.

* Published values; the phosphorylated compounds were tested at 1 mM phosphate concentration [16].

† Values were determined from replots of double-reciprocal plots obtained at 1.0 mM phosphate concentration.

The diphosphate derivatives of 2'NDG had higher affinities than the triphosphate derivative which, in turn, was superior to the monophosphate. These results are similar to those reported for the phosphorylated derivatives of acyclovir. The phosphorylated analogs of 2'NDG, unlike those of acyclovir, are optically active, and **18** and **19** are enantiomers. If **18** assumes a conformation in which the phosphates extend from a position analogous to C(3') of ribose, and the heterocyclic base assumes a "normal" nucleosidic position, then it resembles α-L-lyxosylguanine 3'-diphosphate, whereas **19** resembles guanosine 3'-diphosphate. Apparently the diphosphate substituent can be placed in either position without greatly changing the affinity. This was not unexpected in view of the enzyme's tolerance of significant changes at the C(5') position of nucleosides [12, 42]. Addition of a second hydroxymethyl group at the position analogous to C(2') of ribose, as in **20**, resulted in a radical increase in K_i. This may be due to steric hindrance which prevents the diphosphate moiety from reaching the most favorable binding site. *Seco*-guanosine diphosphate had slightly better affinity for PNP than the more rigid natural nucleoside 5'-diphosphate, dGDP, which was reported to have a K_i value of 3.7×10^{-5} M [16].

The binding of 8-(3-hydroxypropyl)-9-methylguanine 3'-monophosphate (**24**) to the enzyme was also dependent on phosphate concentration, suggesting that it acts as a multisubstrate analog. If that is true, the lower affinity of this compound (K_i = 20 μM), in comparison with guanine (K_i = 5 μM), may reflect considerable strain or steric hindrance. In any case, the phosphate group enhances binding of this analog, since its affinity was 5-fold greater than that of **23**, the nonphosphorylated parent compound.

It is significant that 9-(2-phosphonoethyl)guanine (**22**) was a very poor inhibitor in which the phosphonate group did not appear to compete for the phosphate binding site. This observation is consistent with the low degree of inhibition shown by another short-chain alkylphosphonate, 9-(3-phosphonopropyl)hypoxanthine which, however, is blocked at high phosphate concentration [17]. The low affinity of **22** was not attributable to the fact that it is a phosphonate rather than a phosphate, since the longer 9-(phosphonoalkyl)hypoxanthines with 5 or 7 carbon atom spacers were better inhibitors than acyclovir monophosphate.

Of all compounds reported, **22** most closely mimicked the postulated transition state for the PNP reaction in which nucleophilic displacement of the

heterocycle by phosphate occurs at C(1′) of the nucleoside. Possible explanations for this lack of inhibition by **22** include: (a) the transition state requires a particular orientation about the C(1′) atom which is not possible or not preferred in **22**; (b) for tight binding to occur, an oxygen atom of the phosphate or phosphonate analog may have to simulate the nucleophilic oxygen in the displacement reaction, a condition that may not be possible in this short-chain analog; or (c) that the nucleoside and phosphate binding sites are not closely juxtaposed until a conformational change occurs that requires prior occupancy of both sites. Although explanation (c) may be supported by our earlier kinetic studies which show that the phosphate and nucleoside induce additive negative cooperativity effects [9], it does not explain the poor inhibition of the 9-(phosphonoalkyl)hypoxanthine with the C_3 spacer.

With respect to base modifications, previous results indicated that an amino substituent at C(8) of bases or nucleosides markedly increases affinity for the enzyme [12]. This enhancement of binding also held for the acyclonucleosides. 8-Aminoacyclovir is a much better PNP inhibitor than the parent compound. The K_i value for 8-amino-2′NDG is also an order of magnitude lower than that of its parent compound and is, therefore, comparable to that of 8-aminoguanine.

Generally, other C(8) modifications of guanine or hypoxanthine examined previously did not increase affinity. However, in the current study an 8-thio group slightly improved the binding of 2′NDG, whereas it decreased the affinity of guanine by about 10-fold [12]. An analogous trend was found with the 8-hydroxy substituent in acyclovir by Tuttle and Krenitsky [16]. 8-Hydroxyacyclovir had a K_i value approximately 20-fold lower than acyclovir, whereas 8-hydroxyguanine had a K_i value four times higher than guanine, suggesting a difference in binding of the base to the enzyme when it is coupled to an incomplete sugar moiety. Thus, the affinities of acyclonucleosides can be enhanced by substituents that hinder the optimal binding of the natural substrates. The effects of 8-thio and hydroxy substituents on analogs with a complete pentose ring need to be explored.

The greater flexibility of acyclonucleosides is further demonstrated by a comparison of 8-bromo-2′NDG with 8-bromoguanosine. The bromine did not alter the affinity of 2′NDG but dramatically reduced the binding of guanosine. This bulky substituent restricts free rotation about the glycosidic bond in the ribonucleoside, but it apparently does not hinder those movements of the acyclic substituent that are needed for optimal binding.

In conclusion, acyclonucleosides and -tides inhibited PNP as effectively, or more so, than analogs that incorporate a complete pentose ring. With regard to certain features, such as the beneficial effect of the extra hydroxymethyl group in 2′NDG or the influence of an 8-amino base substituent, their behavior was consistent with that of nucleoside analogs. In other respects, e.g. the lack of a requirement for the ether oxygen (which is essential in nucleosides [42]) or the effects of 8-thio and 8-hydroxy base substituents, acyclonucleosides are unusual and offer exciting opportunities for the development of biologically useful PNP inhibitors.

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