Antitumor Activity of *C*-Methyl-*β*-D-ribofuranosyladenine Nucleoside Ribonucleotide Reductase Inhibitors

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A series of adenosine derivatives substituted at the 1′-, 2′-, or 3′-position of the ribose ring with a methyl group was synthesized and evaluated for antitumor activity. From this study 3′-*C*-methyladenosine (3′-Me-Ado) emerged as the most active compound, showing activity against human myelogenous leukemia K562, multidrug resistant human leukemia K562IU, human promyelocytic leukemia HL-60, human colon carcinoma HT-29, and human breast carcinoma MCF-7 cell lines with IC_{50} values ranging from 11 to 38 μM. Structure−activity relationship studies showed that the structure of 3′-Me-Ado is crucial for the activity. Substitution of a hydrogen atom of the N^6-amino group with a small alkyl or cycloalkyl group, the introduction of a chlorine atom in the 2-position of the purine ring, or the moving of the methyl group from the 3′-position to other ribose positions brought about a decrease or loss of antitumor activity. The antiproliferative activity of 3′-Me-Ado appears to be related to its ability to deplete both intracellular purine and pyrimidine deoxynucleotides through ribonucleotide reductase inhibition.

Introduction

Nucleoside analogues are a pharmacologically diverse family that includes cytotoxic compounds, antiviral agents, and immunosuppressive molecules. Considerable progress has been made in the search for novel nucleoside structures with anticancer and/or antiviral activity by modifications in the base or in the sugar portion of the molecule. Several branched-chain sugar pyrimidine nucleosides such as 1-(2-deoxy-2-methylene-*β*-D-*erythro*-pentofuranosyl)cytosine,[1] (*E*)-2′-deoxy-2′-(fluoromethylene)cytidine,[2] and other *C*-branched 2′-deoxynucleosides have shown potent antitumor activity both in vitro and in vivo. Among the *β*-D-*ribo*-pentofuranosyl nucleosides, 1-(3-*C*-ethynyl-*β*-D-*ribo*-pentofuranosyl)cytosine and its uracil congener have also been reported to have a broad spectrum antitumor activity.[3] On the basis of these findings, we became interested in the investigation of the antitumor activity of purine nucleosides substituted at the carbon atoms of the ribose ring with a methyl group. From a survey of the literature it was found that all four possible adenosine derivatives containing a methyl group at the 1′-*C*-, 2′-*C*-, 3′-*C*-, and 4′-*C*-position have been synthesized.[4−8] Of these, 2′- and 3′-*C*-methyladenosine showed the ability to inhibit the growth of KB cells in culture,[6] while 4′-*C*-methyladenosine proved to be noncytotoxic up to a 100 μM concentration[9] and 1′-*C*-methyladenosine was never tested as a cytotoxic agent. Since the antitumor potential of these nucleoside analogues has not been further explored, and on the basis of the knowledge that adenosine derivatives such as 2-chloroadenosine and 2-chloro-2′-deoxyadenosine are potent cytotoxic and apoptotic agents,[10] we have synthesized and tested as antitumor agents a series of 1′-, 2′-, and 3′-*C*-methyl-substituted adenosine and 2-chloroadenosine analogues and N^6-substituted derivatives (Chart 1).

Chemistry

The ribose-modified nucleoside analogues **3** and **5**−**9** were synthesized by catalyzed glycosylation of 6-chloro- or 2,6-dichloropurine with the suitable protected sugars. 1′-*C*-Methyladenosine (1′-Me-Ado, **1**), 2′-*C*-methyladenosine (2′-Me-Ado, **2**), and 2-chloro-2′-*C*-methyladenosine (2-Cl-2′-MeAdo, **4**) were synthesized as previously reported.[4,5] 2′-*C*-Methyladenosine derivatives **6** and **8** were obtained by ammination of 6-chloro-9*H*-[(2-*C*-methyl)-2,3,5-tri-*O*-benzoyl-*β*-D-ribofuranosyl]purine (**10**)[4] with aqueous methylamine or cyclopropylamine (Scheme 1).

The synthesis of 3′-*C*-methyl-derivatives was carried out starting from 1,2,3-tri-*O*-acetyl-5-*O*-benzoyl-3-*C*-methyl-D-ribofuranose (**11**, mixture of α and *β* anomers) as described in Scheme 2. Compound **11** was synthesized following the method described in the literature[11a] with minor modifications (see the Supporting Information).

Coupling of **11** with 6-chloropurine (**12**) or 2,6-dichloropurine (**13**) was performed by trimethylsilyl trifluoromethanesulfonate (TMSiOTf) mediated *N*-glycosylation in acetonitrile and in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to obtain the protected compounds **14** and **15**, respectively, in high yields.

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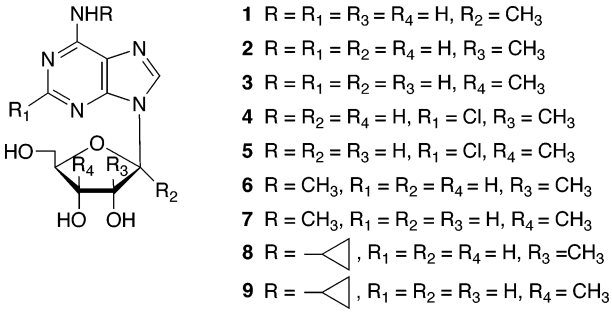
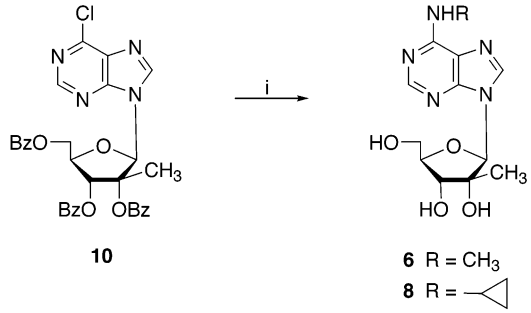
Chart 1



Scheme 1[a]



[a] Reagents and conditions: (i) aqueous methylamine or cyclopropylamine, rt or Δ.

The synthesis of derivatives **14** and **15** resulted in a stereoselective ribosylation (β/α, 99:1).

Nucleophilic displacement of 6-chlorine atom in protected compounds **14** and **15** with liquid ammonia, aqueous methylamine, or cyclopropylamine gave the deblocked nucleoside analogues **3**, **5**, **7**, and **9**. N^6-Methyladenosine (N^6-Me-Ado), N^6-cyclopropyladenosine (N^6-Cp-Ado), and 2-chloroadenosine (2-Cl-Ado) were also synthesized as reported in the literature.[12,13]

Assignment of the β-anomeric configuration of nucleosides **3**, **5**, **7**, and **9** was performed by proton NOE data. In particular, the β-anomeric configuration was determined by selective irradiation of the H-1′ signal that increased the intensity of the H-4′ signal; this indicates that H-1′ and H-4′ are located on the same face of the ribosyl ring.[14]

Biological Evaluation

The synthesized nucleosides were evaluated for their cytotoxicity against human myelogenous leukemia K562, multidrug resistant human leukemia K562IU, human colon carcinoma HT-29, and human breast carcinoma MCF-7 cell lines, and the results are shown in Table 1. 2-Cl-Ado, N^6-Me-Ado, and N^6-Cp-Ado were used as reference compounds.

Table 1. In Vitro Activities of Nucleosides **1**−**9** (IC_{50} in μM)[a] against Human Myelogenous Leukemia K562, Multidrug Resistant Human Leukemia K562IU, Human Colon Carcinoma HT-29, and Human Breast Carcinoma MCF-7 Cell Lines

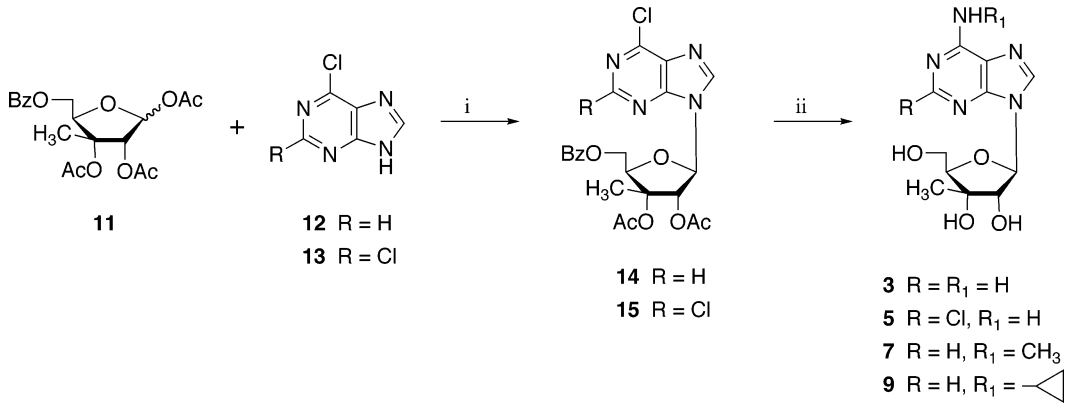
Compd	K562	K562IU	HT-29	MCF-7
1 (1′-Me-Ado)	53	>100	>100	>100
2 (2′-Me-Ado)	16	>100	>100	>100
3 (3′-Me-Ado)	18.2	38.3	23.2	17.5
4 (2-Cl-2′-Me-Ado)	>100	>100	>100	>100
5 (2-Cl-3′-Me-Ado)	>100	>100	>100	>100
6 (N^6-Me-2′-Me-Ado)	>100	>100	>100	>100
7 (N^6-Cp-2′-Me-Ado)	43.9	93.8	74.3	68.1
8 (N^6-Cp-2′-Me-Ado)	>100	>100	>100	>100
9 (N^6-Cp-3′-Me-Ado)	73.0	>100	>100	>100
2-Cl-Ado	15.4	19.5	85.6	72.6
N^6-Me-Ado	>100	>100	>100	>100
N^6-Cp-Ado	>100	>100	>100	93.4

[a] IC_{50} values represent the drug concentration required to inhibit cancer cell replication by 50%. The compounds were tested up to a concentration of 100 μM.

Among the tested nucleosides, 3′-Me-Ado (**3**) showed the best activity in all cell lines with IC_{50} values ranging from 17.5 to 38.3 μM. When compared with 2-Cl-Ado, compound **3** displayed a similar activity against both human leukemia cell lines and exhibited more pronounced cytotoxic properties against both human carcinoma HT-29 and MCF-7. Substitution of the N^6-amino group of **3** with a methylamino one (compound **7**) brings about a reduction of activity. However, the cytotoxicity of **7** against both colon and breast carcinoma cell lines was similar to that of 2-Cl-Ado.

The N^6-substitution with a cyclopropylamino group (compound **9**) resulted in a decrease of the activity against K562 cells and in the loss of the activity against the other tumor cell lines, while the 2′-methyl analogue **8** was found to be not cytotoxic. 1′-Me-Ado (**1**) and 2′-Me-Ado (**2**) showed a cell growth inhibitory activity only against human leukemia K562. Finally, the lack of cytotoxic activity of 2-chloro derivatives **4** and **5** indicated that substitution at the 2-position with a chlorine atom in 2′- and 3′-*C*-methyladenosine is not tolerated. This is a surprising result, because a similar substitution in adenosine and 2′-deoxyadenosine (2-Cl-Ado and

Scheme 2[a]



[a] Reagents and conditions: (i) TMSiOTf, CH_3CN, DBU; (ii) liquid ammonia or aqueous RNH_2, Δ.

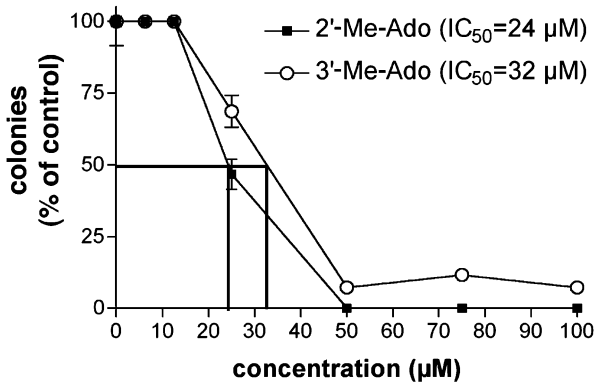


Figure 1. Inhibition of colony formation in CCL-228 cells by 2′-Me-Ado and 3′-Me-Ado after 7 days of incubation.

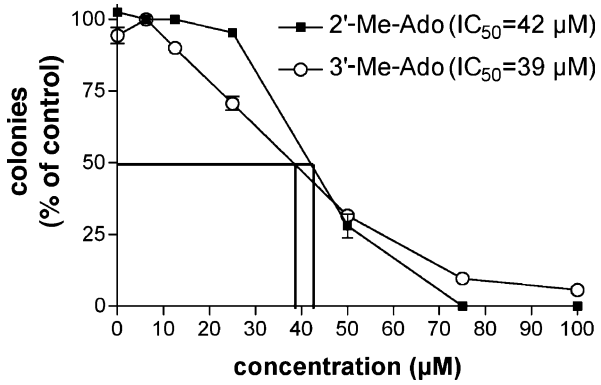


Figure 2. Inhibition of colony formation in HT-29 cells by 2′-Me-Ado and 3′-Me-Ado after 7 days of incubation.

2-Cl-dAdo, respectively) gives rise to very potent cytotoxic and apoptotic compounds.[10]

Adenosine-induced cell death has been claimed to involve either activation of extracellular adenosine receptors or intracellular actions. However, since 3′-Me-Ado was found to be a very poor agonist at adenosine receptors,[15] activation of extracellular receptors probably did not play any significant role in the cytotoxic effect of this nucleoside.

We have further investigated the antitumor activity of 2′-Me-Ado and 3′-Me-Ado by testing their cytotoxicity against HL-60 human promyelocytic leukemia cells. In this type of cell, the cytostatic effect of 3′-Me-Ado proved to be slightly higher compared to that of the 2′-methyl analogue after 3 days of incubation (IC_{50} = 11 and 24 μM, respectively).

The clonogenic activity of 2′-Me-Ado and 3′-Me-Ado in human colorectal adenocarcinoma CCL-228 and human colon adenocarcinoma HT-29 cells was also tested. In CCL-228 cells, 2′-Me-Ado was shown to be slightly more effective, with an IC_{50} value of 24 μM after 7 days of incubation, as compared to the IC_{50} value of 3′-Me-Ado (32 μM), while in the HT-29 cell line both nucleosides reached similar IC_{50} values (42 and 39 μM, respectively) (results are shown in Figures 1 and 2).

The mechanism by which these nucleosides affect human tumor cells could be related to the inhibition of ribonucleotide reductase (RR). In fact, it was reported that 2′- and 3′-Me-Ado, as 5′-diphosphates, behave as mechanism-based inhibitors of ribonucleotide reductase of *Corynebacterium nephridii*.[11] To check this hypothesis, the effect of these nucleosides on cellular RR was

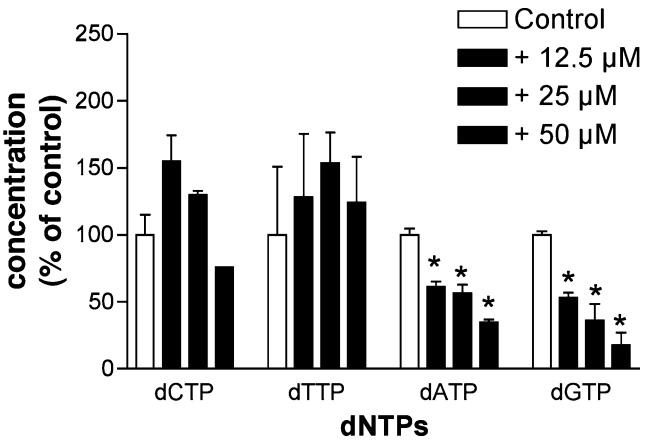


Figure 3. Concentration of dNTPs in HL-60 cells after treatment with 2′-Me-Ado as measured by HPLC. Values marked with an asterisk (*) are significantly different from the control ($P < 0.05$).

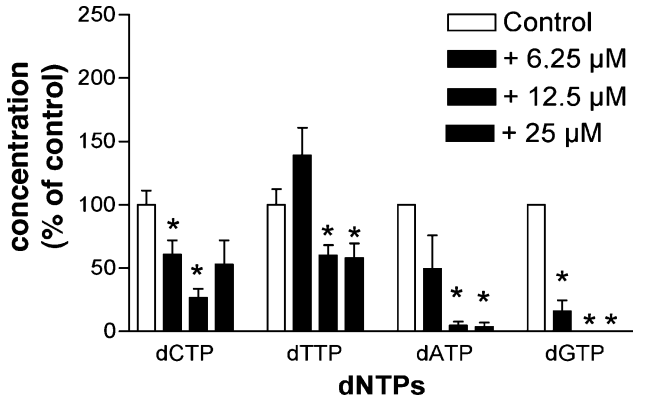


Figure 4. Concentration of dNTPs in HL-60 cells after treatment with 3′-Me-Ado as measured by HPLC. Values marked with an asterisk (*) are significantly different from the control ($P < 0.05$).

evaluated indirectly by measuring the level of intracellular deoxyribonucleotide triphosphate pools in HL-60 cells by HPLC analysis before and after treatment with 2′- and 3′-Me-Ado.

Treatment of HL-60 cells with 2′-Me-Ado significantly depleted intracellular dATP and dGTP pools (Figure 3). Incubation for 24 h with 12.5, 25, and 50 μM 2′-Me-Ado decreased dATP pools to 61%, 56%, and 35% of control values, respectively. The corresponding values for dGTP pools were 53%, 45%, and 23%. Changes in intracellular dCTP and dTTP concentrations were not significant.

In contrast to 2′-Me-Ado, 3′-Me-Ado was able to significantly decrease all dNTP concentrations (Figure 4). After treatment with 6.25 μM 3′-Me-Ado, dCTP, dATP, and dGTP concentrations were decreased to 61%, 49%, and 15% of control values, respectively, whereas intracellular dTTP concentrations showed an increase to 130% of control values, perhaps due to reduction in DNA synthesis in response to the restricted availability of other deoxyribonucleoside triphosphates. Treatment with 12.5 and 25 μM 3′-Me-Ado caused a marked decrease in all dNTP concentrations. Intracellular dCTP and dTTP pools experienced a decrease to 26%, 53%, 61%, and 58% of control values, respectively. These values for dATP were 5% and 3%. Intracellular dGTP pools were depleted beyond detectability by treatment with 3′-Me-Ado.

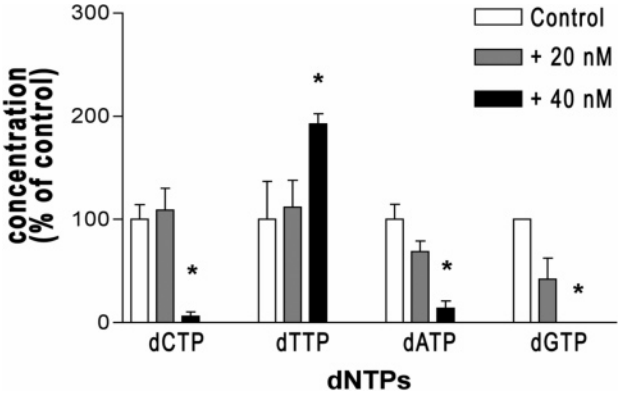


Figure 5. Concentration of dNTPs in HL-60 cells after treatment with gemcitabine (dFdC) as measured by HPLC. Values marked with an asterisk (*) are significantly different from the control.

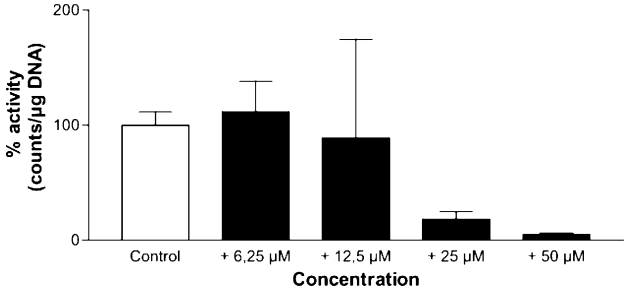


Figure 6. Incorporation of [^{14}C]cytidine into DNA of HL-60 cells after treatment with 3′-Me-Ado. HL-60 cells were treated with 3′-Me-Ado for 24 h and then pulsed with [^{14}C]cytidine for 30 min. Total DNA from the cells was extracted and radioactivity was determined using a scintillation detector. Data are means ± SEM of two measurements.

The effects of gemcitabine (dFdC), a clinically well-established inhibitor of RR on intracellular dNTP pool concentrations, were comparable to those of 3′-Me-Ado. Treatment of HL-60 cells with 40 nM dFdC significantly decreased intracellular dCTP and dATP pools to 6.1% and 13.9% of control values, respectively, while dGTP pools were depleted beyond detectability. In contrast, dTTP pools experienced a significant increase to 192.6% of control values, as shown in Figure 5.

These finding strengthen the hypothesis that the cytotoxicity of these nucleosides is due to the inhibition of RR. The higher activity of 3′-Me-Ado could be explained by its greater ability to reduce the level of all dNTPs, and in particular those of dGTP.

We have also determined the incorporation of [^{14}C]-cytidine into the DNA of HL-60 cells after incubation with 3′-Me-Ado (Figure 6) and 2′-Me-Ado (Figure 7). Cells were first preincubated with various concentrations of these compounds. Incubation with 6.25 and 12.5 μM 3′-Me-Ado did not influence significantly the incorporation of [^{14}C]cytidine into DNA. After treatment of HL-60 cells with 25 and 50 μM 3′-Me-Ado for 24 h, [^{14}C]-cytidine incorporation into DNA was significantly decreased to 14.2% and 5% of control values, respectively. In contrast, incubation with 2′-Me-Ado did not have a significant effect on the incorporation of [^{14}C]cytidine into DNA, although values measured have shown a meaningful increase.

Since RR is the rate-limiting enzyme catalyzing de novo DNA synthesis, the inhibition of the incorporation

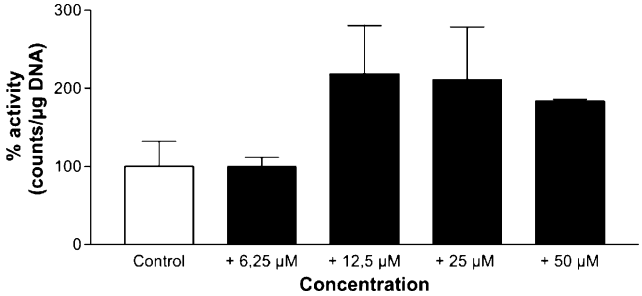


Figure 7. Incorporation of [^{14}C]cytidine into DNA of HL-60 cells after treatment with 2′-Me-Ado. HL-60 cells were treated with 2′-Me-Ado for 24 h and then pulsed with [^{14}C]cytidine for 30 min. Total DNA from the cells was extracted and radioactivity was determined using a scintillation detector. Data are means ± SEM of two measurements.

of [^{14}C]cytidine into DNA elicited by 3′-Me-Ado confirms the hypothesis that this nucleoside is able to inhibit the enzyme.

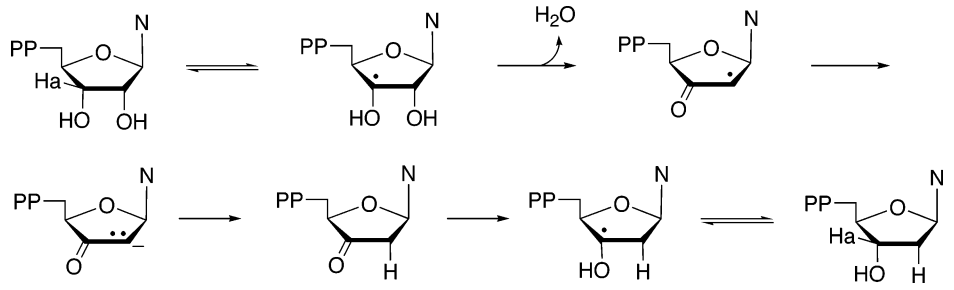
It is known that the initial step in the mechanism of action of RR is the homolytic cleavage of the 3′-carbon−hydrogen bond mediated by a protein radical (Scheme 3).[11,16] Such carbon−hydrogen bond cleavage is not possible in the 3′-*C*-methyladenosine 5′-diphosphate (3′-Me-ADP); thus, this ADP analogue cannot function as a substrate of the enzyme but behaves as an inhibitor of ADP reduction.[11] 3′-Me-Ado is the first example of a β-D-ribonucleoside able to inhibit human RR functioning as mechanism-based inhibitor of the enzyme.

The lower activity of the regioisomeric 2′-*C*-methyladenosine might be due to its instability in the cell culture medium. It is already known that this nucleoside is susceptible to enzymatic conversions by adenosine deaminase and purine nucleoside phosphorylase.[17] We have compared the stability of 2′-Me-Ado and 3′-Me-Ado in MEM medium containing 10% fetal bovine serum for 72 h at 37 °C. Under these conditions 2′-Me-Ado proved to be quite unstable, with a half-life of 4.5 h, while the half-life of 3′-Me-Ado was 43.5 h. Other factors that could negatively affect the activity of 2′-Me-Ado are (a) inefficient intracellular metabolism to the 5′-diphosphate derivative and (b) reduced inhibitory activity of 2′-Me-ADP against human RR. Further studies are underway to evaluate these hypotheses.

Conclusion

In summary, a series of adenosine derivatives substituted at the 1′-, 2′-, or 3′-position of the ribose ring with a methyl group have been synthesized and evaluated as antitumor agents. 3′-*C*-Methyladenosine emerged as the most active compound, showing activity against human myelogenous leukemia K562, multidrug-resistant human leukemia K562IU, human promyelocytic leukemia HL-60, human colon carcinoma HT-29, and human breast carcinoma MCF-7 cell lines with IC_{50} values in the micromolar range. Structure−activity relationships studies have pointed out that the structure of 3′-Me-Ado is crucial for the antitumor activity. Thus, the substitution of a hydrogen atom of the amino group in 6-position with a small alkyl or cycloalkyl group, the introduction of a chlorine atom in the 2-position, or the moving of the methyl group from the 3′-position to the other positions of the ribose ring brings about a decrease or loss of activity. The antitumor activity of 3′-Me-Ado

Scheme 3. Mechanism of Reduction of Ribonucleosides to 2′-Deoxyribonucleosides Catalyzed by Ribonucleotide Reductase (RR) As Proposed by Stubbe and Ackles[16] and Confirmed by Ong et al.[11]



appears to be related to its ability to deplete intracellular purine and pyrimidine deoxynucleotides through ribonucleotide reductase inhibition. Owing to the significant antitumor activity of 3′-Me-Ado, its resistance to the enzymatic degradation by adenosine deaminase,[18] and its prolonged stability in fetal calf serum, additional evaluation of the cytotoxic effects and in vivo activity of this nucleoside in a panel of human tumors is warranted.

Experimental Section

Chemistry. All reagent and solvents were purchased from Aldrich Chemical Co. Thin-layer chromatography (TLC) was run on silica gel 60 F_{254} plates; silica gel 60 (70−230 and 230−400 mesh, Merck) for column chromatography was used. Nuclear magnetic resonance spectra were recorded on a Varian VXR-300 spectrometer with TMS as the internal standard for ^{1}H NMR and external H_3PO_4 for ^{31}P NMR. Chemical shift are reported in part per million (δ) as s (singlet), d (doublet), t (triplet), dd (double doublet), q (quartet), m (multiplet), or br s (broad singlet). Stationary NOE experiments were run on degassed solutions at 25 °C. Mass spectroscopy was carried out on an HP 1100 series instrument. All measurements were performed in the positive ion mode using atmospheric pressure electrospray ionization (API-ESI).

6-Chloro-9*H*-(2,3-di-*O*-acetyl-5-*O*-benzoyl-3-*C*-methyl-β-D-ribofuranosyl)purine (14). To a stirred mixture of **11** (800 mg, 2.03 mmol), 6-chloropurine (**12**) (340 mg, 2.25 mmol), and DBU (0.86 mL, 6.08 mmol) in anhydrous acetonitrile (4 mL) at 0 °C was added TMSiOTf (1.47 mL, 8.11 mmol). After 3 h at 60 °C, the mixture was cooled at room temperature, poured into aqueous $NaHCO_3$ (1 M, 15 mL) and extracted with CH_2Cl_2 (3 × 10 mL). The combined organic phase was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography on silica gel (*n*-hexane/EtOAc, 75:25) to give **14** as a white foam (720 mg, 72%). ^{1}H NMR ($CDCl_3$): δ 1.82 (s, 3H, CH_3), 2.05, 2.15 (2s, 6H, 2 Ac), 4.56 (dd, *J* = 4.6, 12.6 Hz, 1H, H-5′), 4.82 (dd, *J* = 3.3, 12.8 Hz, 1H, H-5′), 5.0 (t, *J* = 3.8 Hz, 1H, H-4′), 6.10 (d, *J* = 7.3 Hz, 1H, H-2′), 6.28 (d, *J* = 7.3 Hz, 1H, H-1′), 7.50, 7.65, 8.05 (3m, 5H, arom.), 8.25 (s, 1H, H-2), 8.63 (s, 1H, H-8). Anal. ($C_{22}H_{21}ClN_4O_7$) C, H, N.

9*H*-(3-*C*-Methyl-β-D-ribofuranosyl)adenine (3). A mixture of **14** (700 mg, 1.43 mmol) and liquid ammonia was reacted in a stainless steel bomb at 60 °C for 30 h. After evaporation the residue was purified by flash chromatography on silica gel eluting with $CHCl_3$/MeOH (95:5) to give **3** as a white solid (390 mg, 97%). ^{1}H NMR (DMSO-d_6): δ 1.30 (s, 3H, CH_3), 3.46−3.74 (m, 2H, H-5′), 3.88 (t, *J* = 2.6 Hz, 1H, H-4′), 4.46 (pseudo t, 1H, H-2′), 4.85 (s, 1H, OH-3′), 5.42 (d, *J* = 6.6 Hz, 1H, OH-2′), 5.82 (d, *J* = 8.1 Hz, 1H, H-1′), 5.95 (dd, *J* = 3.5, 8.3 Hz, 1H, OH-5′), 7.42 (br s, 2H, NH_2), 8.12 (s, 1H, H-2), 8.34 (s, 1H, H-8). MS: *m/z* 282 [M + H]$^+$. Anal. ($C_{11}H_{15}N_5O_4$) C, H, N.

2,6-Dichloro-9*H*-(2,3-di-*O*-acetyl-5-*O*-benzoyl-3-*C*-methyl-β-D-ribofuranosyl)purine (15). The title compound was prepared starting from 2,6-dichloropurine (**13**) (660 mg, 3.49 mmol) and **11** (1.0 g, 2.53 mmol) as described for **14**. Purification by flash chromatography on silica gel (*n*-hexane/$CHCl_3$, 95:5) gave **15** as a white solid (1.03 g, 78%). ^{1}H NMR ($CDCl_3$): δ 1.84 (s, 3H, CH_3), 2.17, 2.23 (2 s, 6H, 2 Ac), 4.56 (dd, *J* = 4.0, 12.5 Hz, 1H, H-5′), 4.85 (dd, *J* = 3.5, 12.6 Hz, 1H, H-5′), 5.06 (t, *J* = 3.7 Hz, 1H, H-4′), 5.94 (d, *J* = 7.3 Hz, 1H, H-2′), 6.26 (d, *J* = 7.3 Hz, 1H, H-1′), 7.45, 7.60, 8.03 (3m, 5H, arom.), 8.24 (s, 1H, H-8). Anal. ($C_{22}H_{20}Cl_2N_4O_7$) C, H, N.

2-Chloro-9*H*-(3-*C*-methyl-β-D-ribofuranosyl)adenine (5). The title compound was obtained as described for **3** starting from **15** (reaction time 22 h). Chromatography on a silica gel column ($CHCl_3$/MeOH, 90:10) gave **5** as a white solid (yield 55%). ^{1}H NMR (DMSO-d_6): δ 1.29 (s, 3H, CH_3), 3.50−3.71 (m, 2H, H-5′), 3.88 (t, *J* = 3.1 Hz, 1H, H-4′), 4.38 (pseudo t, 1H, H-2′), 4.87 (s, 1H, OH-3′), 5.20 (dd, *J* = 4.4, 5.7 Hz, 1H, OH-5′), 5.42 (d, *J* = 7.0 Hz, 1H, OH-2′), 5.78 (d, *J* = 8.1 Hz, 1H, H-1′), 7.85 (br s, 2H, NH_2), 8.40 (s, 1H, H-8). MS: *m/z*, 316 [M + H]$^+$. Anal. ($C_{11}H_{14}ClN_5O_4$) C, H, N.

General Procedure for the Ammination of 10 and 14 into Compounds 6−9. To 6-chloro-9*H*-(2-*C*-methyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)purine (**10**)[4] or 6-chloro-9*H*-(3-*C*-methyl-2,3-di-*O*-acetyl-5-*O*-benzoyl-β-D-ribofuranosyl)purine (**14**) was added the suitable 40% aqueous amine (molar ratio 1:6), and the mixture was stirred at room temperature or at 40 °C. The solution was evaporated in vacuo and the residue was purified by column chromatography.

***N*^{6}-Methyl-9*H*-(2-*C*-methyl-β-D-ribofuranosyl)adenine (6).** Reaction of **10** with 40% aqueous methylamine at room temperature for 3 h, followed by chromatography on a silica gel column ($CHCl_3$/MeOH, 90:10), gave **6** as a white solid (79% yield). ^{1}H NMR (DMSO-d_6): δ 0.75 (s, 3H, CH_3), 2.90 (br s, 3H, C*H*$_3$NH), 3.60−3.90 (2m, 3H, H-4′, H-5′), 4.05 (m, 1H, H-3′), 5.20 (m, 3H, OH), 5.95 (s, 1H, H-1′), 7.75 (br s, 1H, NH), 8.20 (s, 1H, H-2), 8.45 (s, 1H, H-8). MS: *m/z* 296.30 [M + H]$^+$. Anal. ($C_{12}H_{17}N_5O_4$) C, H, N.

***N*^{6}-Cyclopropyl-9*H*-(2-*C*-methyl-β-D-ribofuranosyl)adenine (8).** Reaction of **10** with 40% aqueous cyclopropylamine at 40 °C for 22 h, followed by chromatography on a silica gel column (EtOAc/$CHCl_3$/MeOH, 70:25:5), gave **8** as a white solid (76% yield). ^{1}H NMR (DMSO-d_6): δ 0.50−0.75 (2m, 4H, cyclopropyl), 0.75 (s, 3H, CH_3), 3.0 (br s, 1H, C*H*NH), 3.65−3.90 (m, 3H, H-4′, H-5′), 4.05 (pseudo t, 1H, H-3′), 5.20 (m, 3H, OH), 5.98 (s, 1H, H-1′), 7.98 (br s, 1H, NH), 8.25 (s, 1H, H-2), 8.48 (s, 1H, H-8). MS: *m/z* 322.34 [M + H]$^+$. Anal. ($C_{14}H_{19}N_5O_4$) C, H, N.

***N*^{6}-Methyl-9*H*-(3-*C*-methyl-β-D-ribofuranosyl)adenine (7).** Reaction of **14** with 40% aqueous methylamine at room temperature for 1 h, followed by chromatography on a silica gel column ($CHCl_3$/MeOH/NH_4OH, 92:7:1), gave **7** as a white solid (72% yield). ^{1}H NMR (DMSO-d_6): δ 1.30 (s, 3H, CH_3), 2.95 (br s, 3H, C*H*$_3$NH), 3.60 (m, 2H, H-5′) 3.88 (t, *J* = 2.6 Hz, 1H, H-4′), 4.45 (d, *J* = 7.7 Hz, 1H, H-2′), 4.85 (s, 1H, OH-3′), 5.4 (br s, 1H, OH-2′), 5.83 (d, *J* = 8.1 Hz, 1H, H-1′), 5.95 (dd, *J* = 3.3, 8.4 Hz, 1H, OH-5′), 7.90 (br s, 1H, NH), 8.20 (s, 1H, H-2), 8.30 (s, 1H, H-8). MS: *m/z* 296.30 [M + H]$^+$. Anal. ($C_{12}H_{17}N_5O_4$) C, H, N.

***N*^{6}-Cyclopropyl-9*H*-(3-*C*-methyl-β-D-ribofuranosyl)adenine (9).** The title compound was obtained from **14** as reported for **8**, as a white solid (60% yield). ^{1}H NMR (DMSO-d_6): δ 0.60−0.80 (2m, 4H, cyclopropyl), 1.30 (s, 3H, CH_3), 3.07

(br s, 1H, C*H*NH), 3.60 (m, 2H, H-5′), 3.88 (t, $J = 2.6$ Hz, 1H, H-4′), 4.45 (t, $J = 7.3$ Hz, 1H, H-2′), 4.85 (s, 1H, OH-3′), 5.40 (d, $J = 6.6$ Hz, 1H, OH-2′), 5.85 (d, $J = 8.1$ Hz, 1H, H-1′), 5.92 (dd, $J = 3.5$, 7.9 Hz, 1H, OH-5′), 8.08 (br s, 1H, NH), 8.22 (s, 1H, H-2), 8.35 (s, 1H, H-8). MS: *m/z* 322.34 $[M + H]^+$. Anal. ($C_{14}H_{19}N_5O_4$) C, H, N.

Biological Assays. Cells and Culture. The cell lines human myelogenous leukemia K562, human promyelocytic leukemia HL-60, human colon carcinoma HT-29, human colorectal adenocarcinoma CCL-228, and human breast carcinoma MCF-7 were obtained from the American Type Culture Collection (ATCC, Manasses, VA). Multidrug resistant K562 (K562IU) cells were developed by exposing sensitive K562 cells to sublethal concentrations of tiazofurin over a period of 60 generations. K562IU exhibited resistance to tiazofurin, benzamide riboside, and related nucleoside analogues. K562 and K562IU cells were maintained in RPMI 1640 medium (Gibco/Life Technologies, Gaithersburg, MD) containing 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA) and 10 000 U/L penicillin and 50 mg/L streptomycin.[19] HT-29 and MCF-7 cells were maintained in minimal essential medium alpha (MEM) with Earl's balanced salts, 10% FBS, penicillin, and streptomycin as above. Logarithmically growing HT-29 and MCF-7 cells were incubated with 0.05% trypsin containing 1 mM EDTA at 37 °C for about 5 min until cells were nonadherent and formed a single cell suspension. Trypsin activity was neutralized by adding a 20-fold excess of serum-containing medium. Cells were cultured at 37 °C in an atmosphere of air and 5% CO_2.

Antitumor Activity. Antitumor activity of the compounds was determined by their cytotoxic action on cultured tumor cells. Cytotoxicity assays were conducted by tetrazolium reduction of MTS with PMS (CellTiter Assay, Promega, Madison, WI). Logarithmically growing cells were plated in 0.1-mL aliquots in 96-well microtiter plates. Cells were plated at an initial density of about 50 000 cells/mL and allowed to acclimatize for 24 h. Cell suspensions were treated with various dilutions of compounds in triplicate, mixed well, and allowed to incubate for 48 h at 37 °C in an atmosphere of air and 5% CO_2. To the cell suspension was added 20 μL of tetrazolium reagent, the mixture was incubated for 3 h at 37 °C in an atmosphere of air and 5% CO_2, and absorbance at 490 nm was read by microplate reader. Control plates with serial dilutions of cell types were counted as a control for the assay. In all cases, controls indicated a linear response versus cell number, $R^2 \geq 0.99$.

Clonogenic Assay. The inhibition of colony formation by 2′-Me-Ado and 3′-Me-Ado was also investigated in HT-29 and CCL-228 cells. A total of 10^3 cells/well were plated in 24-well plates and allowed to attach overnight. The next day, the medium was replaced by fresh medium containing various concentrations of the drugs. The plates were incubated with the drugs for 7 days, then the medium was removed and the wells were stained with crystal violet solution. Colonies (>50 cells) were counted using a VWR Merck colony counter. All experiments were run in triplicate.

Analysis of Intracellular dNTP Pools by High Performance Liquid Chromatography (HPLC). The effects of 2′-Me-Ado and 3′-Me-Ado on intracellular dNTP pools were analyzed in HL-60 cells. The effects of these nucleosides were compared with that of a clinically well-established inhibitor of ribonucleotide reductase (RR) on intracellular dNTP pool concentrations, with dFdC as a reference substance. The extraction of dNTPs from the cells was done by the method described by Garrett et al.[20] Cells (7×10^7) were seeded at a concentration of 1×10^6/mL in 70 mL of complete medium and were incubated with various concentrations of 2′-Me-Ado, 3′-Me-Ado, or dFdC for 24 h. After the incubation period, the cell count was determined, and 7.5×10^7 cells were separated for dNTP extraction. All steps of the extraction were carried out on ice. The corrected number of cells was centrifuged at 600*g* for 5 min and then resuspended in 100 μL of phosphate-buffered saline. In this suspension, cells were lysed by addition of 10 μL of trichloroacetic acid, and the mixture was vortexed for 1 min. The lysate was rested on ice for 30 min and then the proteins were separated by centrifugation at 15 000*g* for 10 min in an Eppendorf microcentrifuge. The supernatant was removed and neutralized by adding 1.1 vol of Freon containing 0.5 M tri-*n*-octylamine. Aliquots (100 μL) of this sample were periodated by adding 30 μL of 4 M methylamine solution and 10 μL of periodate solution (concentration 100 g/L). After incubation at 37 °C for 30 min, the reaction was stopped by adding 5 μL of rhamnose solution. The extracted dNTPs were measured by using a Merck "La Chrom" HPLC system equipped with an L-7200 autosampler, an L-7100 pump, an L-7400 UV detector, and a D-7000 interface unit. Samples were eluted with a 3.2 mol/L ammonium phosphate buffer, pH 3.8 containing 20 mol/L acetonitrile using a 4.6 × 250 mm Partisil 10 SAX column. Separation was performed at constant ambient temperature with a flow rate of 2 mL/min. The concentration of dNTPs was calculated as a percent of the control. The initial intracellular dNTP concentrations of untreated control cells were determined by calibration to a standard 100 μM dNTP solutions. Intracellular concentrations of dNTPs in untreated control cells were 7.8, 46.1, 4.4, and 8.2 μM for dCTP, dTTP, dATP, and dGTP, respectively.

Incorporation of ^{14}C-Labeled Cytidine into DNA. To analyze the effect of 2′-Me-Ado and 3′-Me-Ado incubation on the activity of DNA synthesis, an assay was performed as previously described.[21] HL-60 cells in the logarithmic phase of growth (1×10^6 cells/mL) were incubated with various concentrations of 2′-Me-Ado and 3′-Me-Ado for 24 h. After incubation, cells were counted and pulsed with [^{14}C]cytidine (0.3125 μCi, 5 nM) for 30 min at 37 °C. Afterward, cells were collected by centrifugation and washed with PBS. Total DNA was extracted from 5×10^6 cells and the specific radioactivity of the samples was determined using a Wallac 1414 liquid scintillation counter (Perkin-Elmer, Boston, MA).

Stability of 2′-Me-Ado and 3′-Me-Ado in Culture Medium. Stability of 2′-Me-Ado and 3′-Me-Ado was evaluated by incubation of the compounds (50 μM) in MEM medium containing 10% fetal bovine serum (Sigma Chemical Co.) for 72 h at 37 °C. At various times, 200 μL of the medium was removed and frozen. The collected samples were thawed and passed through 0.22-μm syringe filters and then subjected to HPLC. Samples were eluted with a linear gradient from water to 100% methanol using a 4.6 × 250 mm Beckman Ultrasphere 5 μm C_{18} column. A HP1090 system was used. 2′-Me-Ado and 3′-Me-Ado were also analyzed without serum containing medium and found to be stable.

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Supporting Information Available: Experimental details for the synthesis of compound **11** and analysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Niitsu, N.; Ishii, Y.; Matsuda, A.; Honma, Y. Induction of differentiation of acute promyelocytic leukemia cells by a cytidine deaminase-resistant analogue of 1-β-D-arabinofuranosylcytosine, 1-(2-deoxy-2-methylene-β-D-*erythro*-pentofuranosyl)-cytosine. *Cancer Res.* **2001**, *61*, 178−185.

(2) Sun, L. Q.; Li, Y. X.; Guillou, L.; Mirimanoff, R. O.; Coucke, P. A. Antitumor and radiosensitizing effects of (*E*)-2′-deoxy-2′-(fluoromethylene)cytidine, a novel inhibitor of ribonucleoside diphosphate reductase, on human colon carcinoma xenografts in nude mice. *Cancer Res.* **1997**, *57*, 4023−4028.

(3) Hattori, H.; Nozawa, E.; Iino, T.; Yoshimura, Y.; Shuto, S.; Shimamoto, Y.; Nomura, M.; Fukushima, M.; Tanaka, M.; Sasaki, T.; Matsuda, A. Nucleosides and nucleotides. 175. Structural requirements of the sugar moiety for the antitumor activities of new nucleoside antimetabolites, 1-(3-*C*-ethynyl-β-D-ribo-pentofuranosyl)cytosine and -uracile. *J. Med. Chem.* **1998**, *41*, 2892−2902.

(4) Franchetti, P.; Cappellacci, L.; Marchetti, S.; Trincavelli, L.; Martini, C.; Mazzoni, M. R.; Lucacchini, A.; Grifantini, M. 2′-*C*-Methyl analogues of selective adenosine receptor agonists: Synthesis and binding studies. *J. Med. Chem.* **1998**, *41*, 1708−1715.

(5) Cappellacci, L.; Barboni, G.; Palmieri, M.; Pasqualini, M.; Grifantini, M.; Costa, B.; Martini, C.; Franchetti, P. Ribose-modified nucleosides as ligands for adenosine receptors: Synthesis, conformational analysis, and biological evaluation of 1′-*C*-methyl adenosine analogues. *J. Med. Chem.* **2002**, *45*, 1196−1202.

(6) Walton, E.; Jenkins, S. R.; Nutt, R. F.; Zimmerman, M.; Holly, F. W. Branched-chain sugar nucleosides. New type of biological active nucleoside. *J. Am. Chem. Soc.* **1966**, *88*, 4524−4525.

(7) Nutt, R. F.; Dickinson, M. J.; Holly, F. W.; Walton, E. Branched-chain sugar nucleosides. III. 3′-*C*-Methyladenosine. *J. Org. Chem.* **1968**, *33*, 1789−1795.

(8) Waga, T.; Nishizaki, T.; Miyakawa, I.; Ohrui, H.; Meguro, H. Studies on sugar-modified nucleosides. Part I. Synthesis of 4′-*C*-methylnucleosides. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 1433−1438.

(9) Griffon, J.-F.; Dukhan, D.; Pierra, C.; Benzaria, S.; Loi, A. G.; La Colla, P.; Sommadossi, J.-P.; Gosselin, G. 4′-*C*-Methyl-β-D-ribofuranosyl purine and pyrimidine nucleosides revisited. *Nucleosides, Nucleotides Nucleic Acids* **2003**, *22*, 707−709.

(10) Ceruti, S.; Franceschi, C.; Barbieri, D.; Malorni, W.; Camurri, A.; Giammarioli, A. M.; Ambrosini, A.; Racagni, G.; Cattabeni, F.; Abbracchio, M. P. Apoptosis induced by 2-chloro-adenosine and 2-chloro-2′-deoxy-adenosine in a human astrocytoma cell line: differential mechanisms and possible clinical relevance. *J. Neurosci. Res.* **2000**, *60*, 388−400.

(11) (a) Ong, S. P.; Nelson, L. S.; Hogenkamp, H. P. Synthesis of 3′-*C*-methyladenosine and 3′-*C*-methyluridine diphosphates and their interaction with the ribonucleoside diphosphate reductase from *Corynebacterium nephridii*. *Biochemistry* **1992**, *31*, 11210−11215. (b) Ong, S. P.; McFarlan, S. C.; Hogenkamp, H. P. 2′-*C*-Methyladenosine and 2′-*C*-methyluridine 5′-diphosphates are mechanism-based inhibitors of ribonucleoside diphosphate reductase from *Corynebacterium nephridii*. *Biochemistry* **1993**, *32*, 11397−11404.

(12) Kusachi, S.; Thompson, R. D.; Bugni, W. J.; Yamada, N.; Olsson, R. A. Dog coronary artery adenosine receptor: structure of the N^6-alkyl subregion. *J. Med. Chem.* **1985**, *28*, 1636−1643.

(13) Thompson, R. D.; Secunda, S.; Daly, J. W.; Olsson, R. A. Activity of N^6-substituted 2-chloroadenosine at A1 and A2 adenosine receptors. *J. Med. Chem.* **1991**, *34*, 3388−3390.

(14) Rosemeyer, H.; Toth, G.; Seela, F. Assignment of anomeric configuration of D-ribo-, arabino-, 2′-deoxyribo-, and 2′,3′-dideoxyribonucleosides by NOE difference spectroscopy. *Nucleosides Nucleotides* **1989**, *8*, 587−597.

(15) Cappellacci, L.; Franchetti, P.: Pasqualini, M.; Petrelli, R.; Vita, P.; Lavecchia A.; Novellino, E.; Costa B.; Martini, C.; Klotz K.-N.; Grifantini M. Synthesis, biological evaluation and molecular modeling of ribose-modified adenosine analogues as adenosine receptor agonists. *J. Med. Chem.* **2005**, *48*, 1555−1562.

(16) (a) Stubbe, J.; Ackles D. On the mechanism of ribonucleoside diphosphate reductase from *Escherichia coli*. Evidence for 3′-C−H bond cleavage. *J. Biol. Chem.* **1980**, *265*, 8027−8030.

(17) (a) Eldrup, A. B.; Allerson, C. R.; Bennett, C. F.; Bera, S.; Bhat, B.; Bhat, N.; Bosserman, M. R.; Brooks, J.; Burlein, C.; Carrol, S. S.; Cook, P. D.; Getty, K. L.; MacCoss, M.; MacMasters, D. R.; Olsen, D. B.; Prakash, T. P.; Prhavc, M.; Song, Q.; Tomassini, J. E.; Xia, J. Structure-activity relationship of purine ribonucleosides for inhibition of hepatitis C virus RNA-dependent RNA polymerase. *J. Med. Chem.* **2004**, *47*, 2283−2295. (b) Eldrup, A. B.; Prhavc, M.; Brooks, J.; Bhat, B.; Prakash, T. P.; Song, Q.; Bera, S.; Bhat, N.; Dande, P.; Cook, P. D.; Bennett, C. F.; Carroll, S. S.; Ball, R. G.; Bosserman, M.; Burlein, C.; Colwell, L. F.; Fay, J. F.; Flores, O. A.; Getty, K.; LaFemina, R. L.; Leone, J.; MacCoss, M.; McMasters, D. R.; Tomassini, J. E.; Von Langen, D.; Wolanski, B.; Olsen, D. B. Structure−activity relationship of heterobase-modified 2′−C-methyl ribonucleosides as inhibitors of hepatitis C virus RNA replication. *J. Med. Chem.* **2004**, *47*, 5284−5297.

(18) Kalinichenko, E. N.; Beigel'man, L. N.; Mikhailov, S. N.; Mikhailopulo, I. A. Substrate specificity of adenosine deaminase. The role of methyl groups at 2′, 3′, and 5′-carbon atoms of adenosine. *Bioorg. Khim*. **1988**, *14*, 1157−1161.

(19) Yalowitz, J. A.; Pankiewicz, K.; Patterson, S. E.; Jayaram, H. N. Cytotoxicity and cellular differentiation activity of methylenebis(phosphonate) analogues of tiazofurin and mycophenolic acid adenine dinucleotide in human cancer cell lines. *Cancer Lett.* **2002**, *181*, 31−38.

(20) Garrett, C.; Santi, D. V. A Rapid and sensitive high-pressure liquid chromatography assay for deoxynucleoside triphosphates in cell extracts. *Anal. Biochem.* **1979**, *99*, 268−273.

(21) Szekeres, T.; Gharehbaghi, K.; Fritzer, M.; Woody, M.; Srivastava, A.; van't Riet, B.; Jayaram, H. N.; Elford, H. L. Biochemical and antitumor activity of trimidox, a new inhibitor of ribonucleotide reductase. *Cancer Chemother. Pharmacol*. **1994**, *34*, 63−66.

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