Inhibition of Adenosine Deaminase by Analogues of Adenosine and Inosine, Incorporating a Common Heterocyclic Base, 4(7)-Amino-6(5)*H*-imidazo[4,5-*d*]pyridazin-7(4)one

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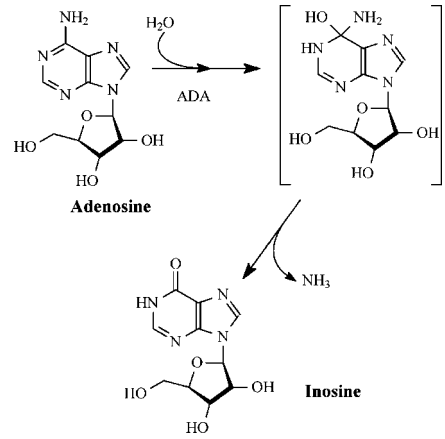
Four nucleoside analogues (**1**−**4**) containing a common heterocyclic base, 4(7)-amino-6(5)*H*-imidazo[4,5-*d*]pyridazin-7(4)one, were screened against calf-intestine adenosine deaminase. Compounds **1** and **3** with K_i values of 10–12 μM are more than four times as potent inhibitors of ADA compared with **2** and **4**, with K_i values of 51–52 μM. Also, **3** is not a substrate of ADA. Nucleosides **3** and **4** also exhibit moderate in vitro activity against breast cancer cell lines, while all four are only minimally or nontoxic to the normal cells.

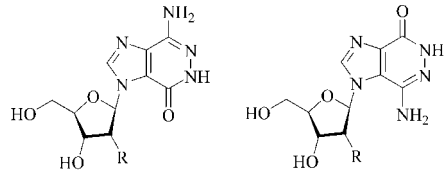
Introduction

Adenosine deaminase (ADA) is an important metabolic enzyme that plays a crucial role in many human disorders involving the immune system and function.[1–4] Inhibition of adenosine deaminase for immunosuppression has been the objective of many investigations for the selective treatment of lympho- proliferative malignancies.[5–7] However, because ADA is also an important essential enzyme, its inhibition cannot be for an unlimited duration or extent. Therefore, the most desirable inhibitors of ADA would not only be potent, but also be considerably short-acting and easily reversible.[8–12] In this context, while there exist a host of known ADA inhibitors,[2,13–20] many, if not all, suffer from one or the other drawbacks, including too high potency with total irreversibility or too long duration of action.[10,11,21] For example, coformycin[22–24] and pentostatin (2′-deoxycoformycin),[20,25,26] the two naturally occurring, strongest known inhibitors of ADA with K_i values in the 10^{-11-12} M range,[27–30] have not been clinically as successful as anticipated because of their associated toxicity,[11,12,31] with an exception of pentostatin in the treatment of hairy cell leukemia.[32–36] The coformycins are believed to be extremely tight-binding, transition state analogue inhibitors of ADA,[27–30] which lead to the total demise of the enzyme upon binding.[10] So, the search must continue for ADA inhibitors with optimal potency combined with a short duration of action and ready reversibility. Our laboratory has been involved in such a quest for some time,[2,8,9,37–39] and this work represents our continued effort in that direction.

We have recently reported the synthesis and self-complementary base-pairing properties of two novel nucleoside analogues **1** and **2** containing the title heterocyclic ring system.[40] Because of their structural resemblance to adenosine and inosine, respectively, compounds **1** and **2** were considered as potential substrate and product analogues of the reaction catalyzed by ADA (see Scheme 1). Therefore, it seemed logical to biochemically screen **1** and **2**, along with their respective ribose counterparts **3** and **4**, as likely substrates or inhibitors (antimetabolites) of ADA. ADA catalyzes the hydrolysis of both adenosine and 2′-deoxyadenosine into their respective inosines with nearly equal efficiency. As we have already reported the synthesis of **1** and **2** recently,[40] only the synthesis of ribose analogues **3** and **4** have been described here.

Scheme 1. Hydrolysis of Adenosine to Inosine Catalyzed by Adenosine Deaminase (ADA)





1, R = H,
3, R = OH

2, R = H,
4, R = OH

Chemistry. The synthesis of the target nucleosides **3** and **4** (Scheme 2) commenced with ethyl 5(4)-cyano-1*H*-imidazole-4(5)carboxylate (**5**).[41] The Vorbrüggen ribosylation[42,43] of the latter with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**6**) at 5 °C yielded the two regioisomeric nucleosides **7** and **8** in 67 and 18%, respectively. The isomers were separated by flash chromatography using a mixture of 5:1 hexanes/ethyl acetate, but their structures were not assigned until after ring closure with hydrazine in the next step. It was easier to distinguish them from each other via a structural match of one of the isomers to the known compound **3**, which was independently synthesized by an altogether different route by Berry et al.[44] several years ago, starting from an imidazole-fused oxadiazole. Compounds

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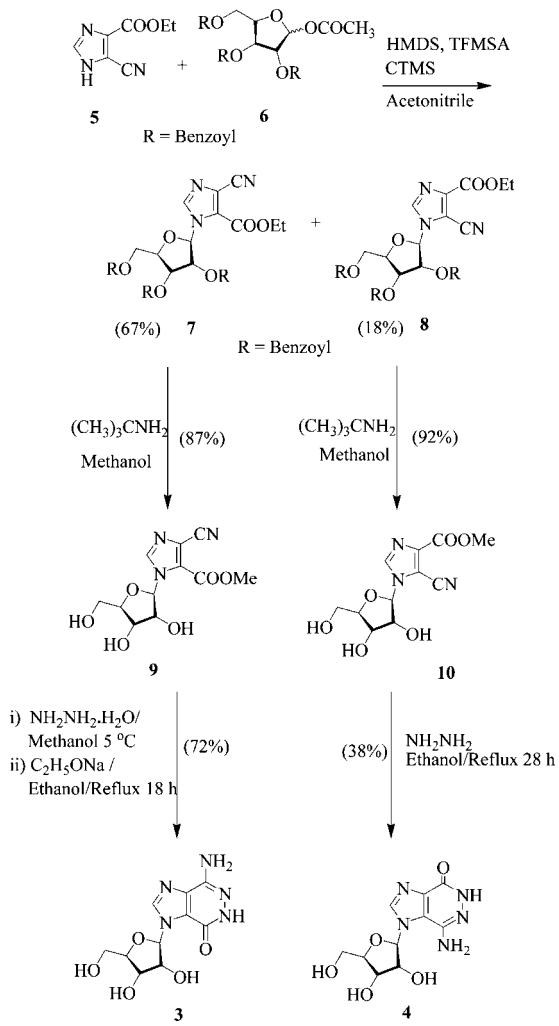
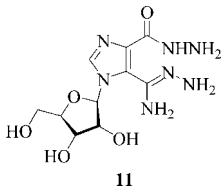
Scheme 2. Synthesis of Nucleosides **3** and **4**



7 and **8** were independently reacted with excess *t*-butylamine in anhydrous methanol at room temperature to obtain **9** and **10** in 87 and 92% yield, respectively. The two compounds were separately reacted with hydrazine at 5 °C for 30 min, followed by heating with sodium ethoxide in ethanol at reflux for 18–28 h to yield the respective target nucleosides **3** and **4**. While the use of excess hydrazine with **9** did not affect the outcome of the reaction, it produced an unexpected bis-adduct **11** in the case of **10**.



Therefore, only 1 equiv of hydrazine had to be employed in the reaction with **10** to obtain the desired product **4**. Both **3** and **4** were fully characterized by ^{1}H NMR, ^{13}C NMR, IR, HRMS, and elemental microanalytical data. The physicochemical data for **3** are completely consistent with those reported by Berry et al.[44]

Results and Discussion

Compounds **1**−**4** and **11** were assessed for inhibitory activity against ADA from calf intestine (Sigma), employing the protocol

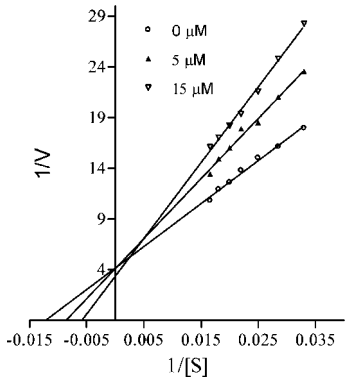


Figure 1. Lineweaver–Burk plot of compound **1**.

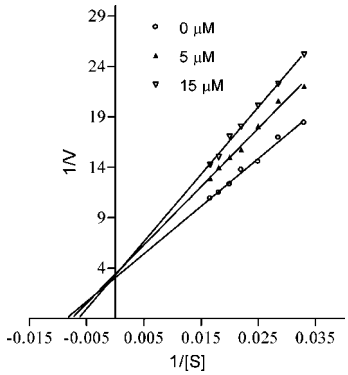


Figure 2. Lineweaver–Burk plot of compound **2**.

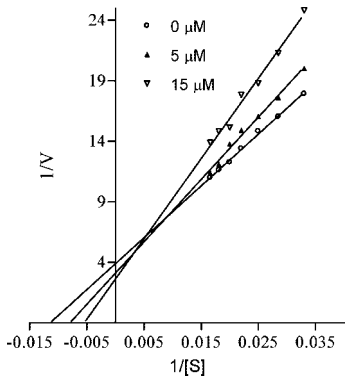


Figure 3. Lineweaver–Burk plot of compound **3**.

that we reported earlier.[8,9,37,39] All studies were carried out at 25 °C and pH 7.4 by spectroscopic measurements of the rate of hydrolysis of the substrate adenosine into product inosine at λ_{max} 265 nm. The change in optical density at λ_{max} 265 nm per unit time was used as a measure of the enzyme activity. Seven different concentrations of the substrate adenosine, ranging from 30 to 60 μM, were employed for each inhibitor concentration that was either 5 or 15 μM, while the amount of enzyme in each assay was 2×10^{-3} unit. The Lineweaver–Burk plots ($1/V$ vs $1/S$), shown in Figures 1–4, were used to calculate the K_i values, which are listed in Table 1.

The biochemical screening results clearly suggest that, out of the four nucleosides **1**–**4**, two of them, namely, **1** and **3**, which structurally resemble the substrate adenosine, have K_i values ranging 10–12 μM, while the other two, namely, **2** and **4**, which mimic inosine, the product of ADA-mediated hydrolysis, possess K_i values in the 51–52 μM range, suggesting that **1** and **3** are more than **4** times as potent as **2** and **4**. Also, the ribose

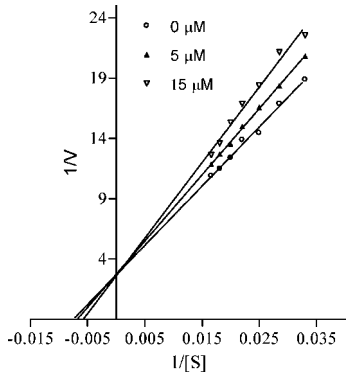


Figure 4. Lineweaver–Burk plot of compound **4**.

Table 1. Inhibition Constants (K_i) for Compounds **1**−**4** and **11** against Calf Intestine ADA

cmpd	**1**	**2**	**3**	**4**	**11**
K_i (μM)	12.14 ± 0.31	52.01 ± 1.6	10.62 ± 0.89	51.44 ± 3.26	26.54 ± 0.39

Table 2. Cytotoxic Effects of Nucleosides 1–4 in Cancer (MCF7) and Normal (MCF10A) Breast Cell Lines

cmpd No.	**MCF7** (human breast cancer cell line) IC_{50} (μM)	**MCF10A** (normal human breast cell line) IC_{50} (μM)
1	>100 ± 0	>100 ± 0
2	97.5 ± 3.54	>100 ± 0
3	27.5 ± 3.54	82.5 ± 3.54
4	23.5 ± 2.1	82.5 ± 3.54

analogues **3** and **4** were only marginally superior to their respective 2′-deoxy counterparts **1** and **2**, as anticipated.

Finally, because of its close structural similarity to adenosine, it was of interest to see if **3** also acted as a substrate of ADA to form the corresponding dicarbonyl compound **HMC-HO5**, whose synthesis, along with its 2′-OMe analogue **HMC-HO4**, was reported by us several years ago.[45] In that case, **3** would be considered an antimetabolite of the enzyme's natural substrate adenosine. It was convenient to monitor the product formation of the ADA reaction with **3** through a simple comparison with the UV λ_{max} of the authentic product. Our results show that while the λ_{max} (264.9 nm) of the starting material had changed to a slightly higher wavelength in the product, it fell significantly short of the expected λ_{max} (275 nm) of the product. Furthermore, the general UV profile of the ADA reaction product was noticeably different than that of the expected product. Therefore, we conclude that **3** is not a substrate of ADA or an antimetabolite of adenosine.

Finally, nucleosides **1**–**4** were screened in vitro for anticancer activity as well as toxicity in human breast cell lines. The MTT assay was used to determine the drug effects on cell proliferation using the breast cancer cell lines MCF7, while toxicity was assessed using the normal breast cell lines MCF10A, following the assay procedure as described by Alley et al.[46] The results are collected in Table 2 and are graphically represented in Figure 5(A−D). The data reveal that while **3** and **4**, both of which are ribose analogues, show moderate anticancer activity, all four nucleosides are minimally or nontoxic to the normal breast cell lines.

Experimental Section

Ethyl 4-Cyano-1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)imidazole-5-carboxylate (7) and Ethyl 5-Cyano-1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)imidazole-4-carboxylate (8). A solution of 5-cyano-1*H*-imidazole-4-carboxylic acid ethyl ester (**5**; 3.30 g, 20 mmol) and 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (10.08 g, 20 mmol) in anhyd acetonitrile (150 mL) was placed into a flame-dried, three-necked, round-bottomed flask under an argon atmosphere. The solution was stirred at 5 °C for 15 min. Then hexamethyldisilazine (10.69 g, 14 mL, 33 mmol), chlorotrimethylsilane (7.85 g, 9.2 mL, 36 mmol), and trifluoromethane sulfonic acid (10.8 g, 6.39 mL, 36 mmol) were consecutively added to the above solution. The resulting solution was stirred at 5 °C for 1.5 h. The reaction mixture was brought to room temperature and acetonitrile was evaporated under reduced pressure. The residue was dissolved in chloroform (500 mL), and the solution was washed with 10% sodium bicarbonate solution (3 × 150 mL) and water (3 × 100 mL). The chloroform layer was dried in anhydrous sodium sulfate, and finally, regioisomers **7** and **8** were separated through column chromatography using 5:1 hexanes/ethyl aceate solvent system. Characterization data of **7** and **8** was as follows. **Compound 7**: Yield 8.17 g, 67%; mp 153 °C; R_f 0.23 (3:1 hexane/ethyl acetate); IR 2244, 1726, 1601, 1262, 1247 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.15 (s, 1H, imidazole CH), 8.06–7.25 (m, 15H, ArH), 6.89–6.88 (d, J = 2.4 Hz, 1H, 1′-H), 5.85–5.84 (d, J = 3.6 Hz, 2H, 4′,3′-H), 4.88–4.84 (m, 2H, 5′, 5′H), 4.74–4.68 (dd, J = 4.2 Hz, 1H, 2′H), 4.39–4.32 (q, J = 7.5 Hz, 2H, CH_2), 1.65–1.34 (t, J = 6.9 Hz, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.5, 164.5, 164.1, 157.3, 138.5, 133.2, 133.2, 133.1, 129.3, 129.2, 129.1, 128.4, 128.01, 127.9, 127.9, 127.3, 121.1, 112.6, 88.4, 80.1, 76.8, 76.4, 76.0, 75.2, 69.6, 62.3, 62.0; Mass spectrum (FAB) m/z 610 (MH^+); Anal. ($C_{33}H_{27}N_3O_9$) C, H, N. **Compound 8**: Yield 2.2 g, 18%; mp 81 °C; R_f 0.12 (3:1 hexane/ethyl acetate); IR 2236, 1723, 1601, 1262, 1179 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.07 (s, 1H,

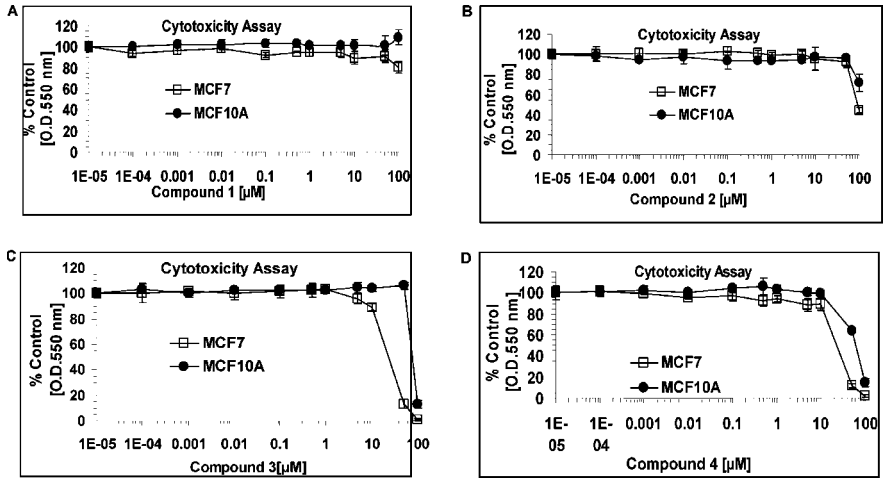


Figure 5. Cytotoxic effects of nucleosides **1**−**4** in cancer (MCF7) and normal (MCF10A) breast cell lines.

imidazole CH), 8.05–7.34 (m, 15H, ArH), 6.30 (d, $J = 6.3$ Hz, 1H, 1′H), 5.95–5.92 (dd, $J = 3.3$ Hz, 1H, 4′-H), 5.82 (t, $J = 6.3$ Hz, 1H, 3′-H), 4.92–4.83 (m, 2H, 5′, 5′H), 4.77–4.72 (dd, $J = 3.3$ Hz, 1H, 2′H), 4.43–4.35 (q, $J = 6.9$ Hz, 2H, CH_2), 1.39–1.35 (t, $J = 7.2$ Hz, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.4, 164.6, 164.4, 159.3, 141.5, 137.6, 133.5, 133.3, 133.1, 129.4, 129.2, 129.1, 128.4, 128.2, 128.1, 127.8, 127.2, 108.7, 87.8, 81.5, 76.8, 76.9, 76.5, 76.1, 74.9, 70.8, 62.9, 61.3; Mass spectrum (FAB) *m/z* 610 (MH^+); Anal. ($C_{33}H_{27}N_3O_9$) C, H, N.

Methyl 4-Cyano-1-(*β*-D-ribofuranosyl)imidazole-5-carboxylate (9). Ethyl 4-cyano-1-(2,3,5-tri-*O*-benzoyl-*β*-D-ribofuranosyl)imidazole-5-carboxylate (**7**; 6.1 g, 0.01 mol) was taken in anhyd methanol (50 mL), and *t*-butylamine (7.3 g, 10.5 mL, 0.10 mol) was added. The reaction mixture was stirred at room temperature for 48 h. Methanol was removed under vacuum and the residue was purified by silica gel flash chromatography. First chloroform was used to remove the deprotected benzoyl groups and then 10:1 chloroform/methanol was used to collect the methyl ester **9**. Yield 2.47 g, 87%; mp 159 °C; R_f 0.21 (10:1 chloroform/methanol); IR 3322, 3142, 2247, 1713, 1537, 1479, 1226 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 8.69 (s, 1H, imidazole CH), 6.19 (d, $J = 2.3$ Hz, 1H, 1′H), 5.56 (d, $J = 5.0$ Hz, 1H, OH), 5.26 (t, $J = 4.6$ Hz, 1H, OH), 5.04 (d, $J = 6.4$ Hz, 1H, OH), 4.13–4.05 (m, 2H, CH_2), 3.95–3.93 (m, 1H, CH), 3.89 (s, 3H, CH_3), 3.79–3.76 (s, 1H, CH), 3.64–3.60 (m, 1H, CH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 158.2, 141.3, 128.6, 119.4, 114.6, 91.7, 84.7, 76.3, 68.5, 59.8, 53.25; Mass spectrum (FAB) *m/z* 284 (MH^+); Anal. ($C_{11}H_{13}N_3O_6$) C, H, N.

Methyl 5-Cyano-1-(*β*-D-ribofuranosyl)imidazole-4-carboxylate (10). Ethyl 5-cyano-1-(2,3,5-tri-*O*-benzoyl-*β*-D-ribofuranosyl)imidazole-4-carboxylate (**8**; 3.5 g, 0.005 mol) was taken in anhyd methanol, *t*-butylamine (3.65 g, 5.25 mL, 0.05 mol) was added, and the reaction mixture was stirred at room temperature for 48 h. Methanol was evaporated under reduced pressure and the pale yellow residue was purified by silica gel flash column chromatography. Initially, chloroform was used to remove the deprotected benzoyl groups and then 10:1 chloroform/methanol was used to collect the title product **10**. Yield 1.3 g, 92%; mp 56 °C; R_f 0.10 (10:1 chloroform/methanol); IR 3322, 3141, 2247, 1712, 1537, 1479, 1226 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 8.48 (s, 1H, imidazole CH), 5.72 (d, $J = 3.6$ Hz, 1H, 1′H), 5.70 (d, $J = 2.7$ Hz, 1H, OH), 5.34 (d, $J = 5.0$ Hz, 1H, OH), 5.09 (t, $J = 5.4$ Hz, 1H, OH), 4.35–4.32 (m, 1H, CH), 4.08–4.05 (m, 2H, CH_2), 3.8 (s, 3H, CH_3), 3.64–3.60 (m, 2H, CH + CH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 160.9, 141.0, 140.6, 110.4, 107.8, 90.8, 86.8, 75.6, 70.4, 61.1, 52.7; Mass spectrum (FAB) *m/z* 284 (MH^+); Anal. ($C_{11}H_{13}N_3O_6$. 0.125 CH_3OH) C, H, N.

4-Amino-1-(*β*-D-ribofuranosyl)-3*H*-imidazo[4,5-*d*]pyridazin-7-(6*H*)-one (3). To a solution of methyl 4-cyano-1-(*β*-D-ribofuranosyl)imidazole-5-carboxylate (**9**; 0.568 g, 0.002 mol) in anhyd methanol (15 mL), hydrazine hydrate (2 mL) was added. The reaction mixture was stirred at ice cold temperature for 30 min. Then methanol and unreacted hydrazine hydrate were removed under high vacuum. Residue was refluxed in ethanol with a catalytic amount of sodium methoxide (8 mg in 2 mL ethanol). After 16 h of refluxing with vigorous stirring, the reaction mixture was brought to room temperature, and the separated solid was filtered and washed with ethanol. Yield 0.41 g, 72%; mp 242–244 °C; R_f 0.56 (1:1:0.3 chloroform/methanol/ammonium hydroxide); IR 3427, 3337, 3266, 3192, 1669, 1630, 1569, 1475, 1414, 1287, 1050 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 11.62 (s, 1H, NH), 8.64 (s, 1H, imidazole CH), 6.38 (d, $J = 4.1$ Hz, 1H, 1′H), 5.83 (s, 2H, NH_2), 5.55 (s, 1H, OH), 5.20 (bs, 2H, OH + OH), 4.37 (t, $J = 5.0$ Hz,1H, CH), 4.13 (t, $J = 4.1$ Hz,1H, CH), 3.94–3.93 (m, 1H, CH), 3.69–3.54 (m, 2H, CH + CH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 154.6, 145.5, 143.08, 136.18, 125.61, 89.36, 86.01, 75.9, 70.2, 61.4; Anal. ($C_{10}H_{13}N_5O_5 \cdot H_2O$) C, H, N; HRMS (FAB) calcd for $C_{10}H_{14}N_5O_5$, 284.0995 (MH^+); found, *m/z* 284.0995 (MH^+).

5-Carbamimidoyl-1-(*β*-D-ribofuranosyl)-1*H*-imidazole-4-carbohydrazide (11). To a solution of methyl 5-cyano-1-(*β*-D-ribofuranosyl)imidazole-4-carboxylate (**10**; 0.284 g, 0.001 mol) in anhyd methanol (15 mL), hydrazine hydrate (2 mL) was added. The reaction mixture was stirred at ice-cold temperature for 30 min. Then methanol and unreacted hydrazine hydrate were removed under high vacuum. The residue was refluxed in ethanol with a catalytic amount of sodium methoxide (8 mg in 2 mL ethanol). After 75 h of refluxing with vigorous stirring, the reaction mixture was brought to room temperature and the separated gummy solid was filtered and washed with ethanol. Yield 0.17 g, 54%; mp 194–196 °C; IR 3487, 3352, 3324, 3271, 1643, 1621, 1571, 1476, 1403, 1282, 1055 cm^{-1}; R_f 0.25 (1:1:0.3 chloroform/methanol/ammonium hydroxide); ^1H NMR (DMSO-d_6, 400 MHz) δ 9.39 (s, 1H, NH), 8.27 (s, 1H, imidazole CH), 6,63 (s, 2H, NH_2), 6.15 (d, $J = 2.9$ Hz, 1H, 1′H), 5.50 (s, 1H, OH), 5.20 (bs, 2H, NH_2), 5.14 (s, 1H, OH), 4.89 (s, 1H, OH), 4.45 (d, $J = 3.6$ Hz,1H, CH), 4.03 (s, 2H, NH_2), 3.89 (m,1H, CH), 3.75 (m, 1H, CH), 3.60 (m, 2H, CH + CH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 163.0, 140.1, 137.0, 132.3, 128.6, 90.4, 84.3, 69.7, 60.4, 41.2; Anal. ($C_{10}H_{17}N_7O_5 \cdot 0.75$ CH_3OH) C, H, N; HRMS (FAB) calcd for $C_{10}H_{18}N_7O_5$, 316.1369 (MH^+); observed, *m/z* 316.1363 (MH^+).

7-Amino-1-(*β*-D-ribofuranosyl)-1*H*-imidazo[4,5-*d*]pyridazin-4-(5*H*)-one (4). To a solution of hydrazine (0.016 g, 0.5 mmol) in anhyd ethanol (10 mL), methyl 5-cyano-(1-*β*-D-ribofuranosyl)-imidazole-4-carboxylate (**10**; 0.142 g, 0.5 mmol) was added and the reaction solution was refluxed for 28 h. The separated solid was filtered and washed with ethanol and recrystallized from methanol. Yield 53 mg, 38%; R_f 0.51 (1:1:0.3 chloroform/methanol/ammonium hydroxide); mp 234–236 °C; IR 3503, 3421, 3356, 3238, 1661, 1627, 1564, 1481, 1416, 1290, 1052 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 11.63 (s, 1H, NH), 8.65 (s, 1H, imidazole CH), 6.38 (d, $J = 5.04$ Hz, 1H, 1′H), 5.84 (s, 2H, NH_2), 5.50 (d, $J = 5.04$ Hz, 1H, OH), 5.16 (m, 2H, OH + OH), 4.38 (dd, $J = 5.04$ Hz, 1H, CH), 4.12 (dd, $J = 4.6$ Hz,1H, CH), 3.98 (dd, $J = 3.68$ Hz, 1H, CH), 3.70 (m, 1H, CH), 3.59 (m, 1H, CH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 154.6, 146.9, 145.6,136.1, 125.5, 88.6, 85.9, 70.5, 61.4, 41.1; Anal. ($C_{10}H_{13}N_5O_5 \cdot 2H_2O$) C, H, N; HRMS (FAB) calcd for $C_{10}H_{14}N_5O_5$, 284.0995 (MH^+); observed, *m/z* 284.0997 (MH^+).

In Vitro Cell Cytotoxicity Assay. The MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO) assay was used to measure drug effects on normal and cancer cell proliferation following the procedure as described by Alley et al.[46] In brief, 2000 cells/well were seeded into 96-well plates (Nunc, U.S.A.) in complete RPMI1640 media and cells allowed to attach overnight at 37 °C in 5% CO_2. Drugs were dissolved in DMSO (as 20 mM stock solutions) and added in final concentrations ranging between 0.0001 to 100 μM in replicates of eight. Assays were terminated after 5 days of continuous exposure to drug by adding MTT. The resulting purple formazan was measured at 550 nm using a Synergy HT Multi-Detection Microplate Reader and KC4 software (Bio-Tek, Winooski, VT). Growth inhibition was assessed as inhibitory concentration 50% (IC_{50}) and 100% (IC_{100}) compared to vehicle treated controls and relative to cell growth at the time of drug addition (day 0). Three independent experiments were performed.

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Supporting Information Available: General Experimental Procedure and elemental microanalyical data on all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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