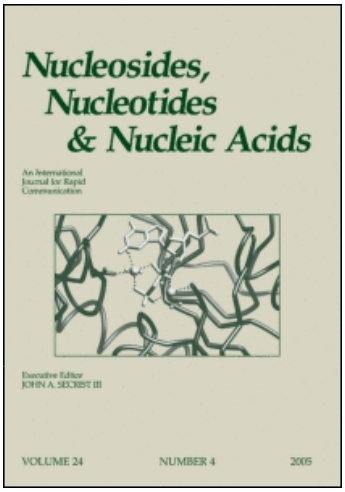

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Interactions of Calf Spleen Purine Nucleoside Phosphorylase with Antiviral Acyclic Nucleoside Phosphonate Inhibitors: Kinetics and Emission Studies

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INTERACTIONS OF CALF SPLEEN PURINE NUCLEOSIDE PHOSPHORYLASE WITH ANTIVIRAL ACYCLIC NUCLEOSIDE PHOSPHONATE INHIBITORS: KINETICS AND EMISSION STUDIES

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ABSTRACT: Association between calf spleen purine nucleoside phosphorylase and a series of phosphonylalkoxyalkyl derivatives of purine bases was studied by inhibition kinetics and fluorimetric titrations. Dissociation constants, determined by fluorimetric titration in phosphate-free conditions, were lower than inhibition constants in 1 mM phosphate, and inhibition was still weaker in 50 mM phosphate, in accord with the postulated bisubstrate analogue character of this class of inhibitors.

Purine nucleoside phosphorylase (PNP, E.C. 2.4.2.1) is of interest as a drug target in some immunological diseases and in the intracellular degradation of some antitumor and antiviral drugs[1]. We have demonstrated previously[2] that one new class of PNP inhibitors, specific for mammalian, but not *E. coli*, PNP[2], comprises phosphonylalkoxyalkyl analogues of purine nucleotides[3] which are broad-spectrum antiviral agents.

Steady-state kinetic studies were conducted on inhibition of human, calf spleen and E. coli PNPs by a series of 9-(2-phosphonylmethoxy)ethyl- (PME), 9-(2-phosphonyl-methoxy)propyl- (PMP), and 9-(3-hydroxy-2-phosphonylmethoxy)propyl- (HPMP) derivatives of adenine (A), guanine (G), 2,6-diaminopurine (DAP), hypoxanthine (Hx) and their base-modified analogues. Kinetics of inhibition of calf PNP in 1 mM phosphate are consistent with simple competition *vs.* nucleoside substrate, with K_i^{app} in the micromolar range[2] (see also Table 1). In 50 mM phosphate (the second substrate of PNP) inhibitory activity is much weaker[2], as expected for bisubstrate analogue inhibitors.

Fluorimetric titration of calf spleen PNP by selected good inhibitors in phosphate-free Hepes buffer, with typical protein concentrations of 1-5 μM per monomer, and ligand

TABLE 1. Dissociation constants (K_d) for complexes of purine alkoxyalkylphosphonate inhibitors and calf spleen PNP in 20 mM HEPES, pH 7.5, at 20 °C, and binding site concentrations ([B]), relative to enzyme subunits ([E]), as determined by fluorimetric titration. Apparent inhibition constants (K_i^{app}), determined in 1 mM phosphate (reported previously[2]), are included for comparison.

Phosphonylakoxy-alkyl moiety	Base	K_d [μM]	[B]/[E]	K_i^{app} [μM]
PME	8-azaG	0.6±0.2	0.35±0.08	~10
(S)-PMP	DAP	<0.2	0.4±0.1	~6
(S)-HPMP	DAP	<0.2	~0.45	24
cyclic (S)-HPMP	G	3.1±0.5	~0.3	~7

concentrations up to ~30 μM, shown that some fluorescent inhibitors (e.g. with DAP or 8-azaguanine as hetherocyclic moieties) exhibited significant emission enhancement upon binding, allowing determination of dissociation constants. Fluorimetric titration data were fitted nonlinearly to binding isotherms, assuming a single binding site. The results (Table 1) show that (a) all dissociation constants, measured in phosphate-free medium, are lower than the apparent K_i in 1 mM phosphate, and (b) the apparent concentration of binding sites is approximately one-third of the measured enzyme subunit concentration, suggesting association of one inhibitor molecule per enzyme trimer. Similar stoichiometry of binding has been demonstrated, in the case of mammalian PNP, for inosine hydrolysis in phosphate-free medium, and for two tight-binding transition-state analogue inhibitors[4].

Fluorescence difference spectra obtained during titrations reveal maxima close to those of unbound ligands. An exception is cyclic (S)-HPMPG, which, in the presence of PNP, shows an increase in fluorescence at 370-390 nm, resembling that of guanosine anion emission, suggesting that some ligands may be bound to the protein as anionic species.

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