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Phosphoramidate Pronucleotides: A Comparison of the Phosphoramidase Substrate Specificity of Human and *Escherichia coli* Histidine Triad Nucleotide Binding Proteins

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Abstract: To facilitate the delivery of nucleotide-based therapeutics to cells and tissues, a variety of pronucleotide approaches have been developed. Our laboratory and others have demonstrated that nucleoside phosphoramidates can be activated intracellularly to the corresponding 5′-monophosphate nucleotide and that histidine triad nucleotide binding proteins (Hints) are potentially responsible for their bioactivation. Hints are conserved and ubiquitous enzymes that hydrolyze phosphoramidate bonds between nucleoside 5′-monophosphate and an amine leaving group. On the basis of the ability of nucleosides to quench the fluorescence of covalently linked amines containing indole, a sensitive, continuous fluorescence-based assay was developed. A series of substrates linking the naturally fluorogenic indole derivatives to nucleoside 5′-monophosphates were synthesized, and their steady state kinetic parameters of hydrolysis by human Hint1 and *Escherichia coli* hinT were evaluated. To characterize the elemental and stereochemical effect on the reaction, two P-diastereoisomers of adenosine or guanosine phosphoramidothioates were synthesized and studied to reveal a 15−200-fold decrease in the specificity constant (k_{cat}/K_m) when the phosphoryl oxygen is replaced with sulfur. While a stereochemical preference was not observed for *E. coli* hinT, hHint1 exhibited a 300-fold preference for D-tryptophan phosphoramidates over L-isomers. The most efficient substrates evaluated to date are those that contain the less sterically hindering amine leaving group, tryptamine, with k_{cat} and K_m values comparable to those found for adenosine kinase. The apparent second-order rate constants (k_{cat}/K_m) for adenosine tryptamine phosphoramidate monoester were found to be 10^7 M^{-1} s^{-1} for hHint1 and 10^6 M^{-1} s^{-1} for *E. coli* hinT. Both the human and *E. coli* enzymes preferred purine over pyrimidine analogues. Consistent with observed hydrogen bonding between the 2′-OH group of adenosine monophosphate and the active site residue, Asp43, the second-order rate constant (k_{cat}/K_m) for thymidine tryptamine phosphoramidate was found to be 3−4 orders of magnitude smaller than that for uridine tryptamine phosphoramidate for hHint1 and 2 orders of magnitude smaller than that for *E. coli* hinT. Ara-A tryptamine phosphoramidate was, however, shown to be a good substrate with a specificity constant (k_{cat}/K_m) only 10-fold lower than the value for adenosine tryptamine phosphoramidate. Consequently, nucleoside phosphoramidates containing unhindered primary amines and either an α or β 2′-OH group should be easily bioactivated by Hints with efficiencies rivaling those for the 5′-monophosphorylation of nucleosides by nucleoside kinases. The differential substrate specificity observed for human and *E. coli* enzymes represents a potential therapeutic rationale for the development of selective antibiotic phosphoramidate pronucleotides.

Keywords: Histidine triad nucleotide binding proteins (Hints); phosphoramidase; human Hint1; *Escherichia coli* hinT; fluorogenic phosphoramidates

Introduction

Nucleosides are an important class of therapeutics. In particular, nucleosides have been clinically used for decades as both antiviral and anticancer drugs. Unfortunately, because they typically must be converted by intracellular kinases to the corresponding 5′-mono-, di-, and triphosphate, the utility of a given nucleoside will depend on the substrate specificity of these intracellular enzymes. Since in general the substrate requirements for nucleoside kinases are more demanding than for either nucleotidylate or diphosphophosphate kinases, a method of delivering nucleoside 5′-monophosphates would expand the number of potentially therapeutically useful nucleosides. To address this issue, a number of imaginative pronucleotide approaches have been developed.[1] In general,

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these methods rely on either remote chemical or enzymatic activation of the masking moiety, followed by collapse of an unstable intermediate and release of the nucleotide. Early efforts to design phosphate ester pronucleotides were limited in their success. Our laboratory and others have sought to utilize phosphoramidate esters as possible pronucleotides.[2−8] Recently, direct intracellular P−N bond cleavage has been observed by ESI-MS for AZT L-tryptophan phosphoramidate monoesters.[9] Moreover, we have demonstrated that human and bacterial variants of a subfamily of the histidine triad (HIT) protein superfamily are nucleoside phosphoramidases.[10]

HIT enzymes are a ubiquitous superfamily consisting primarily of nucleoside phosphoramidases, dinucleotide hydrolyases, and nucleotidylyl transferases.[11] The distinguishing feature of these enzymes is an active site motif composed of the sequence His-X-His-X-His-XX, where X is a hydrophobic residue. Three primary HIT families have been identified. The first and most ancient is the family of the histidine triad nucleotide binding protein (Hint). Hint homologues isolated from rabbits,[12] humans,[10] chickens,[13] yeast,[12] and *Escherichia coli*[10] have been shown to be purine nucleoside phosphoramidases. Mammalian Hint1 has been suggested to have tumor suppressor activity in a *hint1*-knockout mice model[14,15] and human non-small cell lung carcinoma cells.[16] Related to this observation, the expression of hHint1 has been shown to be a potential regulator of apoptosis.[17] Nevertheless, the cellular function and biochemical relevance of the phosphoramidase activity have remained a mystery. Two less typical members of the Hint family are Aprataxin, which is mutated in ataxia-oculomotor apraxia1,[18,19] and the scavenger mRNA decapping enzyme, DcpS/DCS-1, a 7-methyl GpppG hydrolyase.[20,21] The second family is that of the fragile histidine triad (Fhit), which exhibits characteristic diadenosine polyphosphate hydrolase activity

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and was reported to possess tumor suppressor activity.[22] The third family contains relatives of galactose-1-phosphate uridylyltransferase (GalT), which is a specific nucleoside monophosphate transferase.[23]

The reaction mechanism of the Hint protein family has been proposed to be a two-step double replacement, in which the complex of a phosphoramidate with His112 of hHint1 or His101 of *E. coli* hinT forms a covalent Hint−NMP intermediate (N is A or G) followed by dissociation of the amine. In the second step, the Hint−NMP intermediate undergoes hydrolysis to release NMP and Hint. The mechanism is inspired by the similarities of Hint proteins to Fhit[24] and GalT.[25] Recently, human Fhit has been shown to possess phosphoramidase activity with adenosine 5′-phosphoimidazolide (AMP-Im) and adenosine 5′-phospho-*N*-methylimidazolide (AMP-N-MeIm) as substrates with similar catalytic efficiencies in comparison with its best substrate P^1-5′-*O*-adenosine-P^3-5′-*O*-adenosine triphosphate (Ap_3A).[24,26] The only reported spectroscopic substrate of Hint, adenosine 5′-*O*-*p*-nitrophenylphosphoramidate (AMP-pNA), has relatively poor Michaelis−Menten parameters ($k_{cat} = 0.00187\ s^{-1}$, $K_m = 134$, and $k_{cat}/K_m = 14\ s^{-1}\ M^{-1}$).[27] Other reported methods for detecting Hint-catalyzed reaction are either a complicated coupled assay that can detect only formation of AMP[24] or a discontinuous and labor-intensive HPLC or TLC assay.[12,27]

In this study, we report the development of a continuous fluorescence-based assay for Hint phosphoramidase activity, which we then used to map the substrate specificity for the human and bacterial enzymes. On the basis of these results, we propose a set of parameters for the design of Hint activatible phosphoramidate pronucleotides.

Experimental Procedures

General Synthetic Procedure for Fluorogenic Nucleoside 5′-Phosphoramidates. Nucleoside 5′-phosphoramidates were prepared in a single-step carbodiimide-mediated coupling reaction as described previously[10] with minor modification.

1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (4.5 equiv) was added to a solution of nucleoside 5′-monophosphate (1 equiv) and indole compound (5 equiv) in H_2O (pH ∼7.0, adjusted with sodium hydroxide) at room temperature for 6 h and concentrated under reduced pressure. The resulting solid was then purified by flash chromatography (SiO_2, 5:3:0.5 $CHCl_3$/MeOH/H_2O mixture containing 0.5% NH_4OH) to give the desired products. For some tryptamine analogues, the preparative HPLC purification was carried out using an Alltech Econosphere C8 column (10 mm × 250 mm) with a linear gradient elution of solvent A (HPLC-grade water) and solvent B (HPLC-grade methanol) at a flow rate of 3 mL/min. The gradient is as follows: 0% B for 2 min, from 0 to 5% B from 2 to 12 min, and from 5 to 100% B from 12 to 16 min. The elution peak at 6−7 min was collected to give the white solid.

2(*S*)-[Adenosyl-5′-(phosphorylamino)]-3-(3-indolyl)proprionic Acid Methyl Ester (1). The yield is 62.7% (0.96 g) as a white solid: ^{1}H NMR (D_2O, 300 MHz) δ 8.03 (1H, s), 7.87 (1H, s), 7.07 (2H, m), 6.77 (2H, m), 6.57 (1H, t), 5.73 (1H, d), 4.49 (1H, t), 4.18 (1H, m), 4.05 (1H, m), 3.68 (3H, m), 3.4 (3H, s), 2.74 (2H, m); ^{31}P NMR (D_2O, 300 MHz) δ 7.33; HRMS (ESI) *m*/*z* calcd for $[M - H]^-$ 546.1508, found 546.1503.

[2-(3-Indolyl)-1(*S*)-(methylcarbamoyl)ethyl]phosphoramidic Acid 5′-Adenosyl Ester (2). The yield is 71.9% (0.31 g) as a white solid: ^{1}H NMR (D_2O, 300 MHz) δ 8.07 (1H, s), 7.94 (1H, s), 7.2 (2H, d), 7.12 (2H, d), 6.91 (1H, s), 6.85 (1H, t), 6.65 (1H, t), 5.77 (1H, d), 4.45 (1H, t), 4.13 (1H, t), 4.03 (1H, m), 3.62 (3H, m), 2.49 (3H, s), 1.77 (2H, m); ^{31}P NMR (D_2O, 300 MHz) δ 7.10; HRMS (ESI) *m*/*z* calcd for $[M + H]^+$ 547.1824, found 547.1851.

[2-(3-Indolyl)-1(*S*)-carbamoylethyl]phosphoramidic Acid 5′-Adenosyl Ester (3). The yield is 92% (0.045 g) as a white solid: ^{1}H NMR (D_2O, 300 MHz) δ 7.93 (1H, s), 7.79 (1H, s), 7.16 (2H, d), 6.99 (2H, d), 6.86 (1H, s), 6.71 (1H, t), 6.55 (1H, t), 5.65 (1H, d), 4.33 (1H, t), 4.03 (1H, t), 3.96 (1H, m), 3.56 (3H, m), 2.78 (2H, m); ^{31}P NMR (D_2O, 300 MHz) δ 7.01; HRMS (ESI) *m*/*z* calcd for $[M - H]^-$ 533.1668, found 533.1668.

2(*S*)-[Guanosyl-5′-(phosphorylamino)]-3-(3-indolyl)-proprionic Acid Methyl Ester (6). The yield is 72% (0.66 g) as a white solid: ^{1}H NMR (D_2O, 300 MHz) δ 7.74 (1H, s), 7.19 (2H, m), 6.91 (2H, m), 6.76 (1H, t), 5.58 (1H, d), 4.49 (1H, m), 4.21 (1H, m), 4.04 (1H, m), 3.72 (3H, m), 3.41 (3H, s), 2.82 (2H, m); ^{31}P NMR (D_2O, 300 MHz) δ 7.38; HRMS (ESI) *m*/*z* calcd for $[M - H]^-$ 562.1451, found 562.1468.

[2-(3-Indolyl)-1(*S*)-(methylcarbamoyl)ethyl]phosphoramidic Acid 5′-Guanosyl Ester (7). The yield is 64.2% (0.29 g) as a white solid: ^{1}H NMR (D_2O, 300 MHz) δ 7.72

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[2-(3-Indolyl)-1(*S*)-carbamoylethyl]phosphoramidic Acid 5′-Guanosyl Ester (8). The yield is 38.3% (0.21 g) as a white solid: ^{1}H NMR (D_2O, 300 MHz) δ 7.72 (1H, s), 7.28 (1H, d), 7.15 (1H, d), 6.90 (2H, m), 6.73 (1H, t), 5.56 (1H, d), 4.44 (1H, m), 4.11 (1H, m), 3.96 (1H, m), 3.60 (3H, m), 2.85 (2H, m); ^{31}P NMR (D_2O, 300 MHz) δ 7.10; HRMS (ESI) *m*/*z* calcd for $[M - H]^-$ 547.146, found 547.144.

2(*R*)-[Adenosyl-5′-(phosphorylamino)]-3-(3-indolyl)-proprionic Acid Methyl Ester (11). The yield is 40.1% (0.31 g) as a white solid: ^{1}H NMR (D_2O, 300 MHz) δ 8.16 (1H, s), 7.86 (1H, s), 7.17 (2H, m), 6.93 (1H, t), 6.83 (1H, s), 6.78 (1H, t), 5.83 (1H, d), 4.20 (1H, t), 4.12 (1H, m), 3.80 (1H, m), 3.70 (2H, m), 3.19 (3H, s), 2.77 (2H, m); ^{31}P NMR (D_2O, 300 MHz) δ 7.36; HRMS (ESI) *m*/*z* calcd for $[M - H]^-$ 546.1508, found 546.1513.

[2-(3-Indolyl)-1-ethyl]phosphoramidic Acid 5′-Adenosyl Ester (12). The yield is 37.4% (0.26 g) as a white solid: ^{1}H NMR (D_2O, 300 MHz) δ 7.98 (1H, s), 7.86 (1H, s), 6.99 (2H, m), 6.73 (2H, m), 6.55 (1H, t), 5.7 (1H, d), 4.46 (1H, m), 4.23 (1H, m), 4.12 (1H, m), 3.78 (2H, m), 2.77 (2H, m), 2.43 (2H, m); ^{31}P NMR (D_2O, 300 MHz) δ 10.25; HRMS (ESI) *m*/*z* calcd for $[M - H]^-$ 488.1447, found 488.148.

[2-(3-Indolyl)-1-ethyl]phosphoramidic Acid 5′-(1-β-Arabinofuranosyl)adenine Ester (13). The yield is 47% (0.45 g) as a white solid: ^{1}H NMR (D_2O, 300 MHz) δ 8.07 (1H, s), 7.78 (1H, s), 7.11 (1H, d), 7.05 (1H, d), 6.8 (1H, t), 6.75 (1H, s), 6.67 (1H, t), 6.03 (1H, d), 4.43 (1H, m), 4.29 (1H, m), 3.92 (3H, m), 2.80 (2H, m), 2.51 (2H, m); ^{31}P NMR (D_2O, 300 MHz) δ 10.23; HRMS (ESI) *m*/*z* calcd for $[M - H]^-$ 488.1447, found 488.1422.

2(*R*)-[Guanosyl-5′-(phosphorylamino)]-3-(3-indolyl)-proprionic Acid Methyl Ester (14). The yield is 16.3% (0.12 g) as a white solid: ^{1}H NMR (D_2O, 300 MHz) δ 7.88 (1H, s), 7.32 (2H, m), 7.08 (1H, m), 6.95 (2H, m), 5.74 (1H, d), 4.29 (1H, m), 4.14 (1H, m), 3.82 (3H, m), 3.32 (3H, s), 2.90 (2H, m); ^{31}P NMR (D_2O, 300 MHz) δ 7.46; HRMS (ESI) *m*/*z* calcd for $[M - H]^-$ 562.1451, found 562.1462.

[2-(3-Indolyl)-1-ethyl]phosphoramidic Acid 5′-Guanosyl Ester (15). The yield is 16.0% (0.10 g) as a white solid: ^{1}H NMR (D_2O, 300 MHz) δ 7.76 (1H, s), 7.2 (2H, m), 6.98 (1H, m), 6.80 (2H, m), 5.60 (1H, d), 4.27 (1H, m), 4.14 (1H, m), 3.86 (1H, m), 3.79 (1H, m), 2.85 (2H, m), 2.56 (2H, m); ^{31}P NMR (D_2O, 300 MHz) δ 10.21; HRMS (ESI) *m*/*z* calcd for $[M - H]^-$ 504.1397, found 504.1400.

[2-(3-Indolyl)-1-ethyl]phosphoramidic Acid 5′-(7-Benzylguanosyl) Ester (16). The reaction mixture was heated at 55 °C for 6 h. The product mixture was concentrated and the residue chromatographed on silica gel, eluting first with a $CHCl_3$/MeOH mixture (80:20) and then with a $CHCl_3$/MeOH/H_2O mixture (5:3:0.25, containing 0.5% NH_4OH). The solid obtained after evaporation of the solvent was passed through an ion exchange column (Dowex-50W8-200, Na^+ form). The yield is 31% (0.04 g) as a white solid: ^{1}H NMR (D_2O, 300 MHz) δ 7.17−7.06 (m, 7H), 6.91 (s, 1H), 6.81 (t, 1H), 6.62 (t, 1H), 5.65 (d, 1H), 4.96 (d, 2H), 4. 25 (t, 1H), 4.15 (d, 1H), 3.96−3.72 (m, 2H), 2.94−2.67 (m, 2H), 2.61 (t, 2H); ^{31}P NMR (D_2O, 300 MHz) δ 10.44; HRMS (ESI) *m*/*z* calcd for $[M + H]^+$ 618.1837, found 618.1825.

[2-(3-Indolyl)-1-ethyl]phosphoramidic Acid 5′-Inosyl Ester (17). The yield is 4.5% (0.057 g): ^{1}H NMR (D_2O, 300 MHz) δ 8.04 (1H, s), 7.54 (1H, s), 7.14 (2H, m), 6.86 (1H, m), 6.77 (1H, s), 6.69 (1H, m), 5.72 (1H, d), 4.28 (1H, m), 4.16 (1H, m), 3.87 (1H, m), 3.76 (1H, m), 2.75 (2H, m), 2.46 (2H, m); ^{31}P NMR (D_2O, 300 MHz) δ 10.29; HRMS (ESI) *m*/*z* calcd for $[M - H]^-$ 489.1288, found 489.1279.

[2-(3-Indolyl)-1-ethyl]phosphoramidic Acid 5′-Uridyl Ester (18). The yield is 6.3% (0.041 g): ^{1}H NMR (D_2O, 300 MHz) δ 7.37 (2H, m), 7.21 (1H, d), 6.97 (2H, m), 6.86 (1H, m), 5.58 (1H, d), 5.18 (1H, d), 3.96 (3H, m), 3.84 (1H, m), 3.69 (1H, m), 2.95 (2H, m), 2.72 (2H, m); ^{31}P NMR (D_2O, 300 MHz) δ 10.26; HRMS (ESI) *m*/*z* calcd for $[M - H]^-$ 465.1175, found 465.1174.

[2-(3-Indolyl)-1-ethyl]phosphoramidic Acid 5′-Cytidyl Ester (19). The yield is 4.7% (0.031 g): ^{1}H NMR (D_2O, 300 MHz) δ 7.42 (1H, d), 7.33 (1H, d), 7.20 (1H, d), 6.94 (2H, m), 6.83 (1H, m), 5.59 (1H, d), 5.35 (1H, d), 3.90 (4H, m), 3.68 (1H, m), 2.93 (2H, m), 2.7 (2H, m); ^{31}P NMR (D_2O, 300 MHz) δ 10.24; HRMS (ESI) *m*/*z* calcd for $[M - H]^-$ 464.1335, found 464.1335.

[2-(3-Indolyl)-1-ethyl]phosphoramidic Acid 5′-Thymidyl Ester (20). The yield is 21.9% (0.072 g) as a white solid: ^{1}H NMR (D_2O, 300 MHz) δ 7.24 (1H, d), 7.14 (1H, d), 7.09 (1H, s), 6.92 (2H, m), 6.76 (1H, m), 5.93 (1H, t), 4.26 (1H, m), 3.89 (1H, m), 3.78 (1H, m), 3.67 (1H, m), 2.9 (2H, m), 2.66 (2H, m), 1.99 (1H, m), 1.82 (1H, m), 1.23 (3H, s); ^{31}P NMR (D_2O, 300 MHz) δ 10.12; HRMS (ESI) *m*/*z* calcd for $[M - H]^-$ 463.1383, found 463.1396.

***N*-(2-Thiono-1,3,2-oxathiaphospholanyl)-L-tryptophan Methyl Ester (21).** *N*-(2-Thiono-1,3,2-oxathiaphospholanyl)-L-tryptophan methyl ester was prepared as previously described with minor modifications.[28]

To a solution of L-tryptophan methyl ester hydrochloride (0.25 g, 1 mmol) was added 5 mL of dry pyridine elemental sulfur (0.07 g, 2 mmol). Then 2-chloro-1,3,2-oxathiaphospholane (1 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 12 h. Then the solvent was removed under reduced pressure; to the residue was added acetonitrile (10 mL), and an excess of sulfur was filtered off. After evaporation of the solvent, the residue was dissolved in 2−3 mL of chloroform and applied to a silica gel (200−300 mesh) column (2.5 cm × 18 cm). The column was eluted with chloroform. Appropriate fractions were combined and evaporated under reduced pressure to give the desired compound as a mixture of diastereoisomers. The diastereoisomers were separated by crystallization. From the

(28) Baraniak, J.; Kaczmarek, R.; Korczynski, D.; Wasilewska, E. Oxathiaphospholane approach to N- and O-phosphorothioylation of amino acids. *J. Org. Chem.* **2002**, *67*, 7267−7274.

solution of both diastereoisomers in chloroform was deposited the isomer absorbing at lower field in ^{31}P NMR [($CDCl_3$, 121 MHz) δ 96.54]. Mother liquor was concentrated, and the solid residue was dissolved in the solution of chloroform and diethyl ether (10:1). Crystals collected from this solvent mixture constituted the pure diastereomer resonating in ^{31}P NMR at higher field [($CDCl_3$, 121 MHz) δ 95.52]. Crystals of the diastereoisomer absorbing in ^{31}P NMR at lower field have appeared to be appropriate for X-ray analysis,[29] which proved the existence of the *R* configuration at the phosphorus center: yield of 0.27 g (75%); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.09 (bs, 1H), 7.57 (d, 1H), 7.39−7.35 (m, 1H), 7.24−7.06 (m, 3H), 4.38−4.06 (m, 4H), 3.67 (s, 3H), 3.44−3.26 (m, 4H); ^{31}P NMR ($CDCl_3$, 121 MHz) δ 96.54 (R_p), 95.52 (S_p); HRFAB-MS *m*/*z* calcd for [M − H]$^-$ 355.0418, found 355.0419.

General Procedure for the Synthesis of Nucleoside 5′-*O*-(L-Tryptophanylphosphoramidothioates). Nucleoside 5′-*O*-(tryptophanylphosphoramidothioates) were prepared by a similarly described procedure with minor modification.[30]

The individual diastereoisomer of *N*-(2-thiono-1,3,2-oxathiaphospholanyl)tryptophan methyl ester (0.36 g, 1 mmol) was dissolved in dry acetonitrile (5 mL), and into this solution was dropped a solution of N^2-isobutyryl-$O^{2'}$,$O^{3'}$-diacetylguanosine (0.44 g, 1 mmol) or N^6,$O^{2'}$,$O^{3'}$-tribenzoyladenosine (0.58 g, 1 mmol) and DBU (167 μL, 1.1 mmol) in dry acetonitrile (6 mL). The reaction mixture was stirred at room temperature for 12 h and then concentrated under reduced pressure. The residual solid was suspended in 10 mL of 20% aqueous ammonia and left for 24 h at room temperature in a tightly closed vial. Ammonia was then evaporated; the residue was dissolved in water and purified on a Sephadex A-25 column (3 cm × 20 cm), and products were eluted with a linear gradient of triethylammonium bicarbonate buffer (pH 7.5) from 0.05 to 0.4 M. The appropriate fractions were combined and evaporated to yield the oily product, which was dissolved in water and passed through a Dowex 50Wx2 (Na^+) column (1.5 cm × 10 cm). The fractions containing the product were combined and lyophilized to provide the corresponding products as a colorless solid.

[2-(3-Indolyl)-1(*S*)-carbamoylethyl]-(S_p)-thiophosphoramidic Acid 5′-Adenosyl Ester (4). The yield is 71% (0.39 g) as a white solid: ^{1}H NMR (D_2O, 300 MHz) δ 8.20 (1H, s), 8.08 (1H, s), 7.32 (1H, d), 7.12 (1H, d), 7.03 (1H, s), 6.84 (1H, t), 6.67 (1H, t), 5.82 (1H, d), 4.44 (1H, m), 4.12 (2H, m), 3.80 (2H, m), 3.71 (1H, m), 2.97 (2H, m); ^{31}P NMR (D_2O, 121 MHz) δ 55.65; HRFAB-MS *m*/*z* calcd for [M − H]$^-$ 547.1954, found 547.1942.

[2-(3-Indolyl)-1(*S*)-carbamoylethyl]-(R_p)-thiophosphoramidic Acid 5′-Adenosyl Ester (5). The yield is 74.5% (0.41 g) as a white solid: ^{1}H NMR (D_2O, 300 MHz) δ 8.28 (1H, s), 8.06 (1H, s), 7.32 (1H, d), 7.13 (1H, d), 7.02 (1H, s), 6.85 (1H, t), 6.69 (1H, t), 5.82 (1H, d), 4.50 (1H, m), 4.16 (2H, m), 3.94 (1H, m), 3.70 (2H, m), 2.97 (2H, m); ^{31}P NMR (D_2O, 121 MHz) δ 57.96; HRFAB-MS *m*/*z* calcd for [M − H]$^-$ 547.1954, found 547.1940.

[2-(3-Indolyl)-1(*S*)-carbamoylethyl]-(S_p)-thiophosphoramidic Acid 5′-Guanosyl Ester (9). The yield is 76.2% (0.43 g) as a white solid: ^{1}H NMR (D_2O, 300 MHz) δ 7. 86 (1H, s), 7.37 (1H, d), 7.23 (1H, d), 6.96 (2H, t), 6.80 (1H, t), 5.66 (1H, d), 4.52 (1H, m), 4.18 (1H, m), 4.10 (1H, m), 3.86 (2H, m), 3.66 (1H, m), 2.96 (2H, m); ^{31}P NMR (D_2O, 121 MHz) δ 55.63; HRFAB-MS *m*/*z* calcd for [M − H]$^-$ 563.1304, found 543.1236.

[2-(3-Indolyl)-1(*S*)-carbamoylethyl]-(R_p)-thiophosphoramidic Acid 5′-Guanosyl Ester (10). The yield is 66.5% (0.38 g) as a white solid: ^{1}H NMR (D_2O, 300 MHz) δ 7. 93 (1H, s), 7.39 (1H, d), 7.23 (1H, d), 7.03 (1H, s), 6.97 (1H, t), 6.80 (1H, t), 5.65 (1H, d), 4.58 (1H, m), 4.19 (1H, m), 4.12 (1H, m), 3.89 (1H, m), 3.76 (2H, m), 2.96 (2H, m); ^{31}P NMR (D_2O, 121 MHz) δ 57.91; HRFAB-MS *m*/*z* calcd for [M − H]$^-$ 563.1304, found 563.1250.

Continuous Fluorescent Phosphoramidase Activity Assay. Stock solutions of substrates (5 mM) were prepared in deionized water and filtered through a 0.2 μm filter. Hydrolysis of the fluorogenic substrate by Hint proteins was carried out in 600 μL of degassed HEPES buffer [20 mM HEPES (pH 7.2) and 1 mM $MgCl_2$] in quartz cuvettes at 25 °C. The purification of Hint proteins has been described previously.[10] Fluorescence measurements were taken in a Varian/Cary Eclipse fluorimeter with the kinetics program equipped with a thermostated cuvette holder. The excitation wavelength was set at 280 nm; fluorescence emission was measured at 360 nm, and excitation and emission slits were set at 10 nm for substrate concentrations from 50 nM to 2 μM or 5 nm for substrate concentrations from 4 to 50 μM. The fluorescence intensity was monitored for 2 min to obtain the baseline and allow the temperature to stabilize at 25 °C, and then enzyme was added to initiate the reaction. The increase in fluorescence intensity was recorded for 2−30 min on the basis of the rate of hydrolysis. The initial velocities were obtained by converting the slope of the fluorescence spectrum into micromolar substrate hydrolyzed per minute, based on the fluorescence standard curves of substrate and indole product solutions (Figure 1 and Tables I and II of the Supporting Information). Measurements were carried out in duplicate, and variants are given as the standard deviation.

Equilibrium Dialysis. Equilibrium dialysis is figured as a simple but effective tool for the exploration of interactions between molecules in vitro. The interaction between hHint1 and 3′-azido-3′-deoxythymidine-L-phenylalanine phosphoramidate monoester (L-APO) was analyzed with the equilibrium biodialyzer (The Nest Group, Inc.). hHint1 (0.18 μM) and L-APO (0.1∼8.6 mM) were loaded into the sample chamber, and Tris buffer [20 mM Tris (pH 7.5), 1 mM

(29) Wieczorek, M. Unpublished results.
(30) Stec, W. J.; Grajkowski, A.; Karwowski, B.; Kobylanska, A.; Koziolkiewicz, M.; Misiura, K.; Okruszek, A.; Wilk, A.; Guga, P.; Boczkowska, M. Diastereomers of nucleoside 3′-O-(2-Thio-1,3,2-Oxathia(selena)phospholanes): Building blocks for stereocontrolled synthesis of oligo(nucleoside phosphorothioate)s. *J. Am. Chem. Soc.* **1995**, *117*, 12019−12029.

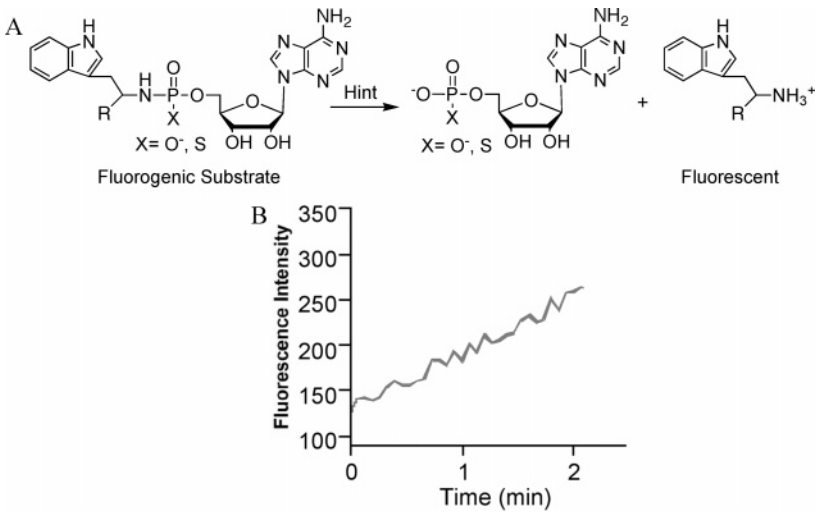


Figure 1. Fluorescence assay. The time course was for conversion of the first 10% of the reaction.

EDTA, and 1 mM DTT] was loaded into the assay chamber. The concentration of free L-APO at equilibrium was determined by the absorbance at 267 nm. The control was performed without adding protein.

Results and Discussion

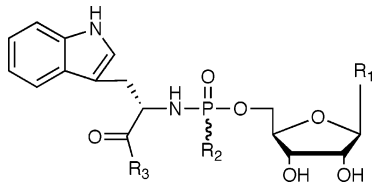
Continuous Fluorescence Assay Development. Since the discovery that Hints are nucleoside phosphoramidases, we have embarked on an effort to map the substrate specificity of these unique enzymes so that we may better understand their native function, as well as profit from their potential as pronucleotide bioactivators. Although both HPLC-based[12] and NMR-based[10] assays of Hint phosphoramidase activity have been developed, only two spectrophotometric assays have been devised. A spectrophotometric assay has been developed that relies on the release of methylcoumarinamine from released *tert*-butoxycarbonyl-L-lysine methylcoumarinamide by trypsin.[13] A continuous spectrophotometric assay that depends on the ability to monitor the release of *p*-nitroaniline (410 nm) after hydrolysis of adenosine 5′−O *p*-nitroaniline phosphoramidate (AMP-pNA) by Hints has also been developed.[27] While each of these spectrophotometric assays has its advantages, the low substrate specificity (vide supra) for AMP-pNA and the discontinuous nature and restriction to lysine-based phosphoramidates of the coumarin-based assay spurred us to investigate the feasibility of developing a simple, sensitive, and efficient continuous fluorescence assay that was also structurally flexible enough to allow the substrate specificity of these enzymes to be probed in greater detail.

Nucleoside phosphoramidates, such as AZT L-tryptophan methyl ester phosphoramidate monoester, that incorporate the fluorescent indole moiety have been shown to have potent antiviral and anticancer activity.[4] As can be seen in Figure 1, because of intramolecular quenching, hydrolysis of the adenosine tryptophan phosphoramidate (**1**) by hHint1 can be easily observed by monitoring the increase in indole fluorescence with time. Due to the magnitude of fluorescence quenching (>10-fold), this assay can be carried out in the low micromolar range, while interfilter effects limited the highest substrate concentration to 50 μM.

Hint Substrate Specificity. From an examination of the Michaelis−Menten parameters for the series of indole-containing nucleoside phosphoramidates, a picture of the substrate specificity for both hHint1 and *ec*hinT begins to emerge (Tables 1−3). First, on the basis of k_{cat}/K_m values, when either adenosine or guanosine phosphoramidate-incorporated L-tryptophan, the human enzyme preferred substitution of the α-carboxylate with a α-carboxamide (Table 1). In contrast, *ec*hinT exhibited no significant preference for either the methyl ester, methyl amide, or amide substitution. This discrepancy is likely due to the closeness of a H-bond donor in the vicinity of the phosphoramidate α-carboxylate. This apparently is not the case for *ec*hinT, since no particular preference for a substitution at the α-carboxylate was observed. When the effect of amino acid stereochemistry was examined, hHint1 was far more sensitive to configurations at the α-carbon of the amino acid, preferring D- over L-tryptophan by approximately 70- and 120-fold for the adenosine and guanosine phosphoramidates, respectively (Table 2). In contrast, the bacterial enzyme exhibited no particular amino acid stereochemical preference, thus revealing an additional difference between the active sites that may be exploited in the development of bacterially specific pronucleotides. The discrepancy between the enzymes was eliminated with the removal of the α-carboxylate. Tryptamine phosphoramidates of adenosine were shown to have significantly higher k_{cat} values and lower K_m values and, therefore, k_{cat}/K_m values that are 240-fold (hHint1) and 44-fold (*ec*hinT) greater than the values for D-tryptophan phosphoramidates (Table 2). Obviously, for both enzymes, the α-carboxylate presents a significant stereochemical barrier to not only substrate binding but also, on the basis of the proposed mechanism, formation of the initial adenylated enzyme.

On the basis of the crystal structure of hHint1 with the stable ADP analogue, AMPCP, the conserved residues His112(hHint1) and His101(*ec*hinT) and His114(hHint1) and

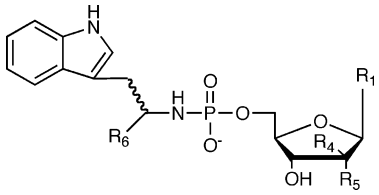
Table 1. Steady State Kinetic Comparison of Hydrolysis of Phosphoramidate and Phosphoramidothioate Substrates by hHint1 and *ec*hinT in HEPES Buffer (pH 7.2)[a]



				k_{cat} (s^{-1})		K_m (μM)		k_{cat}/K_m ($\times 10^{-3}$ s^{-1} M^{-1})	
compound	R_1	R_2	R_3	hHint1	*ec*hinT	hHint1	*ec*hinT	hHint1	*ec*hinT
1	adenine	O^-	OCH_3	0.012 ± 0.001	1.1 ± 0.1	41 ± 4	43 ± 3	0.23 ± 0.04	28 ± 4
2	adenine	O^-	$NHCH_3$	0.02 ± 0.003	0.52 ± 0.06	44 ± 11	63 ± 8	0.5 ± 0.2	8 ± 2
3	adenine	O^-	NH_2	0.45 ± 0.01	1.28 ± 0.09	50 ± 3	94 ± 9	9.2 ± 0.7	14 ± 2
4	adenine	S^- (S_p)	NH_2	0.011 ± 0.007	0.02 ± 0.01	216 ± 15	361 ± 27	0.05 ± 0.04	0.05 ± 0.03
5	adenine	S^- (R_p)	NH_2	0.03 ± 0.008	0.07 ± 0.05	49 ± 14	779 ± 56	0.6 ± 0.3	0.09 ± 0.07
6	guanine	O^-	OCH_3	0.0071 ± 0.002	0.50 ± 0.02	31 ± 1	32 ± 3	0.23 ± 0.01	16 ± 2
7	guanine	O^-	$NHCH_3$	0.024 ± 0.002	0.56 ± 0.07	65 ± 9	67 ± 14	0.37 ± 0.08	8 ± 3
8	guanine	O^-	NH_2	0.23 ± 0.01	0.52 ± 0.04	58 ± 6	64 ± 8	4.0 ± 0.6	8 ± 2
9	guanine	S^- (S_p)	NH_2	0.002 ± 0.0001	0.01 ± 0.003	52 ± 6	190 ± 66	0.04 ± 0.007	0.05 ± 0.03
10	guanine	S^- (R_p)	NH_2	0.012 ± 0.003	0.011 ± 0.003	162 ± 23	137 ± 40	0.07 ± 0.03	0.08 ± 0.04

[a] Measurements were carried out in duplicate, and variants are given as the standard deviation.

Table 2. Steady State Kinetic Parameters of hHint1 and *ec*hinT[a]



					k_{cat} (s^{-1})		K_m (μM)		k_{cat}/K_m ($\times 10^{-3}$ s^{-1} M^{-1})	
compound	R_1	R_4	R_5	R_6	hHint1	*ec*hinT	hHint1	*ec*hinT	hHint1	*ec*hinT
1	adenine	H	OH	*S*-$COOCH_3$	0.012 ± 0.001	1.1 ± 0.1	41 ± 4	45 ± 12	0.23 ± 0.04	28 ± 4
11	adenine	H	OH	*R*-$COOCH_3$	0.27 ± 0.02	1.0 ± 0.2	4.1 ± 0.9	45 ± 12	70 ± 20	20 ± 10
12	adenine	H	OH	H	2.1 ± 0.1	4.5 ± 0.07	0.13 ± 0.02	5.2 ± 0.2	15000 ± 3000	870 ± 50
13	adenine	OH	H	H	1.1 ± 0.03	1.5 ± 0.4	1.0 ± 0.06	60 ± 19	1100 ± 100	30 ± 20
6	guanine	H	OH	*S*-$COOCH_3$	0.0071 ± 0.002	0.50 ± 0.02	31 ± 1	32 ± 3	0.23 ± 0.01	16 ± 2
14	guanine	H	OH	*R*-$COOCH_3$	0.26 ± 0.02	0.37 ± 0.01	3.3 ± 0.6	21 ± 1	80 ± 30	17 ± 2
15	guanine	H	OH	H	2.3 ± 0.07	4.0 ± 0.4	0.21 ± 0.02	6 ± 1	11000 ± 1000	700 ± 200
16	7-benzylguanine	H	OH	H	0.68 ± 0.04	0.15 ± 0.01	14 ± 2	56 ± 6	50 ± 10	2.6 ± 0.7
17	hypoxanthine	H	OH	H	2.6 ± 0.04	4.2 ± 0.1	0.71 ± 0.03	14 ± 1	3700 ± 300	310 ± 40
18	uracil	H	OH	H	2.5 ± 0.3	2.4 ± 0.6	2.2 ± 0.4	42 ± 15	1200 ± 500	70 ± 60
19	cytosine	H	OH	H	1.2 ± 0.1	0.49 ± 0.03	2.3 ± 0.4	30 ± 3	600 ± 200	20 ± 4
20	thymine	H	H	H	0.10 ± 0.01	0.02 ± 0.002	32 ± 5	46 ± 7	3 ± 1	0.4 ± 0.2

[a] Measurements were carried out in duplicate, and variants are given as the standard deviation.

His103(*ec*hinT) are likely to interact directly with the phosphorus oxygens and nitrogen of phosphoramidates[31] (Figure 2). To gain insights into the potential role of these side chains with the phosphoramidate oxygens, each of the oxygens of compounds **3** and **8** were stereospecifically replaced with phosphoramidothioates (Table 1). A marked decrease in the k_{cat} values for both hHint1 and *ec*hinT was observed. The greater effect on k_{cat} observed for both adenosine and guanosine substrates likely reflects the similar importance of specifically aligned active site stabilizing interactions on both the transition state for either enzyme adenylation or deadenylation. While the *ec*hinT K_m values for the phosphoramidothioates increased, the hHint1 K_m value was unchanged for the adenosine R_p analogue (**4**) and the guanosine S_p analogue (**9**). The idiosyncratic differences

(31) Lima, C. D.; Klein, M. G.; Hendrickson, W. A. Structure-based analysis of catalysis and substrate definition in the HIT protein family. *Science* **1997**, *278*, 286−290.

Table 3. *ec*hinT/hHint1 Ratio of the Specificity Constant (k_{cat}/K_m)

compound	ratio of k_{cat}/K_m (*ec*hinT/hHint1)	compound	ratio of k_{cat}/K_m (*ec*hinT/hHint1)
1	122 ± 39	**11**	0.3 ± 0.2
2	16 ± 10	**12**	0.06 ± 0.01
3	1.5 ± 0.3	**13**	0.03 ± 0.02
4	1 ± 1	**14**	0.21 ± 0.1
5	0.2 ± 0.2	**15**	0.06 ± 0.02
6	70 ± 12	**16**	0.05 ± 0.02
7	22 ± 13	**17**	0.08 ± 0.02
8	2.0 ± 0.8	**18**	0.06 ± 0.07
9	1 ± 1	**19**	0.03 ± 0.02
10	1 ± 1	**20**	0.13 ± 0.11

observed for these substrates and the Hints may reflect subtle discrepancies in their binding affinity and/or the rate of active site adenylation by the two enzymes.

Probing the Hint base recognition site, we determined the substrate specificity for both purine and pyrimidine phosphoramidates of tryptamine. Adenosine (**12**) and guanosine (**15**) phosphoramidates were found to have the greatest k_{cat}/K_m values for both enzymes (Table 2). Indeed, the hHint1 k_{cat}/K_m value of 1.5×10^7 s^{-1} M^{-1} for compound **12** is 30-fold greater than the value of 4.9×10^5 s^{-1} M^{-1} reported for the hydrolysis of *N*-ϵ-*N*-Boc-lysine adenosine phosphoramidate by the nearly identical rabbit Hint1.[27] The slight preference by hHint1 and *ec*hinT may be a reflection of a somewhat tighter set of binding interactions between the active site and N-3, as well as the ribose ring (Figure 3A,B). Evidently, the predicted backbone carbonyl interaction with 2-NH_2 (Figure 3C) is significant, since the K_m, and therefore k_{cat}/K_m, for tryptamine phosphoramidate of inosine (**17**) is 3-fold lower than the value for tryptamine guanosine phosphoramidate (**15**). Neither uridine (**18**) nor cytidine (**19**) phosphoramidates can access either of these backbone interactions; thus, purine substrates are preferred by both enzymes, with a uridine analogue enjoying a slight preference over a cytidine analogue. Although the crystal structure studies demonstrated that N-7 for both adenosine and guanosine is solvent accessible, the introduction of a benzyl group at this position seriously compromised both the k_{cat} and K_m value and therefore the k_{cat}/K_m. Nevertheless, modifications on this face are still remarkably well tolerated.

As exhibited by the crystal structure of hHint1 (Figure 3), two significant hydrogen bonding interactions are made with the ribose of either AMP or GMP. The inability of either hHint1 or *ec*hinT to efficiently hydrolyze thymidine tryptamine phosphoramidate (**20**) is consistent with this finding. Furthermore, although we were able to demonstrate by equilibrium dialysis that the potent antiviral AZT phosphoramidate4, L-APO, is able to modestly bind to hHint1 (K_d = 1.0 + 0.2 mM); L-APO proved neither to be an inbibitor nor a substrate for hHint1 (Chou, T.-F. and Wagner, C. R., unpublished results). Thus, phosphoramidates of dideoxynucleosides are unlikely to be substrates for Hints. The configuration of the substituents at this position is not absolute, since the Ara-A phosphoramidate (**13**), in which

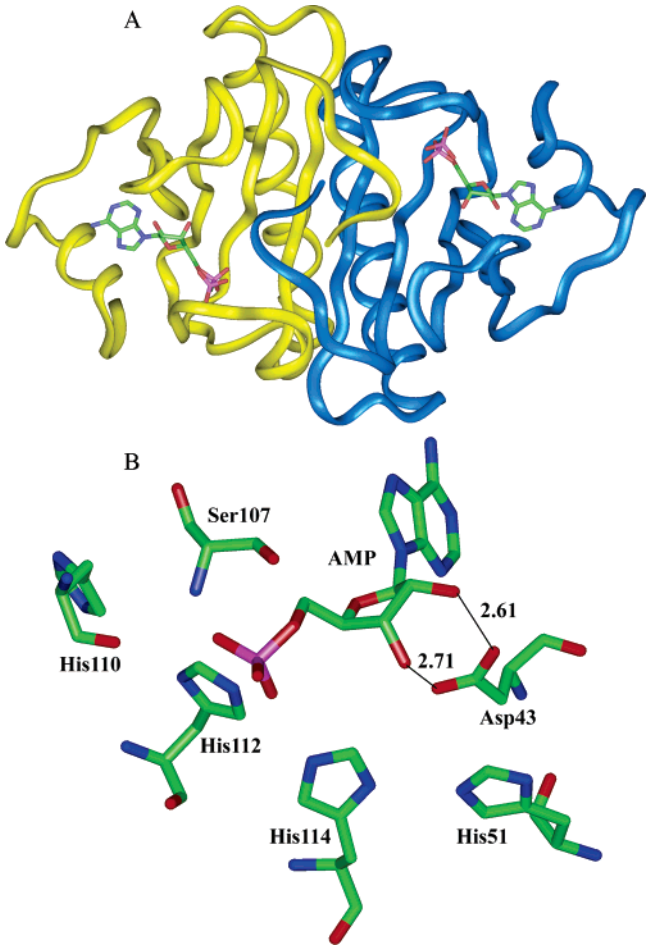


Figure 2. X-ray crystallographic structure of hHint1 with bound AMPCP (A) (PDB entry 1AV5) and of the hHint1 active site with bound AMP (B) (PDB entry 1KPF; ref 31).

the 2′-OH group is in the opposite β-configuration, is tolerated as a substrate (Table 2).

Summary and Concluding Remarks. On the basis of these results, a general outline of the phosphoramidase substrate specificity requirements for Hints can be delineated. First, although there is a clear preference for purine phosphoramidates, pyrimidine-based phosphoramidates can be considered as viable substrates. Second, on the basis of crystal structure studies and our kinetic results with phosphoramidothioates, substitutions of the phosphoramidate oxygens are not likely to be tolerated. Third, regardless of the base, maintenance of an electrophilic or hydrogen-bonding group at the ribose 2′-position is essential. Phosphoramidates containing deoxy- or dideoxynucleosides, such as d4T and AZT, therefore, are not likely to be substrates for mammalian Hints.[2,4,5,7] Nevertheless, as the results with Ara-A demonstrate, the β-configuration is well tolerated, opening the door to the potential for drugs such as fludarabine,[32,33] clofarabine,[34] Ara-C,[35,36] and the recently discovered 2′-methyl nucleosides[37] to be substrates for Hints. Fourth, phosphoramidates containing a primary amine with as few as two methylene groups are significantly favored as substrates over sterically crowded amines. The remarkable

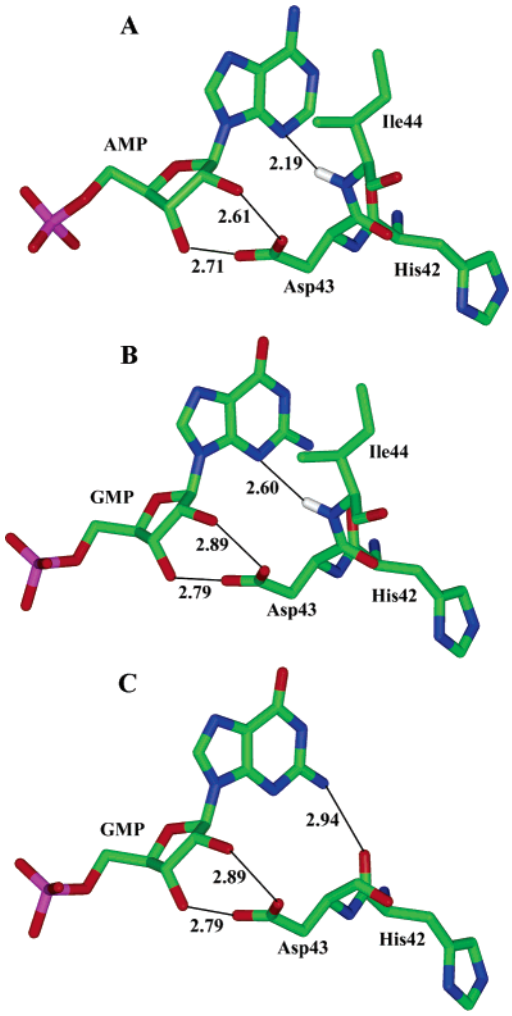


Figure 3. Hydrogen bonding network surrounding the purine ring and ribose of AMP-bound hHint1 (A) (PDB entry 1KPF; ref 31) and ribose GMP-bound rabbit Hint1 (B) (PDB entry 3RHN; ref 43), revealing the chemical basis of discrimination by hHint1 of ara-adenosine, guanosine, and inosine as substrates.

efficiency with which Hints can convert nucleoside phosphoramidates to the 5′-monophosphates is comparable to the values of 10^3-10^6 observed for nucleoside kinases, such as adenosine kinases.[38,39] One can envision, therefore, that the therapeutic utility of nucleoside phosphoramidates could be expanded by improving their cellular uptake, as well as by incorporation of an additional tissue targeting compound or synergistic drug coupled through an alkyl amine linker.

Last, although the phosphoramidase activity of Hints has only recently been discovered, it has been known for decades that both bacterial and mammalian tissues express a ribonucleoside phosphoramidase. Smith and Burrow were the first to demonstrate this activity, when they discovered that *E. coli* extracts were able to efficiently hydrolyze adenosine phosphoramidate (AMP-NH_2).[40] Shabarova and co-workers later partially purified a phosphoramidase from rabbit and rat liver extracts that specifically preferred to hydrolyze phosphoramidate-containing purine bases and D-amino acids.[41] The first phosphoramidase purified to homogenenity was obtained from bacterial extracts by Kumon and coworkers and shown to be a 28 kDa homodimer.[42] Unfortunately, since they did not obtain sequence data for this protein, its identity could not be determined. Consequently, on the basis of our substrate specificity results for both bacterial and human Hints, we believe that the identity of the phosphoramidase previously studied is eukaryotic Hint1 and prokaryotic hinT. Nevertheless, given the inability of hHint1 to hydrolyze L-APO or the related antiviral analog, 3′-azido-3′-deoxythymidine-L-tryptophan methyl amide phosphoramidate (Chou, T.-F. and Wagner, C. R., unpublished results), it is highly unlikely that the phosphoramidase

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activity responsible for the activation of the previously reported antiviral AZT and d4T phosphoramidates is hHint1.[2,4,5,7] The identity of this enzyme(s) remains to be determined.

Abbreviations Used

*ec*hinT, *E. coli* histidine triad nucleotide binding protein; hHint1, human histidine triad nucleotide binding protein 1; AMP-lysine, AMP-*N*-ϵ-(*N*-α-acetyllysine methyl ester) 5′-phosphoramidate; AMPCP, adenosine 5′-(α,*β*-methylene)-diphosphate; GMP, guanosine 5′-monophosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HIT, histidine triad; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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Supporting Information Available: Fluorescence standard curves (Figure 1), fluorescence factors obtained from fluorescence standard curves in HEPES buffer (Table I), and equations used to convert fluorescence intensity changes to velocities (Table II). This material is available free of charge via the Internet at http://pubs.acs.org.

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