Nucleotide and Nucleoside Analogues as Inhibitors of Cytosolic 5′-Nucleotidase I from Heart

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ABSTRACT: Substrate and product specificity studies were used to develop inhibitors of the cytosolic 5′-nucleotidase I (c-N-I) from myocardium. As measured by V_{max}/K_m, c-N-I preferred pyrimidine 2′-deoxyribonucleotides as substrates with thymidine monophosphate (TMP) being the most efficient. In product inhibition studies, thymidine inhibited noncompetitively and inorganic phosphate inhibited competitively, consistent with an ordered release of nucleoside prior to phosphate. Mirroring nucleotide substrate specificities, pyrimidine nucleosides were more potent product inhibitors than purine nucleosides. Thus, pyrimidine nucleotide and nucleoside analogues were developed as inhibitors. Phosphonate analogues of TMP were synthesized by a novel method. The most potent was the 5′-phosphonate of 3′-deoxythymidine (ddT) (apparent K_i value of 63 nM). In addition, pyrimidine nucleoside analogues were inhibitors with 5-ethynyl-2′,3′-dideoxyuridine being the most potent (apparent K_i value of 3.7 μM). The most potent nucleotide and nucleoside inhibitor were both greater than 1000-fold more potent inhibiting c-N-I than the cytosolic 5′-nucleotidase II. The nucleoside analogue was also greater than 1000-fold more potent against c-N-I than the membrane ecto-5′-nucleotidase (e-N). Because the phosphonate analogues measurably inhibited e-N (apparent K_i values of 6−12 μM), the selectivity of the phosphonates for c-N-I versus e-N was less (40−200-fold). Because of the high selectivity for c-N-I versus both of the other 5′-nucleotidases, the nucleoside inhibitors of c-N-I may be useful biochemical tools in discerning the role that c-N-I plays in generating adenosine within myocardium.

During myocardial ischemia, ATP utilization quickly outpaces ATP formation with the eventual consequence of a marked increase in adenosine (*1*). While adenosine release from myocytes is clearly cardioprotective (*2*−*4*), it may also be detrimental (*1*). As a substrate for purine catabolic enzymes, adenosine could be irreversibly lost from the myocyte, resulting in adenine nucleotide depletion within the myocyte, and, in addition, this could lead to the generation of free radicals within heart tissue. Thus, to circumvent ischemia/reperfusion injury, research has paradoxically focused on the use of (1) adenosine itself (*5*−*6*), (2) adenosine precursors (*7*), (3) adenosine transport inhibitors (*8*), and (4) adenosine formation inhibitors (*9*−*10*).

A key enzyme in the myocardial cascade of ATP to adenosine is 5′-nucleotidase (EC 3.1.3.5). Within myocardium tissue, three distinct 5′-nucleotidases have been identified and studied: a membrane-bound ecto-5′-nucleotidase (e-N)[1] and two cytosolic enzymes, c-N-I and c-N-II (for a review, see ref *11*). Separate data support either e-N (*12*), c-N-I (*13*), or c-N-II (*10, 14*) as being largely responsible for the hydrolysis of AMP during ischemia. Specifically, on the basis of its activation by ADP (*15*), which transiently increases during ischemia, c-N-I appears to be well suited to play a role in adenosine production within the ischemic heart.

Inhibitors of adenosine transport (*4*) and of the e-N (*9, 16*) have been used as biochemical tools and some have been developed for potential therapy for ischemia/reperfusion damage. Only one report has described inhibitors of purified c-N-I and c-N-II (*17*); however, these molecules were weak (IC_{50} values in the 2−10 mM range). Furthermore, they showed little if any selectivity between the two cytosolic 5′-nucleotidases and only marginal selectivity between the cytosolic and ecto enzymes. Specific and potent inhibitors may help clarify the location of adenosine formation during ischemia. Additionally, they could potentially be used as therapy to diminish adenine nucleotide depletion during ischemia/reperfusion. We report in this paper the development of potent and selective inhibitors of c-N-I. In addition, we also describe a novel synthesis of nucleoside 5′-phosphonates.

MATERIALS AND METHODS

Materials. Frozen hearts from mature rabbit were obtained from Pel-Freez Biologicals (Rogers, AR). ADP-Agarose

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[1] Abbreviations: c-N-I, heart cytosolic 5′-nucleotidase I with preference for AMP vs IMP; c-N-II, cytosolic 5′-nucleotidase II with preference for IMP and GMP and which also has phosphotransferase activity; e-N, the membrance-bound, ecto-5′-nucleotidase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; ddT, 3′-deoxythymidine; AZT, 3′-azido-3′-deoxythymidine; ddC, 2′,3′-dideoxycytidine; ddU, 2′,3′-dideoxyuridine; d4T, 2′,3′-didehydro-3′-deoxythymidine; AIBN, azoisobutyronitrile; NFSi, *N*-fluorobenzenesulfonimide.

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(A4398) and AMP−Sepharose (A8394), both linked through N-6, were purchased from Sigma (St. Louis, MO). Cellulose phosphate P11 was from Whatman (Hillsboro, OR). DEAE-Sepharose was from Pharmacia LKB Biotechnology (Piscataway, NJ). [8-^{14}C]GMP, [8-^{14}C]Ado, [2-^{14}C]dCMP, [5-^{3}H]CMP, [*methyl*-^{3}H]TMP, and [8-^{14}C]IMP were purchased from Moravek Biochemicals (Brea, CA); [8-^{14}C]AMP was from ICN Radiochemicals (Costa Mesa, CA); and [U-^{14}C]dGMP, and [U-^{14}C] dAMP were from Amersham (Arlington Heights, IL). The 5-substituted analogues of 2′-deoxyuridine and the 5-ethynyl-2′,3′-dideoxyuridine were synthesized by G. Rahim (Glaxo Wellcome, Stevenage, England). Both enantiomers of *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine, 2′,3′-didehydro-3′-deoxythymidine (d_4T), α-D-thymidine, and 5′-deoxythymidine were synthesized at Glaxo Wellcome (Research Triangle Park, NC).

Synthesis of Thymidine 5′-Phosphonates: General. Diethyl 1,1,1-trichloromethylphosphonate was purchased from Fluka (Ronkonkoma, NY). Anhydrous tetrahydrofuran (THF), acetonitrile, toluene, *N*,*N*-dimethylformamide (DMF), and *n*-butyllithium (in hexanes) were obtained from Aldrich (Milwaukee, WI). Azoisobutyronitrile (AIBN) was purchased from Kodak (Rochester, NY). *N*-Fluorobenzenesulfonimide (NFSi) was obtained from Allied Signal (Morristown, NJ). Phosphodiesterase I was purchased from Pharmacia. All NMR spectra were run in DMSO-d_6 unless otherwise indicated. Compounds **8**−**13** each exist as two diastereoisomers and will be reported as such in the peak listings of their proton NMR spectra. The thymidine phosphonic acids were purified by ion-exchange chromatography with either Pharmacia's DEAE-Sephadex or Bio-Rad's AG1-X8 strongly basic anion-exchange resin (formate form). With both resins, the product was applied as a pH 9 solution in deionized water (55−65 mM) and was eluted as the ammonium salt by running a 5−500 mM gradient of ammonium bicarbonate.

Scheme 1

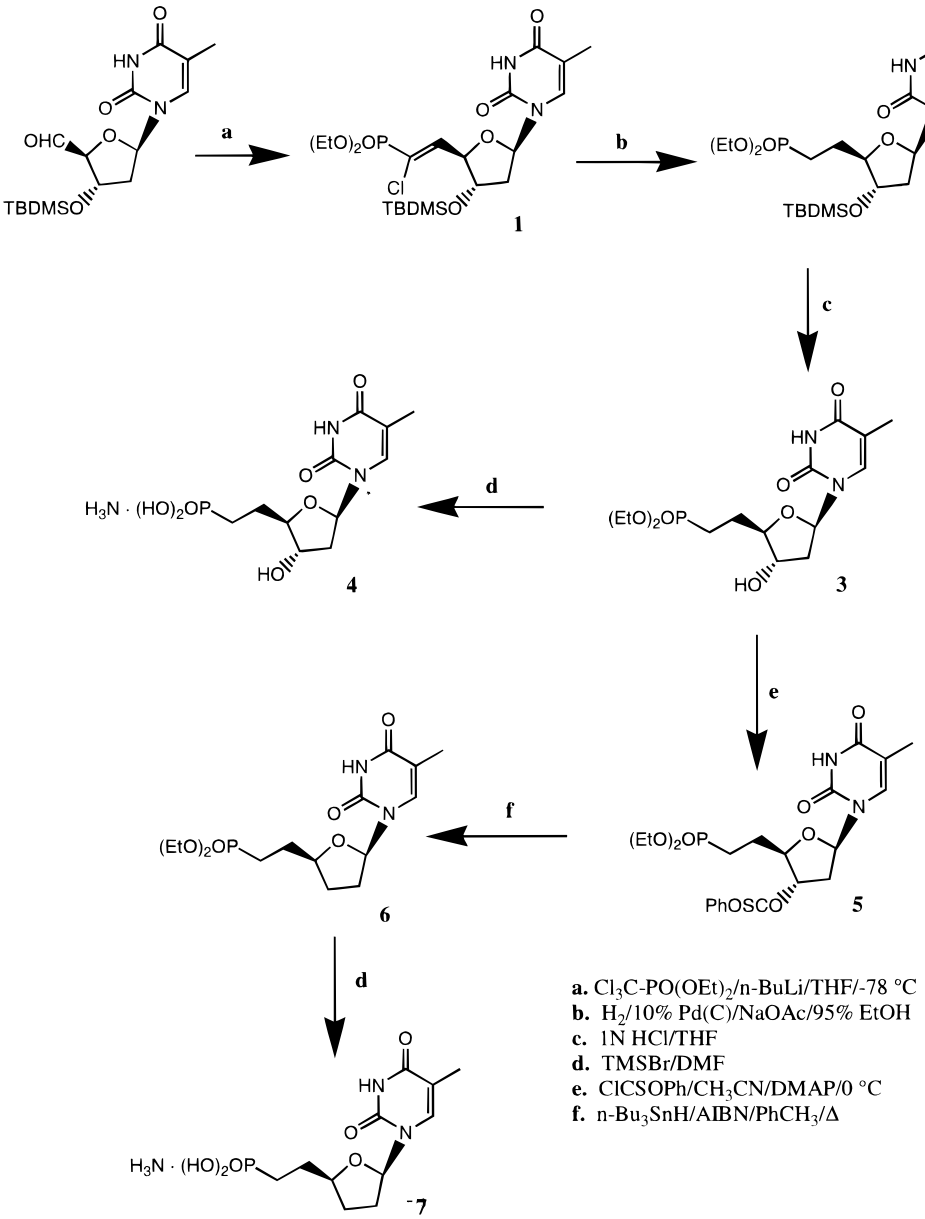
(2R,4S,5R,Z)-1-[4-[(tert-Butyldimethylsilyl)oxy]-5-[2-chloro-2-(diethoxyphosphoryl)vinyl]tetrahydro-2-furyl]thymine (**1**). Under nitrogen, *n*-butyllithium (35.0 mL of a 2.5 M solution, 87.5 mmol) was added dropwise to a −70 °C (internal temperature) solution of diethyl 1,1,1-trichloromethylphosphonate (21.6 g, 84.6 mmol) in THF (200 mL). The resulting red solution was stirred for 1 h at −70 °C before a solution of 1-[3-*O*−(*tert*-butyldimethylsilyl)-2-deoxy-β-D-*erythro*-pentodialdofuranosyl]thymine (*33*) (10.0 g, 28.2 mmol) in THF (150 mL) was added dropwise over 30 min. The reaction was allowed to gradually warm to room temperature over 18 h before being quenched with saturated ammonium chloride (100 mL). The resultant aqueous layer was extracted with ethyl acetate (150 mL). The combined organic layers were concentrated in vacuo and azeotropically mixed with toluene (2 × 200 mL) to remove residual water. Flash chromatography over silica gel with hexane/ethyl acetate (2:1 to 1:5) in increments of increasing polarity provided **1** as an off-white foam (11.8 g, 22.6 mmol, 80%). Proton NMR indicated a single geometric isomer. Since NOE studies of the analogous fluorovinyl phosphonate (not reported in this paper) showed it to be the *Z*-isomer, **1** was similarly designated. ^{1}H NMR (δ) 11.35 (br s, 1 H), 7.61 (s, 1H), 7.01 (dd, J = 13.3 and 8.3 Hz, 1 H), 6.19 (t, J = 6.5 Hz, 1 H), 4.7−4.5 (m, 2 H), 4.2−4.0 (m, 4 H), 2.5−2.4 (m, 1 H), 2.2−2.0 (m, 1 H), 1.82 (s, 3 H), 1.28 (t, J = 7.0 Hz, 6 H), 0.86 (s, 9 H), 0.07 (s, 6 H). Anal. Calcd for $C_{21}H_{36}N_2O_7PClSi$: C, 48.22; H, 6.94; N, 5.36; Cl, 6.78. Found: C, 48.35; H, 6.96; N, 5.28; Cl, 6.67.

(2R,4S,5R)-1-[4-[(tert-Butyldimethylsilyl)oxy]-5-[2-(diethoxyphosphoryl)ethyl]tetrahydro-2-furyl]thymine (**2**). Sodium acetate (3.3 g, 24.4 mmol) and 10% palladium on carbon (2.5 g) were added to a solution of **1** (10.6 g, 20.3 mmol) in 95% ethanol (200 mL) in a 500-mL Parr bottle. The suspension was placed in a Parr apparatus, degassed, and agitated under 40 psi of hydrogen gas for 10 h. The reaction mixture was then filtered through Celite and the cake was washed with ethyl acetate (150 mL). The combined filtrates were concentrated in vacuo to a biphasic residue that was diluted with ethyl acetate (300 mL) and washed with water (100 mL), saturated sodium bicarbonate (50 mL), and brine (50 mL). The organic phase was dried over magnesium sulfate, filtered, and concentrated in vacuo to a pale yellow oil. Flash chromatography over silica gel with chloroform/methanol (98:2 to 90:10) in increments of increasing polarity provided **2** as a white foam (9.0 g, 18.3 mmol, 91%). ^{1}H NMR (δ) 11.40 (br s, 1 H), 7.44 (s, 1 H), 6.13 (t, J = 6.8 Hz, 1 H), 4.3−4.2 (m, 1 H), 4.1−3.9 (m, 4 H), 3.7−3.6 (m, 1 H), 2.4−2.2 (m, 1 H), 2.2−1.6 (m, 5 H), 1.8 (s, 3 H), 1.2 (t, J = 7.0 Hz, 6 H), 0.89 (s, 9 H), 0.11 (s, 6 H). Anal. Calcd for $C_{21}H_{39}N_2O_7PSi$: C, 51.41; H, 8.01; N, 5.71. Found: C, 51.48; H, 8.05; N, 5.69.

(2R,4S,5R)-1-[5-[2-(Diethoxyphosphoryl)ethyl]tetrahydro-4-hydroxy-2-furyl]thymine (**3**). Hydrochloric acid (1 M, 100 mL) was added to a solution of **2** (11.6 g, 23.6 mmol) in THF (300 mL). The resulting solution was stirred for 18 h at room temperature followed by in vacuo removal of the THF. The remaining aqueous phase was neutralized with solid sodium bicarbonate and then saturated with solid sodium chloride. After extraction with ethyl acetate (6 × 50 mL), the combined organic layers were dried over magnesium sulfate, filtered, and concentrated in vacuo to a yellow oil. Flash chromatography over silica gel with chloroform/methanol (97:3 to 90:10) in increments of increasing polarity provided **3** as a white foam (8.55 g, 22.7 mmol, 96%). ^{1}H NMR (δ) 11.35 (br s, 1 H), 7.43 (s, 1 H), 6.14 (t, J = 6.8 Hz, 1 H), 5.32 (d, J = 4.5 Hz, 1 H), 4.1−3.9 (m, 5H), 3.68 (br s, 1 H), 2.3−1.6 (m, 6 H), 1.82 (s, 3 H), 1.24 (t, J = 7.0 Hz, 6 H). Anal. Calcd for $C_{15}H_{25}N_2O_7P$ (0.4 H_2O): C, 46.97; H, 6.78; N, 7.30. Found: C, 46.91; H, 6.58; N, 7.33.

(2R,4S,5R)-1-[Tetrahydro-4-hydroxy-5-(2-phosphonoethyl)-2-furyl]thymine (**4**). Under nitrogen, bromotrimethylsilane (17.2 mL, 130 mmol) was added dropwise to a 0 °C solution of **3** (7.00 g, 18.6 mmol) in DMF (150 mL). The reaction was allowed to reach room temperature and stir for 18 h before the in vacuo removal of DMF. Deionized water (300 mL) was added to the residue along with sufficient 1 M sodium hydroxide to form a pH 9 solution. Ion-exchange chromatography (Bio-Rad) provided **4** as a white powder (4.16 g, 11.3 mmol, 61%). ^{1}H NMR (D_2O) (δ) 7.44 (s, 1 H), 6.22 (t, J = 7.0 Hz, 1 H), 4.3−4.25 (m, 1 H), 3.9−3.85 (m, 1 H), 2.3−2.25 (m, 2 H), 1.85 (s, 3 H), 1.9−1.5 (m, 4 H). Anal. Calcd for $C_{11}H_{17}N_2O_7P$ (1.5 H_2O, 1.2 NH_3): C,

Scheme 1



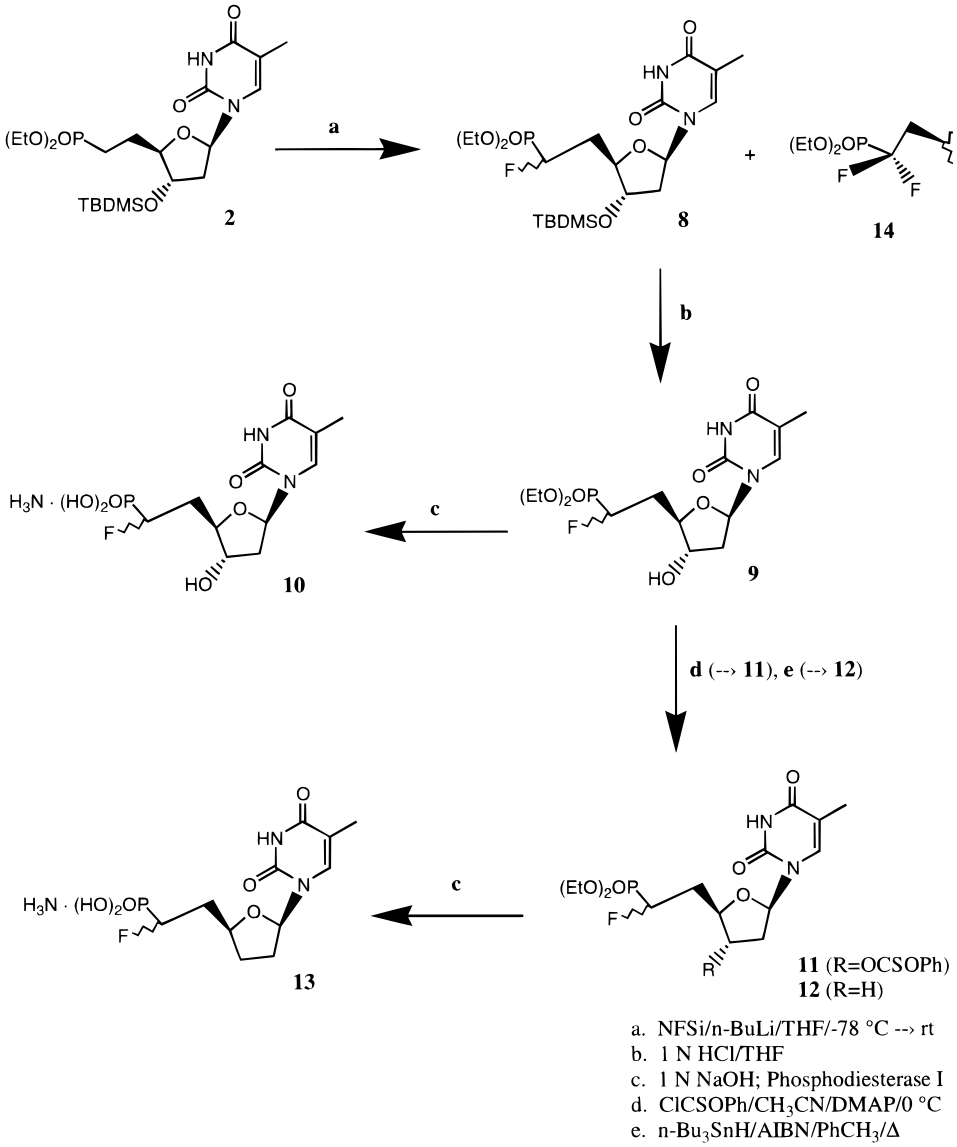
35.93; H, 6.47; N, 12.19. Found: C, 35.80; H, 6.42; N, 12.21.

(2R,4S,5R)-1-[5-[2-(Diethoxyphosphoryl)ethyl]tetrahydro-4-[(phenoxythiocarbonyl)oxy]-2-furyl]thymine (**5**) (method taken from ref *41*). Under nitrogen, 4-(dimethylamino)-pyridine (5.50 g, 45 mmol) was added to a 0 °C solution of **3** (6.76 g, 18.0 mmol) in acetonitrile (250 mL). After 10 min, a solution of phenyl chlorothionoformate (3.73 g, 21.6 mmol) in acetonitrile (20 mL) was added dropwise over 15 min. After 3 h, the resulting yellow suspension was concentrated in vacuo and ethyl acetate (300 mL) and water (100 mL) were added to the residue. The layers were separated and the organic layer was washed sequentially with saturated sodium bicarbonate (50 mL) and brine (50 mL) before being dried over magnesium sulfate, filtered, and concentrated in vacuo. Flash chromatography over silica gel with chloroform/methanol (98:2 to 95:5) in increments of increasing polarity provided **5** as an off-white foam (3.39 g, 6.61 mmol, 37%), which was used immediately in the next step.

(2R,5S)-1-[5-[2-(Diethoxyphosphoryl)ethyl]tetrahydro-2-furyl]thymine (**6**). Under nitrogen, a solution of **5** (3.32 g, 6.48 mmol) in toluene (120 mL) was degassed for 10 min before the addition of tri-*n*-butyltin hydride (4.2 mL, 15.2 mmol) and AIBN (410 mg, 2.50 mmol). The reaction was heated at reflux for 1 h, allowed to cool, and concentrated in vacuo. The oily residue was diluted with acetonitrile (300 mL), washed with hexanes (5 × 100 mL), and concentrated in vacuo. Flash chromatography over silica gel with chloroform/methanol (98:2) gave **6** as a colorless oil (2.06 g, 5.72 mmol, 88%). ^{1}H NMR (δ) 11.31 (s, 1 H), 7.39 (s, 1 H), 6.0−5.95 (m, 1 H), 4.0−3.85 (m, 5 H), 2.4−1.7 (m, 8 H), 1.82 (s, 3 H), 1.24 (t, J = 7.0 Hz, 6 H).

(2R,5S)-1-[Tetrahydro-5-(2-phosphonoethyl)-2-furyl]thymine (**7**). Under nitrogen, bromotrimethylsilane (3.7 mL, 27.5 mmol) was added dropwise to a solution of **6** (1.91 g, 5.30 mmol) in DMF (40 mL). The reaction was allowed to stir for 18 h at room temperature before the in vacuo removal of DMF. Deionized water (85 mL) was added to the residue along with sufficient 1 M sodium hydroxide to form a pH 9

Scheme 2



solution. Ion-exchange chromatography (Bio-Rad) provided **7** as a white powder (631 mg, 1.88 mmol, 35%). Note: The low yield is due to partial glycosidic cleavage of the nucleoside during the acidic conditions of the bromotrimethylsilane reaction. ^{1}H NMR (D_2O) (δ) 7.44 (d, J = 5.2 Hz, 1 H), 6.1−6.0 (m, 1 H), 4.2−4.0 (m, 1 H), 2.5−1.4 (m, 8 H), 1.85 (s, 3 H). Anal. Calcd for $C_{11}H_{17}N_2O_6P$ (0.80 H_2O, 1.00 NH_3): C, 39.36; H, 6.49; N, 12.52. Found: C, 39.42; H, 6.45; N, 12.41.

Scheme 2

(2R,4S,5R)-1-[4-[(tert-Butyldimethylsilyl)oxy]-5-[2-(diethoxyphosphoryl)-2-fluoroethyl]-tetrahydro-2-furyl]thymine (**8**). Under nitrogen, *sec*-butyllithium (31.4 mL of a 1.3 M solution in hexanes, 40.8 mmol) was added dropwise to a −70 °C solution (internal temperature) of **2** (6.68 g, 13.6 mmol) in THF (300 mL) so that the reaction temperature did not exceed −65 °C. After the resulting slurry was stirred for 2 h at −70 °C, a solution of NFSi (12.9 g, 40.9 mmol) in THF (100 mL) was added dropwise over 45 min. The reaction was then allowed to reach room temperature while stirring for 17 h before saturated ammonium chloride (200 mL) was added and the layers were separated. The organic layer was concentrated in vacuo to a residue that was diluted with ethyl acetate (300 mL) and water (100 mL). The layers were separated and the organic layer was washed with brine (50 mL), dried over magnesium sulfate, filtered, and concentrated to a semisolid. Flash chromatography over silica gel with hexane/ethyl acetate (2:1 to 1:4) in increments of increasing polarity provided **8** (2.69 g, 5.29 mmol, 39%) as a viscous oil and the difluoro byproduct **14** (1.89 g, 3.59 mmol, 26%) also as an oil. (The ratios of monofluoro to difluoro products were somewhat different for each reaction run.) ^{1}H NMR (δ) 11.35 (br s, 2 H), 7.52 (s, 1 H), 7.48 (s, 1 H), 6.18 (t, J = 6.7 Hz, 1 H), 6.12 (t, J = 6.7 Hz, 1 H), 5.2−4.8 (m, 2 H), 4.40 (br s, 2 H), 4.2−4.0 (m, 8 H), 3.85 (br s, 2 H), 2.4−2.0 (m, 8 H), 1.81 (br s, 6 H), 1.28 (t, J = 7.0 Hz, 12 H), 0.89 (br s, 18 H), 0.10 (br s, 12 H). ^{31}P NMR (δ) 19.05, 18.92, 18.46, 18.32. Anal. Calcd for $C_{21}H_{38}N_2O_7SiFP$: C, 49.59; H, 7.53; N, 5.51. Found: C, 49.77; H, 7.59; N, 5.41.

(2R,4S,5R)-1-[5-[(RS)-2-(Diethoxyphosphoryl)-2-fluoroethyl]tetrahydro-4-hydroxy-2-furyl]thymine (**9**) was made from **8** in an analogous synthesis as 3 from 2. ^{1}H NMR (δ)

11.33 (br s, 2 H), 7.48 (s, 1 H), 7.43 (s, 1 H), 6.175 (t, J = 7.0 Hz, 1 H), 6.11 (t, J = 7.0 Hz, 1 H) 5.4−5.3 (m, 2 H), 5.2−4.8 (m, 2 H), 4.2−4.0 (m, 10 H), 3.9−3.7 (m, 2 H), 2.4−1.9 (m, 8 H), 1.81 (br s, 6 H), 1.31 (t, J = 7.0 Hz, 12 H). ^{31}P NMR (ppm) 19.27, 19.08, 18.68, 18.49. Anal. Calcd for $C_{15}H_{24}N_2O_7FP$ (0.10 EtOAc): C, 45.88; H, 6.20; N, 6.95. Found: C, 46.10; H, 6.18; N, 7.08.

(2R,4S,5R)-1-[5-(2-Fluoro-2-phosphonoethyl)tetrahydro-4-hydroxy-2-furyl]thymine (**10**). A solution of **9** (0.92 g, 2.33 mmol) in 1 N sodium hydroxide (40 mL) in a 125-mL Erlenmeyer flask was stirred at room temperature for 1.5 h before being washed with ethyl acetate (2 × 25 mL) and acidified with concentrated hydrochloric acid (to pH 9). Phosphodiesterase I from *Crotalus* sp. (300 units) was added and the reaction was stirred for 10 days. During this time, the pH of the reaction was maintained at pH 9 by the occasional addition of 0.1 N sodium hydroxide. Ion-exchange chromatography (Sephadex) provided **10** (0.26 g, 0.77 mmol, 33%) as a white powder. Proton NMR indicated that only one diastereoisomer was present. ^{1}H NMR (D_2O) (one isomer) (δ) 7.45 (s, 1 H), 6.10 (t, J = 6.5 Hz, 1 H), 4.6−4.5 (m, 1 H), 4.4−4.3 (m, 1 H), 4.2−4.1 (m, 1 H), 3.9−3.8 (m, 1 H), 2.4−1.8 (m, 2 H), 1.81 (s, 3 H). Anal. Calcd for $C_{11}H_{16}N_2O_7FP$ (1.75 H_2O, 1.30 NH_3): C, 33.71; H, 6.02; N, 11.79. Found: C, 33.68; H, 5.96; N, 11.73.

(2R,4S,5R)-1-[5-[2-(Diethoxyphosphoryl)-2-fluoroethyl]-tetrahydro-4-[(phenoxythiocarbonyl)oxy]-2-furyl]thymine (**11**) was made from **9** in an analogous synthesis as **5** from **3** and used immediately in the next step.

(2R,5S)-1-[5-[2-(Diethoxyphosphoryl)-2-fluoroethyl]-tetrahydro-2-furyl]thymine (**12**) was made from **11** in an analogous synthesis as **6** from **5**. ^{1}H NMR (δ) 11.30 (br s, 2 H), 7.45 (br s, 1 H), 7.40 (br s, 1 H), 6.0 (m, 2 H), 5.3−5.2 (m, 1 H), 5.1−4.9 (m, 1 H), 4.2−4.0 (m, 10 H), 2.3−1.8 (m, 12 H), 1.81 (br s, 6 H), 1.28 (t, J = 7.0 Hz, 12H). Anal. Calcd for $C_{15}H_{24}N_2O_6FP$ (0.60 H_2O): C, 46.30; H, 6.53; N, 7.20. Found: C, 46.23; H, 6.35; N, 7.05.

(2R,5R)-1-[5-(2-Fluoro-2-phosphonoethyl)tetrahydro-2-furyl]thymine (**13**) was made from **12** in an analogous synthesis as **10** from **9**. Proton NMR indicated that both diastereoisomers were present. ^{1}H NMR (D_2O) (δ) 7.40 (br s, 2 H), 6.0−5.9 (m, 2 H), 4.6−4.5 (m, 1 H), 4.4−4.2 (m, 1 H), 4.2−4.0 (m, 2 H), 2.4−1.6 (m, 12 H), 1.81 (br s, 6 H). Anal. Calcd for $C_{11}H_{16}N_2O_6FP$ (1.20 H_2O, 1.40 NH_3): C, 35.93; H, 6.20; N, 12.95. Found: C, 35.96; H, 6.27; N, 12.94.

Enzyme Purifications. c-N-I from rabbit heart was purified to homogeneity using the procedure of Yamazaki et al. (*15*), except for two minor alterations. The concentration of glycerol was lowered from 25% to 15%, and the crude extract was prepared by homogenization in a Waring blender (3 times 1 min, with cooling between). After the AMP−Sepharose chromatography step, 5′-NTase was greater than 90% homogeneous (as analyzed by SDS−PAGE) and had a specific activity of 71 μmol min^{-1} mg using AMP as a substrate under standard conditions.

e-N was purified from rabbit heart using a modified procedure of Naito and Lowenstein (*18*). The first two steps of extraction of e-N from the membrane fraction and heat and pH 5.4 treatment were the same. The preparation at this stage was desalted through a G-25 Sephadex column to remove the ADP from the previous step. The sample was applied directly to ADP−agarose and eluted as described (*18*). Again, the preparation was desalted through a G-25 Sephadex column to remove the AMP that was needed to elute the e-N. The specific activity after this step was 10 μmol min^{-1} mg^{-1} with AMP as the substrate.

c-N-II was purified from human placenta according to the procedure of Spychala et al. (*19*), as previously modified (*20*).

Enzyme Assays. c-N-I was assayed in 50 mM MOPS−NaOH buffer, pH 6.9, containing 3 mM $MgCl_2$ and 1 mM ADP (as activator), with varying concentrations of radiolabeled nucleoside monophosphate as substrate, in 20-μL volumes, at 37 °C. Aliquots (2 μL) were spotted onto poly(ethylenimine) thin-layer chromatography plates prespotted with 10 nmol of unlabeled nucleoside product. Plates were developed in 50% MeOH. Product and substrate were quantitated by visualizing and cutting out UV spots and counting the radioactivity by liquid scintillation spectrophotometry with ScintiLene (Fisher). All reactions were analyzed within the initial 15% conversion of substrate. One unit of enzyme is the amount that converts 1 μmol of labeled substrate to product/min.

e-N was assayed in 50 mM Tris-HCl (pH 7.5), 1.6 mM sodium cholate, and 5 mM $MgCl_2$, with varying concentrations of radiolabeled nucleoside monophosphate as substrate, as described above. Adenosine phosphotransferase and c-N-II were assayed using radiolabeled nucleoside or nucleotide substrates with thin-layer chromatography analysis as previously described (*21*). Adenosine phosphotransferase activity was assayed with 0.5 mM radiolabeled adenosine, 1 mM AMP (as donor), and 1 mM ADP (as activator). c-N-II was assayed in a dephosphorylation reaction with 0.5 mM radiolabeled GMP and in the phosphorylation reaction with 0.25 mM radiolabeled inosine and 2.5 mM IMP (as donor).

Analysis of Kinetics. c-N-1 is an allosteric enzyme and shows a sigmoidal AMP saturation curve in the absence of activator ADP and a hyperbolic curve in the presence of saturating ADP (*15*). In this study, all kinetic constants were determined at saturating activator ADP (1 mM) and therefore all are apparent constants. Steady-state kinetics to determine kinetic constants were performed with a minimum of five substrate concentrations that spanned the apparent K_m value. The apparent inhibition constant (K_i) of a compound was determined from its ability to inhibit the hydrolysis of AMP (at a single concentration of substrate, 0.5 mM). A minimum of four concentrations of inhibitor were used and the resulting data were analyzed by a Dixon plot (*22*). To determine the type of inhibition pattern, the substrate was varied at a minimum of four concentrations of inhibitor. The fitted data were analyzed (*23*) for conformity to the competitive or noncompetitive model. K_i values were determined from a weighted, least-squares fit (*24*) of the data at each substrate concentration to the rate equation for competitive or noncompetitive inhibition.

RESULTS AND DISCUSSION

Heart 5′-NTase Was Not a Phosphotransferase. On the basis of similiar activation by ADP and a similiar sigmoidal velocity versus substrate curve in the absence of effectors, a formal possibility existed that heart c-N-I was adenosine phosphotransferase (*21*), an enzyme for which an in vivo

Table 1: Substrate Specificity of Heart Cytosolic 5′-Nucleotidase[a]

substrate	relative V_{max} (%)	apparent K_m[b] (mM)	V_{max}/K_m	apparent K_i[c] (mM)
AMP	100	1.2	83	0.87
dAMP	21	0.13	160	0.13
IMP	39	1.0	39	1.1
dIMP				0.21
GMP	26	0.32	81	0.58
dGMP	12	0.12	97	0.19
TMP	14	0.020	710	0.058
CMP	14	0.11	130	0.090
dCMP	18	0.061	300	0.069
UMP				0.080
dUMP				0.040

[a] Standard errors are within ±15%. [b] Apparent K_m values were determined by varying the concentration of nucleotide at a single fixed concentration of activator ADP (1 mM). [c] Determined by varying inhibitor concentration at a fixed concentration of substrate AMP (0.5 mM) and activator ADP (1 mM); except for the apparent K_i value of AMP, which was determined by varying AMP concentration at a fixed concentration substrate TMP (0.02 mM) and activator ADP (1 mM).

function is not known. However, when purified heart c-N-I (at a concentration that would hydrolyzed AMP at a rate of 1.5 nmol/min) was assayed for adenosine phosphotransferase (see Materials and Methods section), no phosphorylation of adenosine by AMP was observed (<0.04% the rate of AMP hydrolysis). Therefore, heart c-N-I was a hydrolase without phosphotransfer activity.

Substrate Specificity. c-N-I from the hearts of rabbit (*15*, *25*), dog (*26*), rat (*27*), and pigeon heart (*28*) have previously been studied. Substrate specificity had been examined mostly by relative rates and, except for the report on pigeon heart 5′-NTase, had been developed only for purine nucleoside monophosphates. From the results of these studies, this enzyme had been designated as AMP-specific.

We examined all the naturally occurring purine and pyrimidine nucleoside monophosphates as substrates of c-N-I or inhibitors of AMP hydrolysis. The apparent kinetic constants are shown in Table 1. Consistent with AMP showing the largest relative rate among purine and pyrimidine nucleoside monophosphates for the pigeon enzyme (*28*), AMP hydrolysis by the rabbit enzyme displayed the greatest V_{max}. However, AMP was not the most efficient substrate, as measured by V_{max}/apparent K_m. Whereas purine nucleoside monophosphates tended to show greater V_{max} values, pyrimidine nucleoside monophosphates displayed even correspondingly smaller apparent K_m values. For example, the apparent K_m value for TMP was 60 times lower than that for AMP; contrasted with a 6-fold lower V_{max} value. Thus, TMP was approximately 10 times more efficient than AMP as a substrate. Consequently, when substrate efficiency was measured, the heart 5′-NTase was not AMP-specific but rather pyrimidine monophosphate-specific. A second characteristic was that deoxyribonucleoside monophosphates had a smaller apparent kinetic constant (K_m or K_i) than their ribonucleoside monophosphate counterparts. This trend continued to even tighter binding for dideoxynucleoside monophosphates and dideoxynuclesides (see below).

The stimulation of AMP hydrolysis by ADP has been reported for c-N-I from rabbit (*15*), dog (*26*), and rat (*27*). When the concentration of ADP was varied from 0 to 1 mM, the same dependence of initial velocity on ADP concentration was observed for TMP hydrolysis as for AMP hydrolysis (data not shown). There was a 6-fold increase in the rate of hydrolysis, with the concentration that produced half-maximal effect being 50 μM. This dependence was the same as that reported previously for ADP stimulation of AMP hydrolysis by rabbit heart c-N-I (*15*).

When either the concentration of AMP was varied at constant concentrations of TMP or the concentration of TMP was varied at constant concentrations of AMP, at a fixed concentration of activator ADP (see Materials and Methods section), competitive inhibition patterns were observed (data not shown). The apparent K_i values from these studies for TMP and AMP were 0.074 and 0.87 mM, respectively. The competitive nature of inhibition, in conjunction with the similiarity between apparent K_m and K_i values for individual nucleoside monophosphates (Table 1), indicated a single binding site for both purine and pyrimidine nucleoside monophosphates.

Product Inhibition Studies. As a prelude to the development of nucleoside analogues as inhibitors of c-N-I (see below), product inhibition studies were performed. TMP was chosen as the substrate due to the relative potency of product inhibition by thymidine (see below). When TMP was varied at fixed concentrations of product thymidine (at a single fixed concentration of activator ADP, 1 mM), noncompetitive inhibition (data not shown) was observed (with an apparent K_I value for thymidine of 0.51 mM). When the product phosphate was the inhibitor, competitive inhibition (data not shown) was observed (with an apparent K_I value for phosphate of 0.51 mM). These kinetic patterns are consistent with an *ordered uni-bi* kinetic mechanism (*29*) with nucleoside product releasing prior to inorganic phosphate. When coupled with a lack of phosphotransferase activity, these patterns are consistent with c-N-I proceeding through a direct displacement by water of phosphate from the nucleoside monophosphate substrate. This is in contrast to the mechanism of a nucleoside phosphotransferase that proceeds through a phosphorylenzyme intermediate (*30, 31*).

Nucleotide Analogues as Inhibitors of Heart c-N-I. We were interested in exploring 5′-phosphonates (isosteric with a nucleoside 5′-monophosphate) as nonhydrolyzable inhibitors of c-N-I. We focused our attention on the 5′-phosphonates of thymidine and its analogues, because TMP had the lowest apparent K_m value. We had recently developed a new method for the synthesis of chlorovinylphosphonates using commercially available diethyl 1,1,1-trichloromethylphosphonate (*32*). When the known aldehyde (*33*) was reacted with 1,1,1-trichloromethylphosphonate and *n*-butyllithium, the chlorovinylphosphonate **1** was generated. This and the remaining chemistry is depicted in Scheme 1.

These phosphonate analogues of TMP were potent inhibitors of the c-N-I-catalyzed hydrolysis of AMP (Table 2). The most potent inhibitor was **7**, which is isosteric to the monophosphate of ddT. Deoxythymidine 5′-phosphonate was competitive with respect to TMP (data not shown), and the apparent K_i value was 63 nM. As noted above, when both the 2′-deoxyribo and 2′,3′-dideoxyribo analogues of the same phosphonate were tested, the dideoxy analogue was more potent. When the negative charges of thymidine 5′-phosphonate were masked with ester linkages (**3**), no inhibition was detectable, suggesting that either the negative charge(s) or correct steric interactions are crucial for binding.

Table 2: Inhibition of Heart Cytosolic 5′-Nucleotidase by 5′-Phosphonates[a]

phosphonates of	apparent K_i (μM)
thymidine	
parent (**4**)	0.15
6′-monofluoro (**10**)	1.7
diethyl ester (**3**)	>10 000
dideoxythymidine	
parent (**7**)	0.063
6′-monofluoro (**13**)	0.46

[a] Inhibition of AMP hydrolysis under standard assay conditions at a single fixed concentration of activator ADP (1 mM). Standard errors are within ±15%.

Table 3: Inhibition of Heart Cytosolic 5′-Nucleotidase by Nucleosides[a]

nucleoside	apparent K_i (mM)
dAdo	5% inhibition at 1 mM
ddA	13% inhibition at 1 mM
dGuo	20% inhibition at 1 mM
ddG	15% inhibition at 1 mM
dIno	13% inhibition at 1 mM
ddI	22% inhibition at 1 mM
Thd	0.42
5′-deoxyThd	0.49
ddT	0.019
d4T	0.10
AZT	0.056
dUrd	0.26
ddU	0.017
dCyd	0.33
ddC	0.020

[a] Inhibition of AMP hydrolysis under standard assay conditions conditions at a single fixed concentration of activator ADP (1 mM). Standard errors are within ±15%.

In considering the effect of the phosphonate's negative charges on binding, we measured the pK_{a2} of thymidine 5′-phosphonate (**4**) to be 7.80 ± 0.04. Therefore, at pH 6.9 (the pH of the assay), the phosphonate will have predominantly one negative charge. Thus, the second negative charge of the phosphonate apparently is not critical for binding.

The effect of lowering the pK_{a2} of the phosphonate on the potency of inhibition was examined by substituting a fluorine for hydrogen on the carbon adjacent to the phosphorus. The measured pK_{a2} for the fluorophosphonate (**10**) was 6.67 ± 0.01. Thus, at pH 6.9 of the assay, approximately equal amounts of singly and doubly charged phosphonate would be present. The apparent K_i value for the phosphonate was 1.7 μM, compared to the apparent K_i value of 0.15 μM of the parent phosphonate. Thus, *if* the fluorine's only effect is on pK_{a2}, it appears that the introduction of the second negative charge on the phosphonate is actually detrimental to binding.

Nucleoside Analogues as Inhibitors of Heart c-N-I. As stated above, thymidine was a product inhibitor of the c-N-I-catalyzed hydrolysis of TMP. To develop more potent nucleoside inhibitors of c-N-I, we examined the naturally occurring nucleosides, deoxyribonucleosides, and their dideoxyribonucleoside analogues as inhibitors of AMP hydrolysis (Table 3). At a concentration of 1 mM nucleoside, no inhibition was observed with any ribonucleoside. However, the natural deoxyribonucleosides produced some inhibition at 1 mM, with the same trend of pyrimidines being more potent than purines as was observed in nucleotide substrate specificity studies. Likewise, as observed with the phosphonate inhibitors, 2′,3′-dideoxyribonucleosides were more potent than the respective deoxyribonucleoside. Thus, all three pyrimidine 2′,3′-dideoxyribonucleosides, ddT, ddU, and ddC, were relatively good inhibitors of heart NTase with apparent K_i values of 17−20 μM. A weakening of binding was observed with either the 2′,3′-ene or the 3′-azido derivative of ddT; d4T and AZT were both 3-fold less potent than ddT. Finally, when the concentration of AMP was varied at constant concentrations of ddT, a noncompetitive inhibition pattern was observed in the reciprocal velocity versus reciprocal substrate plot (data not shown). This pattern was consistent with ddT acting as a product analogue in a *uni-bi ordered* kinetic mechanism.

Table 4: Inhibition of Heart Cytosolic 5′-Nucleotidase by 5-Substituted dUrd Analogues[a]

5-substitution of dUrd	apparent K_i (mM)
ethynyl	0.025
bromovinyl	0.15
propargyl	0.15
vinyl	0.25
fluoro	0.25

[a] Inhibition of AMP hydrolysis under standard assay conditions conditions at a single fixed concentration of activator ADP (1 mM). Standard errors are within ±15%.

It is interesting to note that 5′-deoxythymidine had approximately the same apparent K_i value as thymidine. Therefore, the 5′-hydroxyl does not appear to participate significantly in the binding of the nucleoside product.

As a further development of nucleoside inhibitors, a series of 5-substituted dUrd compounds were examined for inhibition of c-N-I (Table 4). Whereas the 5-substitution of fluoro or vinyl had no effect and bromovinyl or propynyl had little effect, the ethynyl substitution led to significantly tighter binding. This substitution reduced the apparent K_i value to 25 μM, approximately 10-fold more potent than dUrd. The affinity was further increased by substituting 2′-deoxyribose with 2′,3′-dideoxyribose to make 5-ethynyl-ddU. This nucleoside analogue had a apparent K_i value of 3.7 ± 0.5 μM. As observed with ddT, this inhibitor was noncompetitive with the substrate AMP (data not shown).

The stereochemical requirements for nucleoside binding to c-N-I were minimally explored. The two enantiomers of the nucleoside analogue *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine (FTC; ref *34*) were tested as analogues of ddC. Whereas the analogue that corresponded to the natural 1-β-D configuration [(−)-FTC] was slightly more potent than ddC (apparent K_i value of 11 μM), (+)-FTC with a 1-β-L configuration was approximately 60-fold weaker (apparent K_i value of 690 μM). In addition, 1-α-D-thymidine (apparent K_i value of 1.2 mM) was approximately 4-fold weaker than its natural counterpart. On the basis of this limited survey, c-N-I appears to have a preference for the natural 1-β-D configuration.

Selectivity of Inhibitors of Heart c-N-I versus e-N and c-N-II. The selectivity of inhibition by the nucleotide and nucleoside inhibitors of heart c-N-I was examined by surveying the most potent inhibitors against the two other known 5′-nucleotidases found in heart: e-N and c-N-II. e-N from rabbit heart was purified and verified to be the ecto-

5′-nucleotidase by its substrate preference of AMP > GMP > dAMP > TMP (data not shown), and its inhibition by AMPCP, ADP, and ATP (apparent K_i values of 0.09, 1.9, and 37 μM, respectively). When ddT or 5-ethynyl ddU were tested at 1 mM as inhibitors of e-N, no inhibition was observed. Therefore, these nucleoside inhibitors were extremely selective inhibitors of c-N-I vs e-N, with 5-ethynyl-ddU being greater than 1000-fold selective. However, in contrast, thymidine 5′-phosphonate and ddT 5′-phosphonate *were* inhibitors of e-N. Thymidine 5′-phosphonate was a competitive inhibitor with respect with dAMP, and its K_i value was 5.8 μM. The phosphonate of ddT had an apparent K_i value of 12 μM. Thus, these two phosphonate inhibitors were 40- and 200-fold more selective for the heart c-N-I vs e-N.

When c-N-II was assayed in the presence of either 1 mM ddT 5′-phosphonate or 5-ethynyl-ddU, no inhibition of either GMP hydrolysis (in the absence of nucleoside acceptor) or inosine phosphorylation (in the presence of IMP as donor and inosine as acceptor) was observed. Thus, both classes of inhibitors were extremely selective (>1000-fold) for c-N-I compared to c-N-II. c-N-II displays a strong preference for IMP and GMP and their 2′-deoxyribonucleotides. Pyrimidine 2′-deoxyribonucleoside monophosphates are its poorest substrates among the natural nucleotides (*19*). In addition, because c-N-II has phosphotransferase activity (*31*), nucleosides are substrates, not inhibitors, with nucleoside substrate specificity reflective (*35*) of nucleotide specificity (i.e., purine nucleosides are utilized much more efficiently than pyrimidine nucleosides). Therefore, the absence of inhibition by a pyrimidine phosphonate or dideoxynucleoside is consistent with the substrate specificity of c-N-II.

Summary. A fuller understanding of the substrate specificity of heart c-N-I was essential to the development of potent inhibitors of this enzyme. The distinct preference (as measured by apparent K_m values) for pyrimidine 2′-deoxyribonucleoside monophosphates focused direction for both nucleotide and nucleoside inhibitors.

Although nucleoside phosphonates were 300−1000-fold more potent against the c-N-I than the corresponding nucleoside analogues, two characteristics may interfere with their use in cellular or animal studies. First, the charged nature of the phosphonate may hinder cellular penetration. The use of phosphonates as effective antivirals has left an ambiguous answer to the question of their cellular penetration. Some (mostly acyclic nucleoside phosphonates) have biological activity (*36*−*38*); others do not (*39*, *40*). (Inactivity of antiviral phosphonates could also be explained by lack of anabolism to the active triphosphate analogue.) Second, the selectivity of the phosphonate for c-N-I versus e-N most likely is not great enough. The amount of phosphonate necessary to obtain intracellular inhibition of the c-N-I would probably significantly, if not completely, inhibit e-N.

We are currently using our most potent nucleoside and nucleotide inhibitors in experiments to test their biological effects in primary myocytes. The results may clarify the issue of cellular penetration by phosphonates and, we hope, the larger issues of where and how AMP is hydrolyzed in myocardial ischemia.

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