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ARTICLE

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Synthesis and Significant Cytostatic Activity of 7-Hetaryl-7-deazaadenosines

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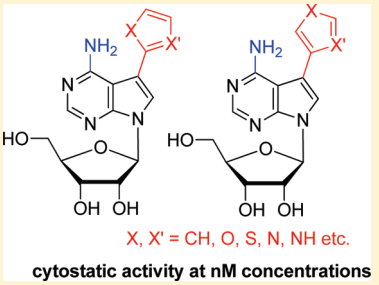
Supporting Information

ABSTRACT: A series of 7-aryl- and 7-hetaryl-7-deazaadenosines were prepared by the cross-coupling reactions of unprotected or protected 7-iodo-7-deazaadenosines with (het)arylboronic acids, stannanes, or zinc halides. Nucleosides bearing 5-membered heterocycles at the position 7 exerted potent in vitro antiproliferative effects against a broad panel of hematological and solid tumor cell lines. Cell cycle analysis indicated profound inhibition of RNA synthesis and induction of apoptosis in treated cells. Intracellular conversion to triphosphates has been detected with active compounds. The triphosphate metabolites showed only a weak inhibitory effect on human RNA polymerase II, suggesting potentially other mechanisms for the inhibition of RNA synthesis and quick onset of apoptosis. Initial in vivo evaluation demonstrated an effect of 7-(2-thienyl)-7-deazaadenine ribonucleoside on the survival rate in syngeneic P388D1 mouse leukemia model.



INTRODUCTION

Purine nucleosides and their analogues display a wide range of biological activities. Their antiviral and antitumor properties are particularly important. Many purine (fludarabine, cladribine, nelarabine, and clofarabine),[1] pyrimidine (gemcitabine, cytarabine, 5-fluoro deoxyuridine, capecitabine, and decitabine),[2] and other (e.g., 2-deoxycoformycin) nucleosides are clinically used for treatment of both solid and hematological malignancies. Despite extensive research over the last five decades, there still remains a space for the design of new purine nucleoside analogues and development of novel nucleoside-based anticancer therapeutics[3] for the treatment of drug-resistant tumors. In our previous works, we have reported on significant cytostatic effects of 6-(het)arylpurine nucleosides **1** (Chart 1).[4] Recently, we have discovered[5] 6-hetaryl-7-deazapurine ribonucleosides **2**−**4** with nanomolar cytostatic activities toward a wide panel of leukemia and cancer cell lines. The most active were derivatives bearing furyl or thienyl groups at the position 6 and either hydrogen or fluorine at position 7 of the 7-deazapurine. Surprisingly, their cyclo-Sal-phosphate and phosphoramidate prodrugs[6] were less active due to increased efflux from the cells.

7-Deazaadenosine (Tubercidin) is a natural cytostatic antibiotic.[7] Numerous studies[8] were devoted to the synthesis of diverse derivatives of Tubercidin and related natural nucleosides Toyocamycin and Sangivamycin. A variety of 7-substituted derivatives of **5** bearing halogens, carboxamides, or alkynes were prepared[9] and many of them exerted significant cytotoxic, antiparasitic, and antiviral activities mostly through inhibition of adenosine kinase. An important group of Tubercidin derivatives are 7-substituted 2′-C-methylribonucleosides that are selective inhibitors of HCV replication.[10] Although some 7-phenyltubercidin derivatives and analogues have been reported,[11] no systematic study of 7-aryl- and 7-hetaryl-7-deazaadenosines has been reported in the literature. Therefore, we report here on the synthesis and significant cytostatic activities of the title 7-hetaryl-7-deazaadenosines.

CHEMISTRY

The synthesis of the majority of the target 7-aryl- and 7-hetaryltubercidins **6** (Chart 1) was envisaged by the cross-coupling reactions of either unprotected 7-iodotubercidin[12] or its sugar-protected derivatives with the corresponding aryl- or hetaryl boronic acids. Whenever possible, we have employed the previously developed aqueous Suzuki−Miyaura cross-couplings[13] for the reactions of 7-iodotubercidin **7** with boronic acids in the presence of $Pd(OAc)_2$, TPPTS ligand, and Na_2CO_3 in acetonitrile/water. In this way, the desired 7-(het)aryl substituted 7-deazaadenosines **6a**−**n** were prepared in a single step mostly in good yields (Scheme 1).

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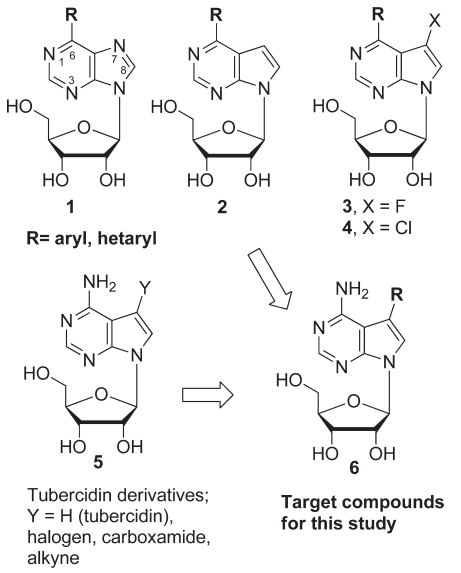
Chart 1. Structures of Cytostatic 6-(Het)arylpurines 1 and -7-Deazapurine Ribonucleosides 2−4 and Tubercidin Derivatives 5



It should be noted that *N*-protecting group in both starting pyrrolyl boronic acids were removed under the conditions of coupling (products **6k** and **6l**). Because of low yield of 4-pyrazolyl derivative **6m** by Suzuki reaction, the compound was alternatively prepared by Stille reaction of 7-iodotubercidin **7** with 1-dimethylsulfamoyl-4-tributylstannylpyrazole[14] and subsequent removal of dimethylsulfamoyl group under acidic conditions (1 M aq HCl) in 68% overall yield after crystallization.

Ethynyl derivative **6o** was prepared by Sonogashira reaction of **7** (Scheme 2) with trimethylsilylacetylene and protodesilylation of TMS-ethynyl derivative **8** under basic conditions. Copper-catalyzed [3 + 2] cycloaddition[15] of ethynyl derivative **6o** with trimethylsilyl azide afforded triazolyl derivative **6p** in 24% yield.

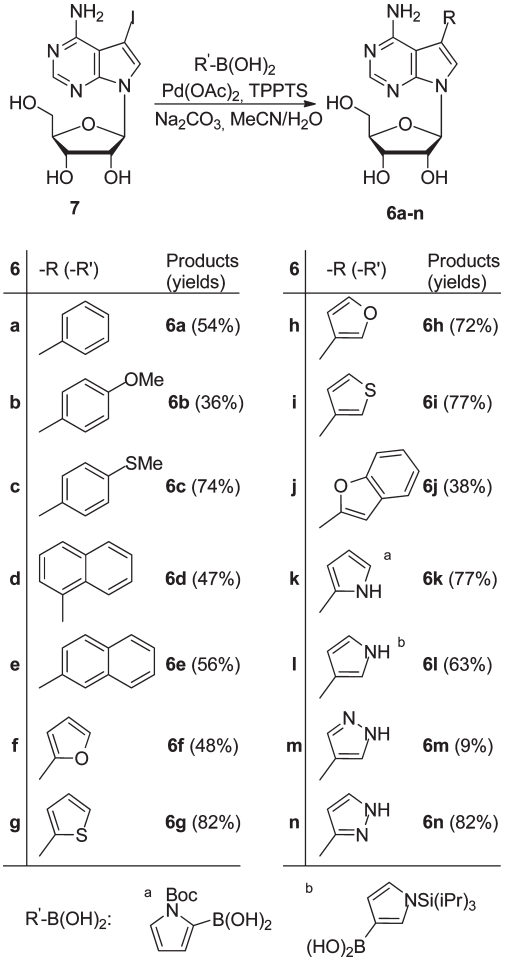
Thiazolyl and imidazolyl derivatives **6q**−**s** were prepared by the Stille or Negishi cross-coupling reactions of per-*O*-silyl protected 7-iodotubercidin **9** (Scheme 3) with thiazolyl-stannane or *N*-protected imidazolyl zinc reagents[16] and subsequent acidic deprotections. It should be noted that attempted Stille cross-coupling of 2-(tributylstannyl)thiazole with *O*-unprotected 7-iodotubercidin **7** failed, presumably due to hydrolytic protodestannylation of organotin by ribose hydroxyl groups under the reaction conditions.

In addition, as standards for metabolism studies, we have prepared several nucleoside 5′-*O*-monophosphate (NMP) and nucleoside 5′-*O*-triphosphate (NTP) derivatives. The target 7-substituted-7-deazaadenosine 5′-*O*-triphosphates **10a**,**f**−**i** and 5′-*O*-monophosphates **11a**,**f**−**i** were synthesized by the aqueous Suzuki−Miyaura reactions[17] (Scheme 4) of 7-iodo-7-deazaadenosine 5′-*O*-triphosphate **12** and 5′-*O*-monophosphate **13** with the corresponding boronic acids. Starting 7-iodotubercidine triphosphate **12** and monophosphate **13** were prepared by convenient phosphorylation methods from 7-iodotubercidin **7**.

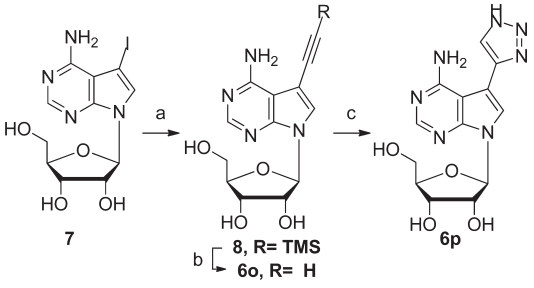
BIOLOGICAL PROFILING

Cytostatic Activity. Cytostatic activity of prepared compounds was initially evaluated against eight different cell lines derived from various human solid tumors including lung (A-549

Scheme 1



Scheme 2[a]



[a] Conditions: (a) trimethylsilylacetylene, $PdCl_2(PPh_3)_2$, CuI, NEt_3, DMF (100%); (b) K_2CO_3, MeOH (96%); (c) $TMSN_3$, CuI, DMF/MeOH (24%).

cells and NCI-H23), prostate (Du145 and PC3), colon (HCT116 and HCT15), and breast (Hs578 and BT549) carcinomas. Concentrations inhibiting the cell growth by 50% (GIC_{50}) were determined using a quantitative cellular staining with sulforhodamine B (SRB)[18] following a 5-day treatment. The SRB method allowed for a quantitative measurement of a net effect on cell growth by subtracting background signal generated by the cell culture inoculum at the beginning of treatment. In

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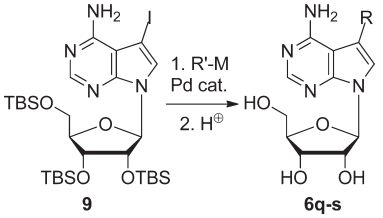
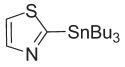
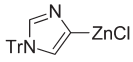
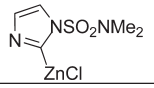
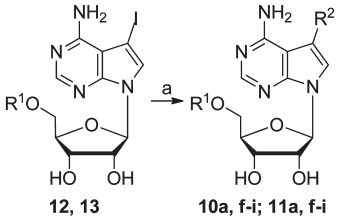
Scheme 3



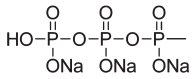
9 **6q-s**

6	-R	R'-M	Products (yields)
q			**6q** (76%)
r			**6r** (77%)
s			**6s** (13%)

Scheme 4



12, 13 **10a, f-i; 11a, f-i**

12, 10a, f-i R^1= 

13, 11a, f-i R^1= 

10, 11	R^2	Products (yields)
a		**10a** (46%) **11a** (94%)
f		**10f** (27%) **11f** (34%)
g		**10g** (53%) **11g** (51%)
h		**10h** (37%) **11h** (45%)
i		**10i** (67%) **11i** (47%)

general, 7-substituted 7-deazaadenine nucleosides modified with a phenyl or naphthyl ring either with or without substitution (compounds **6a**,**c**−**e**) or bulky benzofuryl (**6j**) showed minimal to no cytostatic activity against the tested cell lines (Table 1). The only exception was 7-(4-methoxyphenyl)tubercidin **6b** that exerted submicromolar activities. In contrast, the nucleosides containing 5-member heterocyclic moiety at 7-position (**6f**−**6i**, **6k**−**6n**, and **6p**−**6s**) exhibited potent cytostatic effects (nanomolar GIC_{50}) over a wide range of the tested cell lines comparable to Tubercidin or Doxorubicin and better than Clofarabin. Interestingly, also 7-ethynyltubercidin **6o** exerted nanomolar cytostatic affect.

In addition, the antiproliferative effect of prepared compounds was tested against a human T-lymphoblastic leukemia line CCRF-CEM, promyelocytic leukemia HL-60, and cervical carcinoma HeLa S3 growing in liquid suspension. Cell viability was determined following a 3-day incubation using metabolic 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) based method.[19] The average IC_{50} values for all compounds in the XTT-based assays were higher compared to the SRB-based assays, but the potency ranking followed similar trends as those observed in the SRB assays. Shorter duration of incubation during the XTT-based assay and/or different genetic background of the cells are likely the main reason for the lower activities.

All the nucleosides **6a**−**6s** were also tested for their inhibition of human adenosine kinase (ADK). Most of the compounds were only weak inhibitors with $IC_{50} > 5\ \mu M$. The exceptions were the 7-pyrazolyl- **6m** and 7-ethynyl- **6o** derivatives that showed IC_{50} in submicromolar range. Therefore, we can conclude that the strong cytostatic effect of this class of compounds is not due to the ADK inhibition.

In addition, selected five compounds, **6m**, **6i**, **6g**, **6h**, and **6l**, were subjected for expanded cytotoxic activity profiling against a panel of 26 human or rodent cancer cell lines (Table 2). The most potent (nanomolar) activity was confirmed against leukemia (K562, BV-173), including the drug resistant subline (CEM-DNR-Bulk), breast (BT-549 and MDA-MB 231), colon (HT-29 and HCT116), prostate (LNCaP), cervix (HeLa), leiomyosarcoma (MES-SA), and pancreatic (HPAC) cancers; the cytotoxic activities were comparable or slightly lower than gemcitabine and better than clofarabine. However, low potency was observed in neural (SK−N-As, C6 U-87 MG), androgen refractory, and p53 mutant prostate (PC-3), melanoma (SK-Mel-2), lung (NCi-H146), and ovarian (SK-OV-3) tumors. Importantly, the compounds **6m**, **6i**, **6g**, **6h**, and **6l** were generally noncytotoxic against normal human fibroblasts (BJ), demonstrating promising therapeutic index under in vitro conditions.

Metabolism and Cell-Cycle Studies. As one of the expected mode of action of the cytostatic nucleosides is the phosphorylation to NTPs, which downregulates the RNA synthesis through the inbibition of RNA polymerases, we have studied intracellular phosphorylation of nucleosides **6g** (active) and **6a** (inactive) in drug sensitive Du145 cells (Table 3). Both nucleosides are phosphorylated to the corresponding NMPs (**11g**,**a**) and NTPs (**10g**,**a**). Notably, the more active nucleoside **6g** is phosphorylated significantly better than the inactive one (**6a**) after 24 h. The first phosphorylation step to NMP appears to be significantly more efficient than the formation of triphosphate.

Further, four NTPs (**10f**−**10i**) derived from the corresponding strongly cytostatic nucleosides (**6f**−**6i**) were tested for the inhibition of human RNA polymerase II in a nuclear extract-based assay. The results indicated relatively low inhibition, with the IC_{50} values in the range of 54 to >400 μM (Table 4). Interestingly, Tubercidin triphosphate inhibited the RNA polymerase II with the efficiency similar to that of the other tested analogues. These data suggest that the direct inhibition of mRNA transcript synthesis by RNA polymerase II is unlikely to be the primary mode of action for tubercidine and its 7-substituted derivatives, although a possibility of stable internal incorporation into RNA with subsequent inhibition of translation should be addressed in future studies.

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Table 1. Cytostatic, Cytotoxic, and ADK Inhibition Activities of Nucleosides 6a−6s

	GIC_{50} (μM)[a]									IC_{50} (μM)[b]			IC_{50} (μM)
compd	A549	NCIH23	Du145	PC3	HCT116	HCT15	HS578	BT549	MT-4	HL60	HeLa S3	CCRF-CEM	human ADK
6a	>20	>20	>20	>20	>20	>20	>20	>20	>20	>10	>10	>10	>5
6b	0.701	0.856	0.152	1.106	0.633	0.544	0.811	nd	4.3	>10	>10	>10	>2
6c	nd	0.39	1.87	nd	1.29	nd	1.97	nd	4.4	>10	>10	>10	>10
6d	nd	>10	>10	nd	>10	nd	>10	nd	nd	>10	>10	>10	>10
6e	nd	5.35	1.05	nd	5.67	nd	2.16	nd	nd	>10	>10	>10	>5
6f	0.035	0.294	0.019	0.004	0.048	0.003	0.017	nd	0.086	11.64	0.05	0.10	>2
6g	0.0070	0.0037	0.133	1.62	0.0026	0.011	0.012	nd	0.035	0.95	0.02	0.05	>5
6h	0.034	0.198	0.330	0.418	0.015	nd	0.021	0.028	0.170	9.06	0.02	0.07	>5
6i	0.016	0.073	0.097	1.323	0.007	nd	0.050	0.028	0.124	6.62	0.06	0.11	>5
6j	7.02	5.40	nd	6.29	3.74	nd	6.73	>10	4.8	>10	>10	3.95	>10
6k	0.1604	0.333	nd	0.0878	0.0652	nd	0.1129	0.1292	nd	2.04	1.04	0.16	
6l	0.0053	0.033	nd	0.0290	0.0059	nd	0.0044	0.0080	0.024	0.84	0.07	0.11	>5
6m	0.192	0.144	nd	0.014	0.970	nd	0.012	0.050	0.110	>25	0.22	0.56	0.05
6n	nd	0.035	nd	0.015	0.018	nd	0.008	nd	0.018	>20	0.69	0.13	>10
6o	nd	0.001	nd	0.011	0.002	nd	0.001	nd	0.001	0.09	0.02	0.01	0.2
6p	nd	0.004	nd	0.005	0.004	nd	0.003	nd	0.018	0.31	0.09	0.06	
6q	nd	0.005	nd	0.042	0.093	nd	0.015	nd	0.042	1.61	0.04	0.02	
6r	nd	0.002	nd	0.002	0.003	nd	0.002	nd	0.028	0.13	0.07	0.07	>10
6s	nd	0.006	nd	0.001	0.002	nd	0.001	nd	0.049	0.10	0.44	0.07	>5
Doxorubicin	0.016	0.005	nd	0.021	0.011	nd	0.006	0.010	nd				
Tubercidin	0.001	0.011	0.018	0.048	0.001	0.011	0.098	nd	0.021				
Clofarabin	0.086	0.040	0.125	0.063	0.106	0.180	1.241	0.065	0.051				

[a] Cytostatic activity (GIC_{50}) was determined by SRB assay following a 5-day incubation with tested compounds. Values represent means from two independent experiments. [b] Cytotoxic activity was determined by XTT assay following a 3-day incubation. Values represent means from four independent experiments.

Cell-cycle study of compound **6g** has been performed on CCRF-CEM lymphoblasts at 1× GIC_{50} and at 5× GIC_{50} concentrations within 12 h (Figure 1). At the higher concentration (5×GIC_{50}), the compound induces apoptosis within 4−8 h and at later times also accumulation of cells in G2/M phase was observed. The compound produces potent and fast onset of the inhibition of RNA synthesis at both concentrations, whereas the synthesis of DNA is inhibited only after longer incubations at high concentration. This indicates that the inhibition of DNA synthesis is likely a secondary effect, and the RNA synthesis is primary target of compound **6g**, although the direct inhibition of RNA polymerase II is unlikely to be involved (Table 4).

This study shows that the 7-hetaryltubercidins **6** are strongly cytostatic compounds that inhibit RNA synthesis and induce apoptosis. Although they are phosphorylated to NTPs, the activity is not due to inhibition of RNA polymerase II. Our future studies are scheduled to elucidate the detailed mechanisms of action of 7-hetaryl-7-deazaadenine nucleotides and their potential biological use.

Initial in Vivo Evaluation of Antitumor Activity in Mouse Model. To evaluate anticancer activity of **6g** under in vivo conditions, the P388D1 leukemia survival model was employed. The compound was dosed at maximum tolerated dose (MTD) and appoximately 0.5× MTD, corresponding to 75 and 35 mg/kg, respectively, in two cycles. Administration of **6g** prolonged mean survival time (MST), increased lifespan percentage (ILS), and significantly increased overall survival (Figure 2). These data warrant further more detailed exploration of compound **6g** in additional in vivo pharmacokinetics/pharmacodynamics and anticancer models.

CONCLUSIONS

7-Hetaryl-7-deazaadenine ribonucleosides (bearing 5-membered heterocycles at position 7) have been found to possess cytostatic effects at low nanomolar concentrations with the potency comparable to clofarabine. On the other hand, the corresponding 7-aryl-7-deazaadenine ribonucleosides were much less active. The compounds effectively inhibit RNA synthesis in treated cells and induce apoptosis at their cytotoxic concentrations. The molecular mechanism of this effect requires additional investigation. Thus far, our experiments detected the formation of intracellular triphosphate metabolites in treated cells and ruled out the inhibition of ADK and RNA polymerase II by parent nucleosides and corresponding triphosphates, respectively, as the major targets of the observed antiproliferative effects. Encouraging in vivo activity has been observed in the syngeneic mouse P388D1 leukemia model, warranting additional studies of the mechanism together with further characterization of the in vivo cytostatic activity and pharmacokinetics.

EXPERIMENTAL SECTION

General Methods. Melting points were determined on a Kofler block and are uncorrected. Optical rotations were measured at 25 °C, $[\alpha]_D^{20}$ values are given in 10^{-1} deg cm^2 g^{-1}. NMR spectra were measured at 400 MHz for ^{1}H and 100.6 MHz for ^{13}C nuclei, or at 500 MHz for ^{1}H and 125.8 MHz for ^{13}C, or at 600 MHz for ^{1}H and 151 MHz for ^{13}C in $CDCl_3$ (TMS was used as internal standard), MeOH-d_4 (referenced to the residual solvent signal), or DMSO-d_6 (referenced to the residual solvent signal). Chemical shifts are given in ppm (δ scale),

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Table 2. Cytotoxic Activity Profiling of the Most Potent Compounds 6m, 6i, 6g, 6h, and 6l on Expanded Panel of Cancer Cell Lines and Normal Human Fibroblasts

	IC_{50}[a] (μM)								
	leukemia						breast		
compd	K562	K526-tax	BV-173	CEM-DNR-Bulk	L1210 (mouse)	EL4 (mouse)	MCF-7	BT-549	MDA-MB 231
6g	0.025	0.047	0.105	0.091	0.115	9.30	0.528	0.119	0.026
6h	0.200	0.147	0.068	0.364	0.445	>10	9.74	0.452	0.126
6i	0.067	0.093	0.029	0.110	0.199	>10	7.25	0.120	0.082
6l	0.033	0.009	0.004	0.008	0.084	9.42	6.31	0.006	0.027
6m	9.83	0.092	9.78	0.104	0.258	>10	>10	0.108	>10
Gemcitabine	0.718	0.006	0.001	0.022	0.007	0.007	0.149	0.008	0.245
Cladribine	7.69	0.170	0.0008	0.352	0.393	0.848	2.35	0.123	>10

	neural			colon			prostate		
	SK-N-As	U-87 MG	C6 (rat)	HT-29	HCT116	CT-26 (mouse)	PC-3	LNCaP	Mat-LyLu (rat)
6g	>10	>10	4.54	0.096	0.060	0.124	8.73	0.018	0.180
6h	>10	>10	>10	0.394	0.425	1.67	>10	0.112	1.86
6i	>10	>10	8.29	0.184	0.194	0.225	>10	0.068	0.525
6l	>10	>10	3.68	4.05	nd	0.017	>10	0.021	nd
6m	>10	>10	0.538	0.136	>10	0.396	>10	9.55	>10
Gemcitabine	1.10	1.49	0.504	1.53	nd	0.006	nd	0.512	nd
Cladribine	>10	>10	9.07	9.44	9.43	0.131	8.28	>10	nd

	others							
	NCi-H146 (lung)	MES-SA (leiomyo-sarcoma)	HeLa (cervix)	SK-OV-3 (ovary)	SK-Mel2 (mela-noma)	HPAC (pan-creas)	P388D1 (mouse)	BJ (fibro-blasts)
6g	>10	0.232	0.033	>10	1.98	0.070	0.028	9.85
6h	>10	1.33	0.350	>10	>10	0.133	0.139	>10
6i	>10	0.076	0.124	>10	>10	0.110	0.076	>10
6l	>10	0.081	0.040	>10	8.71	0.068	0.010	6.09
6m	>10	7.74	0.185	>10	>10	0.141	0.093	>10
Gemcitabine	2.78	0.005	4.12	>10	7.11	0.073	0.019	9.88
Cladribine	>10	0.165	>10	>10	>10	9.32	0.285	>10

[a] Cytotoxic activity was determined by MTT assay following a 3-day incubation. Values represent means from four independent experiments.

Table 3. Intracellular Phosphorylation of Nucleosides 6g and 6a

	dosing					
	6 h (pmol/million)			24 h (pmol/million)		
compd	nucleoside	NMP (**11**)	NTP (**10**)	nucleoside	NMP (**11**)	NTP (**10**)
6g	4.66	5.04	0.53	30.4	64.0	1.96
6a	32.91	6.23	0.13	68.8	6.68	0.27

Table 4. Inhibition of RNA Pol II by NTPs 10f−10i

entry	compd	IC_{50} (μM)
1	**10f**	148
2	**10g**	54
3	**10h**	>400
4	**10i**	91
5	Tubercidin triphosphate	93
6	α-amanitin	2.2 ng/mL

coupling constants (J) in Hz. Complete assignment of all NMR signals was performed using a combination of H,H-COSY, H,H-ROESY, H,C-HSQC, and H,C-HMBC experiments. High resolution mass spectra were measured using electrospray ionization. Reverse phase high performance flash chromatography (HPFC) purifications were performed with Biotage SP1 apparatus on with KP-C18-HS columns. All final free nucleosides for testing were >95% pure as determined by combustion analysis. Synthetic procedures and characterization data of compounds **6b**−**e**, **6g**−**s**, **12**, **10f**−**i**, and **11f**−**i** are given in the Supporting Information.

4-Amino-5-phenyl-7-(β-D-ribofuranosyl)-7H-pyrrolo[2,3-d]pyrimidine (***6a***). An argon purged mixture of 7-iodotubercidin **7**[12] (200 mg, 0.51 mmol), phenylboronic acid (93 mg, 0.76 mmol), Na_2CO_3 (502 mg, 4.74 mmol), $Pd(OAc)_2$ (6.6 mg, 0.029 mmol), and TPPTS (42 mg, 0.07 mmol) in water/MeCN (2:1, 3.6 mL) was stirred at 100 °C for 1 h. After cooling, the mixture was neutralized by the addition of

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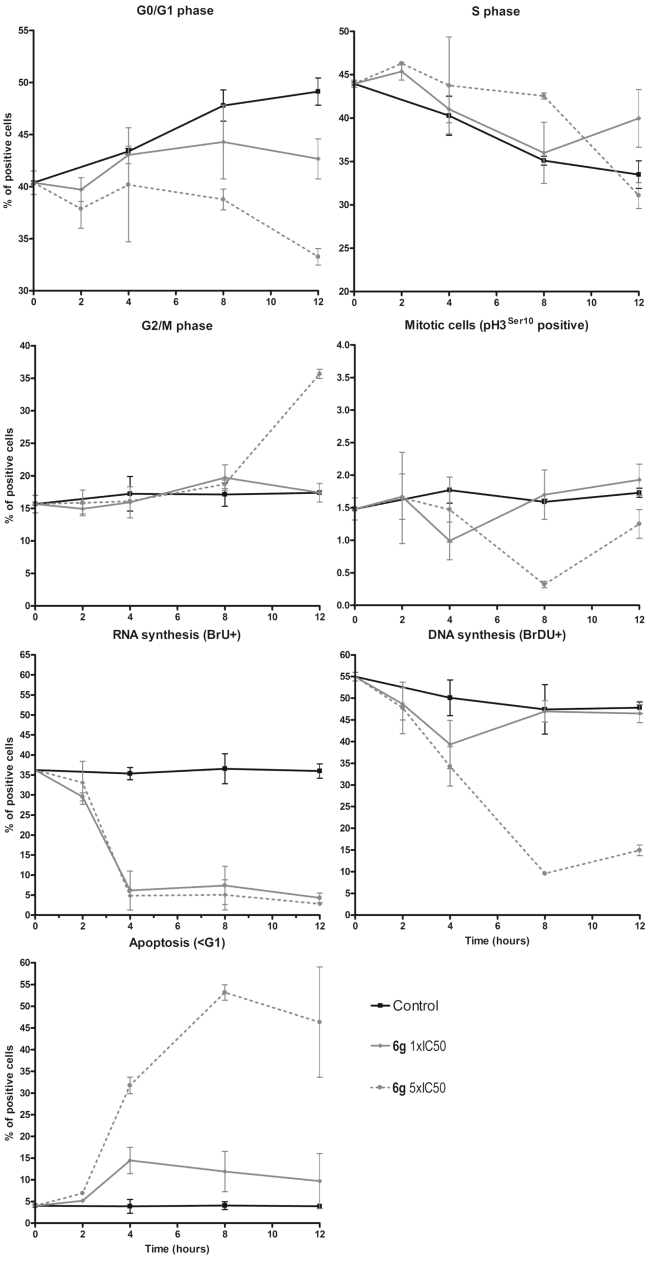

Figure 1. Effects of compound **6g** on cell cycle, apoptosis, and DNA/RNA synthesis in CCRF-CEM lymphoblasts.

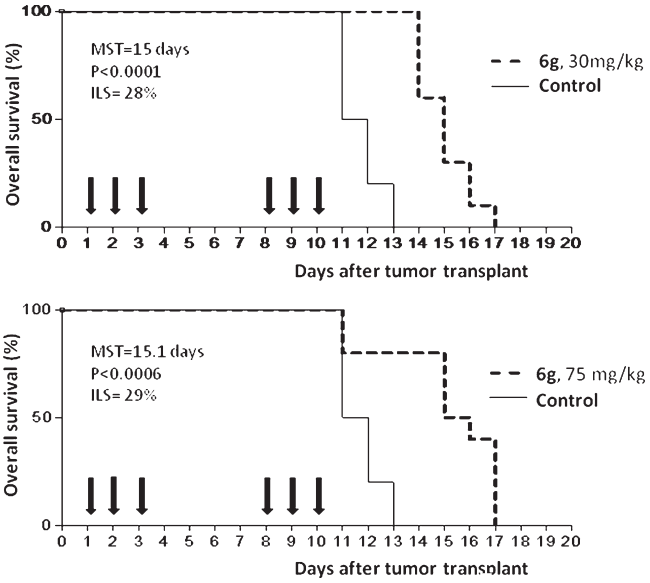

Figure 2. In vivo antitumor activity of the compound **6g** in P388D1 syngeneic tumor model is demonstrated by prolonged MST, ILS and significant increase of overall survival when administered at MTD or 1/2MTD. Arrows indicate administration of active molecule or vehicle in control animals.

aqueous HCl (1M), volatiles were removed in vacuo and the residue was purified by reverse phase chromatography (0→100% MeOH in water), affording title compound **6a** as white solid (94 mg, 54%); mp 119 °C; $[\alpha]^{20}_{D}$ −49.8 (*c* 0.301, MeOH). UV (MeOH): λ_{max} (ε) 282 (12900). ^{1}H NMR (600 MHz, DMSO-d_6): 3.53 (ddd, 1H, J_{gem} = 11.9, $J_{5'b,OH}$ = 6.1, $J_{5'b,4'}$ = 3.9, H-5′b), 3.63 (ddd, 1H, J_{gem} = 11.9, $J_{5'a,OH}$ = 5.2, $J_{5'a,4'}$ = 3.4, H-5′a), 3.90 (ddd, 1H, $J_{4',5'}$ = 3.9, 3.4, $J_{4',3'}$ = 3.5, H-4′), 4.10 (bm, 1H, H-3′), 4.46 (bm, 1H, H-2′), 5.16 (d, 1H, $J_{OH,3'}$ = 3.5, OH-3′), 5.22 (dd, 1H, $J_{OH,5'}$ = 6.1, 5.2, OH-5′), 5.36 (d, 1H, $J_{OH,2'}$ = 4.8, OH-2′), 6.12 (d, 1H, $J_{1',2'}$ = 6.3, H-1′), 7.37 (m, 1H, H-*p*-Ph), 7.47 (m, 2H, H-*o*-Ph), 7.49 (m, 2H, H-*m*-Ph), 7.54 (s, 1H, H-6), 8.15 (s, 1H, H-2). ^{13}C NMR (151 MHz, DMSO-d_6): 61.93 (CH_2-5′), 70.89 (CH-3′), 74.05 (CH-2′), 85.38 (CH-4′), 87.27 (CH-1′), 100.73 (C-4a), 116.57 (C-5), 121.41 (CH-6), 127.20 (CH-*p*-Ph), 128.70 (CH-*o*-Ph), 129.28 (CH-*m*-Ph), 134.71 (C-*i*-Ph), 151.10 (C-7a), 151.95 (CH-2), 157.57 (C-4). MS (FAB) *m*/*z* 343 (M + H), 365 (M + Na). HRMS (FAB) for $C_{17}H_{19}N_4O_4$ [M + H] calcd, 343.1406; found, 343.1409. Anal. ($C_{17}H_{18}N_4O_4 \cdot 0.8H_2O$): C, H, N.

4-Amino-5-(furan-2-yl)-7-(β-D-ribofuranosyl)-7H-pyrrolo[2,3-d]-pyrimidine (***6f***). Compound **6f** was prepared as described for **6a** from compound **7** and furan-2-boronic acid; yield 48%; tan solid after recrystallization from MeOH; mp 118 °C; $[\alpha]^{20}_{D}$ −52.5 (*c* 0.287, MeOH). UV (MeOH): λ_{max} (ε) 288 (13200), 258 (12500). ^{1}H NMR (500 MHz, DMSO-d_6): 3.55 and 3.65 (2 × dd, 2H, J_{gem} = 11.9, $J_{5',4'}$ = 3.9, H-5′), 3.91 (td, 1H, $J_{4',5'}$ = 3.9, $J_{4',3'}$ = 3.4, H-4′), 4.11 (dd, 1H, $J_{3',2'}$ = 5.2, $J_{3',4'}$ = 3.4, H-3′), 4.41 (dd, 1H, $J_{2',1'}$ = 6.1, $J_{2',3'}$ = 5.2, H-2′), 5.00−5.50 (bs, 2H, OH-2′,3′,5′), 6.09 (d, 1H, $J_{1',2'}$ = 6.1, H-1′), 6.61 (dd, 1H, $J_{4,3}$ = 3.3, $J_{4,5}$ = 1.9, H-4-furyl), 6.67 (dd, 1H, $J_{3,4}$ = 3.3, $J_{3,5}$ = 0.8, H-3-furyl), 6.88 (bs, 2H, NH_2), 7.78 (dd, 1H, $J_{5,4}$ = 1.9, $J_{5,3}$ = 0.8, H-5-furyl), 7.83 (s, 1H, H-6), 8.13 (s, 1H, H-2). ^{13}C NMR (125.7 MHz, DMSO-d_6): 61.81 (CH_2-5′), 70.67 (CH-3′), 74.00 (CH-2′), 85.33 (CH-4′), 87.27 (CH-1′), 99.55 (C-4a), 105.50 (CH-3-furyl), 106.34 (C-5), 112.09 (CH-4-furyl), 120.70 (CH-6), 142.16 (CH-5-furyl), 148.77 (C-2-furyl), 151.04 (C-7a), 152.26 (CH-2), 157.45 (C-4). MS (FAB): *m*/*z* 333 (M + H), 355 (M + Na). HRMS (FAB) for $C_{15}H_{17}N_4O_5$ [M + H] calcd, 333.1199; found, 333.1202. Anal. ($C_{15}H_{16}N_4O_5 \cdot H_2O$): C, H, N.

4-Amino-5-phenyl-7-(β-D-ribofuranosyl)-7H-pyrrolo[2,3-d]pyrimidine 5′-O-triphosphate Sodium Salt (***10a***). An argon purged mixture of $Pd(OAc)_2$ (1.5 mg, 6.7 μmol) and TPPTS (17.3 mg, 30 μmol) in water/MeCN (2:1, 1.2 mL) was sonicated to full dissolution, and one-quarter of this preprepared solution (0.3 mL, 1/4 of total amount) was added to an argon purged mixture of compound **12** (20.1 mg, 29 μmol), phenylboronic acid (5.3 mg, 43 μmol), and Cs_2CO_3 (28 mg, 87 μmol) in water/MeCN (2:1, 0.6 mL), and the mixture was stirred at 100 °C for 30 min. After cooling, the mixture was filtered through microfilter and purified by HPLC on C-18 phase (0→100% MeOH in 0.1 M aq TEAB) affording after ion exchange on Dowex 50 (Na^+ form) and lyophilization title compound **10a** as white cotton (8.6 mg, 46%). ^{1}H NMR (500 MHz, D_2O + phosphate buffer, pH = 7.1, $ref_{dioxane}$ = 3.75 ppm): 4.14 (ddd, 1H, J_{gem} = 11.6, $J_{H,P}$ = 4.7, $J_{5'b,4'}$ = 3.5, H-5′b), 4.25 (ddd, 1H, J_{gem} = 11.6, $J_{H,P}$ = 6.5, $J_{5'a,4'}$ = 3.3, H-5′a), 4.35 (m, 1H, $J_{4',5'}$ = 3.5, 3.3, $J_{4',3'}$ = 2.9,

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$J_{H,P}$ = 1.7, H-4′), 4.57 (dd, 1H, $J_{3',2'}$ = 5.4, $J_{3',4'}$ = 2.9, H-3′), 4.73 (dd, 1H, $J_{2',1'}$ = 6.9, $J_{2',3'}$ = 5.4, H-2′), 6.30 (d, 1H, $J_{1'2'}$ = 6.9, H-1′), 7.45 (m, 1H, H-*p*-Ph), 7.49−7.56 (m, 4H, H-*o*,*m*-Ph), 7.57 (s, 1H, H-6), 8.16 (s, 1H, H-2). ^{13}C NMR (125.7 MHz, D_2O + phosphate buffer, pH = 7.1, $ref_{dioxane}$ = 69.3 ppm): 68.22 (d, $J_{C,P}$ = 5, CH_2-5′), 73.26 (CH-3′), 76.24 (CH-2′), 86.31 (d, $J_{C,P}$ = 9, CH-4′), 88.34 (CH-1′), 103.75 (C-4a), 121.56 (C-5), 122.86 (CH-6), 130.41 (CH-*p*-Ph), 131.59 and 131.94 (CH-*o*,*m*-Ph), 136.10 (C-*i*-Ph), 153.15 (C-7a), 153.67 (CH-2), 159.61 (C-4). ^{31}P (^{1}H dec) NMR (202.4 MHz, D_2O + phosphate buffer, pH = 7.1, ref_{H3PO4} = 0 ppm): −21.32 (dd, J = 19.3, 19.0, P_β), −10.45 (d, J = 19.3, P_α), −6.98 (d, J = 19.0, P_γ). MS (ESI, negative mode) m/z 581 (M-3Na + 2H), 603 (M-2Na + H), 625 (M-Na). HRMS (ESI, negative mode)$^-$ for $C_{17}H_{20}N_4O_{13}P_3$ [M-3Na + 2H] calcd, 581.0240; found, 581.0253.

*4-Amino-5-phenyl-7-(β-D-ribofuranosyl)-7H-pyrrolo[2,3-d]pyrimidine 5′-O-monophosphate Sodium Salt (**11a**).* An argon purged mixture of $Pd(OAc)_2$ (1.7 mg, 7.6 μmol) and TPPTS (21.7 mg, 38 μmol) in water/MeCN (2:1, 1.6 mL) was sonicated until dissolution, and one-quarter of resulting solution (0.4 mL, 1/4 of total amount, 1.9 μmol Pd) was added to an argon purged mixture of compound **13** (21 mg, 38 μmol), phenylboronic acid (6.9 mg, 57 μmol), and Na_2CO_3 (12 mg, 114 μmol) in water/MeCN (2:1, 0.8 mL), and the mixture was stirred at 100 °C for 1.5 h. After cooling, the mixture was filtered through microfilter and purified by HPLC on C-18 phase (0→100% MeOH in 0.1 M aq TEAB), affording after ion exchange on Dowex 50 (Na^+ form) and lyophilization title compound **11a** as white solid (15.8 mg, 94%). ^{1}H NMR (500 MHz, D_2O, $ref_{dioxane}$ = 3.75 ppm): 3.91 (dt, 1H, J_{gem} = 11.4, $J_{H,P}$ = $J_{5'b,4'}$ = 4.4, H-5′b), 3.95 (ddd, 1H, J_{gem} = 11.4, $J_{H,P}$ = 5.6, $J_{5'a,4'}$ = 4.2, H-5′a), 4.30 (ddd, 1H, $J_{4',5'}$ = 4.4, 4.2, $J_{4',3'}$ = 2.7, H-4′), 4.46 (dd, 1H, $J_{3',2'}$ = 5.4, $J_{3',4'}$ = 2.7, H-3′), 4.75 (dd, 1H, $J_{2',1'}$ = 7.1, $J_{2',3'}$ = 5.4, H-2′), 6.31 (d, 1H, $J_{1'2'}$ = 7.1, H-1′), 7.46 (m, 1H, H-*p*-Ph), 7.54 (m, 2H, H-*m*-Ph), 7.56 (m, 2H, H-*o*-Ph), 7.58 (s, 1H, H-6), 8.18 (s, 1H, H-2). ^{13}C NMR (125.7 MHz, D_2O, $ref_{dioxane}$ = 69.3 ppm): 66.53 (d, $J_{C,P}$ = 5, CH_2-5′), 73.61 (CH-3′), 76.15 (CH-2′), 86.94 (d, $J_{C,P}$ = 9, CH-4′), 88.22 (CH-1′), 103.97 (C-4a), 121.38 (C-5), 122.79 (CH-6), 130.44 (CH-*p*-Ph), 131.72 (CH-*o*-Ph), 131.91 (CH-*m*-Ph), 136.31 (C-*i*-Ph), 153.35 (C-7a), 154.40 (CH-2), 160.15 (C-4). ^{31}P (^{1}H dec) NMR (202.4 MHz, D_2O, ref_{H3PO4} = 0 ppm): 4.64. MS (ESI) m/z 445 (M + H), 467 (M + Na). HRMS (ESI) for $C_{17}H_{19}N_4NaO_7P$: [M + H] calcd, 445.0884; found, 445.0880.

Biology. *Cytostatic Activity Assays.* All cell lines were obtained from ATCC (Manassas, VA). Colon (HCT116, HCT 15), breast (BT549, HS 578), lung (A549, NCI-H23), and T-lymphoblastic (CCRF-CEM) cell lines were maintained in the RPMI cultivation medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). Prostate cell lines (Du145, PC3) were cultivated in MEM/F12K medium containing 10% FBS, respectively. Doxorubicin, clofarabine, trichloroacetic acid (TCA), and sulforhodamine B (SRB) were from Sigma-Aldrich (St. Louis, MO). Gemcitabine was obtained from Moravek Biochemicals (Brea, CA)

A modified protocol of sulforhodamine B colorimetric assay was used for the cytostatic activity screening.[18] Cells were distributed into the 96-well plates in 150 μL of media (HCT116 and Du145 5300 cell/mL, HCT15 and A549 10600cells/mL, Hs578 and BT549 26600 cells/mL, PC3 16600 cells/mL, NCI-H23 40000 cells/mL) and incubated overnight in humidified CO_2 incubator at 37 °C. Next day, one plate of each cell line was fixed with TCA by removing media and adding 100 μL of cold 10% (v/v) TCA to each well. After 1 h incubation at 4 °C, TCA was discarded and plates were washed four times with tap water. These plates represented cell counts at day zero. The tested compounds were 5-fold serially diluted and distributed to cells in 50 μL of media. After five days of incubation, the plates were fixed with TCA as mentioned above and 100 μL of 0.057% SRB solution in 1% (v/v) acetic acid was added to each well. After 30 min incubation at room temperature, SRB was removed and the plates were rinsed four times 1% (v/v) acetic acid. Next, 200 μL of 10 mM Tris base solution (pH 10.5) was added to each well of completely dried plates and absorbance of cell associated SRB was read at 500 nm. The percentage of cell-growth inhibition was calculated using the following formula: % of control cell growth = 100 × (OD_{sample} − mean OD_{day0})/($OD_{neg\ control}$ − mean OD_{day0}). For GIC_{50} determination, dose−response curves between the compound concentration and percent of growth inhibition were plotted. GIC_{50} values can be derived by fitting dose−response curves using a sigmoidal dose−response equation.

Cytotoxic 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay[20]. The most cytostatic compounds were further tested in cytotoxic MTT assay on extended cancer cell line panel. All cells were purchased from the ATCC (Manassas, VA), unless otherwise indicated. The daunorubicin resistant subline of CEM cells (CEM-DNR bulk) and paclitaxel resistant subline K562-tax were selected in our laboratory by the cultivation of maternal cell lines in increasing concentrations of daunorubicine or paclitaxel, respectively.[21] The cells were cultured in DMEM/RPMI 1640 with 5 g/L glucose, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal calf serum, and $NaHCO_3$.

Cell suspensions were prepared and diluted according to the particular cell type and the expected target cell density (2500−30000 cells/well based on cell growth characteristics). Cells were added by pipet (80 μL) into 96-well microtiter plates. Inoculates were allowed a preincubation period of 24 h at 37 °C and 5% CO_2 for stabilization. Four-fold dilutions, in 20 μL aliquots, of the intended test concentration were added to the microtiter plate wells at time zero. All test compound concentrations were examined in duplicate. Incubation of the cells with the test compounds lasted for 72 h at 37 °C, in a 5% CO_2 atmosphere at 100% humidity. At the end of the incubation period, the cells were assayed using MTT. Aliquots (10 μL) of the MTT stock solution were pipetted into each well and incubated for a further 1−4 h. After this incubation period, the formazan produced was dissolved by the addition of 100 μL/well of 10% aq SDS (pH = 5.5), followed by a further incubation at 37 °C overnight. The optical density (OD) was measured at 540 nm with a microplate reader. Tumour cell survival (IC_{50}) was calculated using the following equation: IC = ($OD_{drug\text{-}exposed\ well}$/mean $OD_{control\ wells}$) × 100%. The IC_{50} value, the drug concentration lethal to 50% of the tumor cells, was calculated from appropriate dose−response curves.

Human ADK Inhibition[6]. The standard reaction mixture (50 μL) contained 50 mM HEPES pH 6.2, 10 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 80 μg BSA, 1 μCi of [^{3}H]-adenosine (20 Ci/mmol), 1 μM unlabeled adenosine, various concentration of tested compounds, and 0.94 ng of adenosine kinase. The mixtures were incubated at 37 °C and separated on PEI cellulose plate (prespotted with 0.01 AMP). The plates were developed in the solvent system 2-propanol−NH_4OH−water (7:1:2). The spots were visualized under UV light (254 nm) and cut out for radioactivity determination in the toluene-based scintillation cocktail.

Intracellular Metabolism. Du145 cells were seeded into T25 flasks at 60% confluence in the MEM medium supplemented with 10% FBS. Next day, the medium was replaced with fresh media containing the tested compounds at 10 μM concentration. After 6 and 24 h of incubation, cells were washed with phosphate buffer and detached with trypsin. Trypsin was neutralized by adding cultivation medium, and the cells were spun for 5 min at 500*g*. The supernatants were removed, and cell pellets were resuspended in 0.5 mL of media. Cell suspension was layered onto 0.25 mL of Nyosil M25 oil and centrifuged for 3 min. The media was aspirated off, and the top of the oil layer was washed with water. Both water and oil were aspirated off without disturbing the cell pellet. The cells were extracted with 500 μL of 70% MeOH, and cell lysates were centrifuged, supernatants collected, dried by vacuum, and samples were resuspended in 1 mM Ammonium phosphate pH 8.5.

Transient ion-pairing high-performance liquid chromatography using dimethylhexylamine coupled to positive ion electrospray tandem mass spectrometery (LC/MS/MS) was used to quantitate intracellular nucleotides.[22] Standard curves and quality control samples were generated for all analytes using extracts from untreated cells. Methods were adapted from those described for the acyclic phosphonate nucleotide analogue adefovir, its phosphorylated metabolites, and natural nucleotides.[23]

RNA Polymerase II Inhibition Assay. The assay was performed using a HeLaScribe nuclear extract (Promega). The reaction mixture (25 μL) contained 7.5 μL 1× HeLaScribe transcription buffer, 3 mM $MgCl_2$, 100 ng CMV(+) control DNA, 400 μM CTP, GTP, UTP, and 25 μM [^{33}P]ATP. The mixture was preincubated with various concentrations of the inhibitor for 5 min at 30 °C, followed by the addition of 3.5 μL of HeLaScribe nuclear extract. After 1 h incubation at 30 °C, the polymerase reaction was stopped by adding Proteinase K, SDS, and EDTA. After additional incubation at 37 °C for 3 h and denaturation at 75 °C, the reaction mixture was separated on 6% polyacrylamide gel with 8 M urea. The full-length product of transcription was quantified using Typhoon Trio imager and Image Quant TL software (GE Healthcare), and the IC_{50} value was defined as the concentration of inhibitor at which a 50% decrease in the product formation was observed.

Cell Cycle and Apoptosis Analysis. Subconfluent CCRF-CEM cells (ATCC), seeded at the density of 5×10^5 cells/ml in 6-well panels, were cultivated with the 1× or 5× GIC_{50} of tested compounds in a humidified CO_2 incubator at 37 °C in RPMI 1640 cell culture medium containing 10% fetal calf serum, 10 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Controls containing vehicle were harvested at the same time points (2−24 h). Cells were washed with cold PBS and fixed in 70% ethanol overnight at −20 °C. The next day, the cells were washed in hypotonic citrate buffer, treated with RNase (50 μg/mL), stained with propidium iodide, and analyzed by flow cytometry using a 488 nm single beam laser (Becton Dickinson). Cell cycle was analyzed in the program ModFitLT (Verity), and apoptosis was measured in logarithmic mode as a percentage of the particles with propidium content lower than cells in G0/G1 phase (sub-G1) of the cell cycle. Half of the sample was used for phospho-histon $H3^{Ser10}$ antibody (Sigma) labeling and subsequent flow cytometry analysis of mitotic cells.[24]

BrdU Incorporation Analysis. Cells were cultured as for cell cycle analysis. Before harvesting, they were pulse-labeled with 10 μM 5-bromo-2′-deoxyuridine (BrdU) for 30 min. The cells were trypsinized, fixed with ice-cold 70% ethanol, incubated on ice for 30 min, washed with PBS, and resuspended in 2 M HCl for 30 min at room temperature to denature their DNA. Following neutralization with 0.1 M $Na_2B_4O_7$, the cells were washed with PBS containing 0.5% Tween-20 and 1% BSA. They were then stained with primary anti-BrdU antibody (Exbio) for 30 min at room temperature in the dark. Cells were than washed with PBS and stained with secondary antimouse-FITC antipody (Sigma). The cells were then washed with PBS and incubated with propidium iodide (0.1 mg/mL) and RNase A (0.5 mg/mL) for 1 h at room temperature in the dark and finally analyzed by flow cytometry using a 488 nm single beam laser (FACSCalibur, Becton Dickinson).[25]

BrU Incorporation Analysis. Cells were cultured as for cell cycle analysis. Before harvesting, they were pulse-labeled with 1 mM 5-bromouridine (BrU) for 30 min. The cells were fixed in 1% buffered paraformaldehyde with 0.05% of NP-40, incubated in room temperature for 15 min, and then in the refridgerator overnight. They were then washed in 1% glycin in PBS, washed in PBS, and stained with primary anti-BrdU antibody crossreacting to BrU (Exbio) for 30 min at room temperature in the dark. Cells were than washed with PBS and stained with secondary antimouse-FITC antibody (Sigma). Following the staining, the cells were washed with PBS and fixed with 1% PBS buffered paraformaldehyde with 0.05% of NP-40. The cells were then washed with PBS, incubated with propidium iodide (0.1 mg/mL) and RNase A (0.5 mg/mL) for 1 h at room temperature in the dark, and finally analyzed by flow cytometry using a 488 nm single beam laser (FACSCalibur, Becton Dickinson).[25]

In Vivo Activity in P388D1 Leukemia Model. The P388D1 mouse leukemia cells were obtained from ATCC (Manassas, VA) and grown in the RPMI cultivation medium (Sigma Aldrich, Prague, Czech Republic) supplemented with 10% fetal bovine serum. DBA/2 mice were obtained from Charles River Laboratories (Sulzfeld, Germany) and maintained in specific pathogen free facility. Then 10^5 of P388D1 leukemia cells per mice were transplanted intraperitoneally on day 0 to 18−22 g female mice, and animals were randomized into control and treatment groups (10 mice per group). The testing regiment included administering **6g** once a day intraperitoneally dissolved in 50% solution of PEG400/deionized water (pH 7.0) in two cycles on day 1−3 and 8−10. Animals were treated at MTD and 1/2 MTD (70 and 35 mg/kg of **6g**, respectively), and control mice received volume equivalent of the vehicle. Animal weights and deaths were recorded daily. Survival parameters (MST and percentage of ILS) were calculated. Kaplan−Meier survival curves were constructed and the significant difference in overall survival (control versus treatment groups) was evaluated by log-rank test (Figure 2).

■ ASSOCIATED CONTENT

Supporting Information. Synthetic procedures and characterization data of compounds **6b**−**e**, **6i**−**n**, **10h**,**i**, and **11h**,**i** and analytical characterization data for the final nucleosides. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

ADK, adenosine kinase; BrdU, 5-bromo-2′-deoxyuridine; BrU, 5-bromouridine; HPFC, high performance flash chromatography; ILS, increased lifespan percentage; MTD, maximum tolerated dose; MST, mean survival time; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMP, nucleoside 5′-*O*-monophosphate; NTP, nucleoside 5′-*O*-triphosphate; SRB, sulforhodamine B; TCA, trichloroacetic acid; TDA-1, tris(2-(2-methoxyethoxy)ethyl)amine; TMS, trimethylsilyl; TPPTS, tris(3-sulfonatophenyl)phosphine; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

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