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6-(Het)aryl-7-Deazapurine Ribonucleosides as Novel Potent Cytostatic Agents

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A series of novel 7-deazapurine ribonucleosides bearing an alkyl, aryl, or hetaryl group in position 6 and H, F, or Cl atom in position 7 has been prepared either by Pd-catalyzed cross-coupling reactions of the corresponding protected 6-chloro-(7-halogenated-)7-deazapurine ribonucleosides with alkyl- or (het)-arylorganometallics followed by deprotection, or by single-step aqueous phase cross-coupling reactions of unprotected 6-chloro-(7-halogenated-)7-deazapurine ribonucleosides with (het)arylboronic acids. Significant cytostatic effect was detected with a substantial proportion of the prepared compounds. The most potent were 7-H or 7-F derivatives of 6-furyl- or 6-thienyl-7-deazapurines displaying cytostatic activity in multiple cancer cell lines with a geometric mean of 50% growth inhibition concentration ranging from 16 to 96 nM, a potency comparable to or better than that of the nucleoside analogue clofarabine. Intracellular phosphorylation to mono- and triphosphates and the inhibition of total RNA synthesis was demonstrated in preliminary study of metabolism and mechanism of action studies.

Introduction

Purine nucleosides and their analogues represent an important class of biologically active compounds that have been extensively explored in the last five decades for their antiviral and antitumor properties. A number of active compounds in this class are being used as clinical drugs for the treatment of viral infections such as HIV[a] (abacavir, didanosine) and chronic hepatitis B (entecavir). Many purine (fludarabine, cladribine, and clofarabine)[1] and other (gemcitabin, ara-C, 5-F-dU, 2-deoxycoformycin, capecitabine, nelarabine, and decitabine)[2] nucleosides are clinical therapeutics for variety of hematological malignancies. Despite systematic studies of modified purine nucleosides, there still remains a space for the design of new analogues and development of novel nucleoside-based therapeutics. In particular, there is a need for new cytostatics[3] for the treatment of drug-resistant tumors and antivirals for therapeutic interventions against infections by various RNA viruses, particularly hepatitis C virus (HCV).[4] In our prior studies, we have discovered a new type of biologically active nucleosides **1**: purine ribonucleosides bearing aryl or hetaryl substituents in position 6.[5] This group of compounds exhibits[5] low micromolar cytostatic activities toward leukemia cell-lines. Moreover, some 6-hetarylpurine ribonucleosides also exert[6] strong anti-HCV activities. Although the mechanism of action remains to be fully identified, potent inhibition of both viral and host RNA synthesis[6] was observed, suggesting that these nucleosides are phosphorylated to triphosphates that may subsequently interfere with the activity of viral and/or host RNA polymerases. However, the lack of selectivity toward HCV prevented further development of these compounds as antivirals. Therefore, novel modifications of 6-hetaryl nucleosides were pursued with a goal to achieve either selective antiviral activity through the specific inhibition of HCV RNA polymerase or more potent cytostatic activity that could potentially lead to novel anticancer therapeutics. Sugar-modified derivatives (2′- and 5′-deoxyribonucleosides,[7] 3′-deoxyribonucleosides,[8] as well as 2′-*C*-methyl-ribonucleosides[9]) of the 6-aryl- or 6-hetarylpurine series are all inactive, while some carbocyclic homonucleosides were reported[10] to still exert cytostatic effects. Some L-ribonucleosides were found[11] to exhibit weak anti-HCV effect in replicon assay, but their triphoshates did not inhibit HCV RNA polymerase. In addition, nucleoside with modified purine ring such as 2-substituted[12] and 8-substituted[13] 6-arylpurine ribonucleosides as well as purine ribonucleosides bearing partially or fully saturated hetaryl groups[14] do not exhibit any notable biological activities. Replacing purine-ring N-atoms by carbon to form deazapurine analogues has also been explored. While some 6-aryl-1-deazapurine nucleosides[15] possessed moderate cytostatic activities, the corresponding 6-aryl-3-deazapurine nucleosides **2** were devoid of cytostatic and antiviral activities.[16] This shows that the N-3 nitrogen is crucial for the interaction of these compounds with the target biological system (presumably a kinase or RNA polymerase), while the N-1 nitrogen is not. Therefore, the next logical step was to assess the role of N-7 nitrogen, which is engaged neither in H-bonds with the complementary pyrimidine nucleobase during biosynthesis of RNA nor in minor groove interactions in the active site of RNA polymerase. We

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[a] Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; BrU, bromouridine; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HPFC, high performance flash chromatography; NMP, nucleoside 5′-*O*-monophosphate; NTP, nucleoside 5′-*O*-triphosphate; SRB, sulforhodamine; TCA, trichloroacetic acid; TDA-1, tris(2-(2-methoxyethoxy)-ethyl)amine; TIPS, triisopropylsilyl, TTPTS, tris(3-sulfonatophenyl)phosphine.

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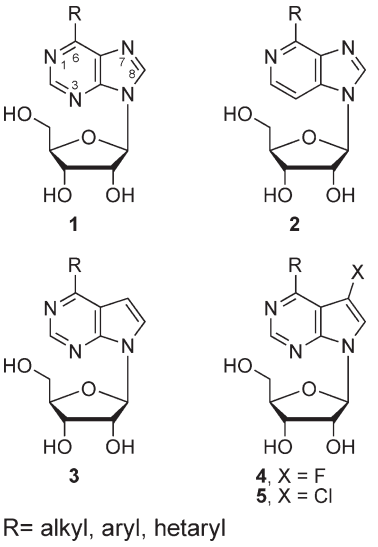
Chart 1



were interested whether the replacement of the N-7 nitrogen by C−H, C−F, or C−Cl would result in improved selectivity toward viral RNA polymerase or enhanced cytostatic effect, presumably through the inhibition of cellular RNA polymerases. Here we report on the synthesis and evaluation of cytostatic and anti-HCV activity of novel 7-unsubstituted or 7-halogenated 6-aryl-7-deazapurine ribonucleosides **3**−**5** (Chart 1). Because 6-ethyl-[17] and 6-(hydroxymethyl)purine[18] ribonucleosides are known to be potent cytostatics, we have also included 6-ethyl and 6-hydroxymethyl substituents into the series.

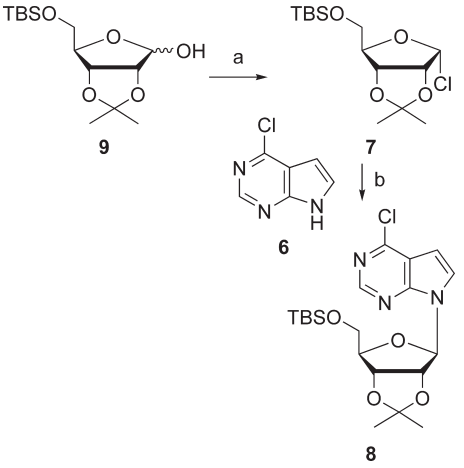
7-Deazapurine (correct IUPAC name is pyrrolo[2,3-*d*]-pyrimidine but purine numbering will be used throughout the paper) nucleosides are important compounds showing broad spectrum of activities.[19] Tubercidin (7-deazaadenosine)[20] is an antibiotic exerting potent cytotoxicity and antiviral activity. Tubercidin and some other 7-deazapurine nucleosides are known to be inhibitors of adenosine kinases[21] and display antiparasitic activity.[22] 2′-*C*-Me-ribonucleosides derived from 7-deazaadenine (including 7-fluoro- and 7-chloro derivatives) were found to be highly potent inhibitors of HCV and are being evaluated as potential antiviral therapeutics.[23]

Chemistry

Our synthesis of novel 6-substituted 7-deazapurine ribonucleosides **3a**−**s** relied on palladium catalyzed cross-coupling reactions of either protected or unprotected 6-chloro-7-deazapurine ribonucleosides. The glycosylation (Scheme 1) of sodium[24] or (more preferably) potassium salt[25] of 6-chloro-7-deazapurine **6** with "nonparticipating" α-D-halogenose **7**[26] affording protected riboside **8** represents hitherto the only practically usable access to 6-chloro-7-deazapurine ribonucleosides unsubstituted in position 7. Unstable halogenose **7** is generated from lactol **9** by Appel's chlorination and is immediately used in the displacement reaction. In accordance with literature reports,[22] the protocol[25] utilizing (KOH/TDA-1/MeCN-THF) provided the β-anomer **8** contaminated with unseparable amounts of α-anomer in an overall yield of up to 40% from **9**.

During extensive glycosylation studies utilizing pyrrolopyrimidines base anions with a series of analogous α-D-ribofuranosyl chlorides, Ugarkar[21] developed modified conditions

Scheme 1[a]



[a] Conditions: (a) CCl_4, $P(NMe_2)_3$, THF or toluene; (b) KOH, TDA-1, MeCN, or toluene (40% from **9**).

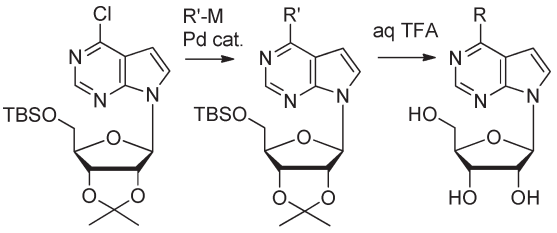
using toluene as a solvent in both steps (chlorination and glycosylation). Our application of the same conditions for glycosylation of 6-chloro-7-deazapurine **6** with chlorose **7** gave the desired 6-chloro-7-deazapurine riboside **8** as a single β-anomer in an overall yield of 63% from **9**.

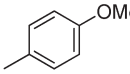
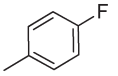
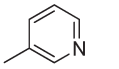
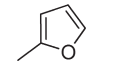
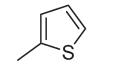
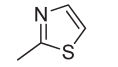
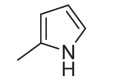
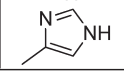
Palladium catalyzed cross-coupling reactions[27] of compound **8** (Scheme 2) with the corresponding alkyl- or arylboronic acids, -zinc, -tin, and -aluminum reagents were performed to provide protected 6-substituted 7-deazapurines ribonucleosides **10a**−**l** in good yields. The reaction conditions were derived from previously reported procedures for the modification of purine derivatives.[5,18,28] The subsequent deprotection step was performed by treatment with 90% aqueous trifluoroacetic acid to afford the desired ribonucleosides **3a**−**3l**. *N*-Protecting Boc (**10k**) and trityl (**10l**) groups were also simultaneously removed under the acidic deprotection conditions. In the case of 6-hydroxymethyl derivative (**10b**), the benzoyl group was quantitatively deprotected with sodium methoxide in methanol before final acidic deprotection.

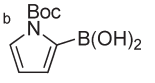
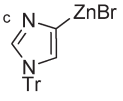
Alternative 6-hetaryl-7-deazapurine ribosides **3m**−**3s** (Scheme 3) were prepared directly from unprotected 6-chloro-7-deazapurine riboside **11**, using aqueous-phase Suzuki cross-coupling reaction[29] (**3m**−**3r**) or Stille coupling (**3s**). In the case of 3-pyrrolyl derivative, the *N*-protecting triisopropylsilyl moiety was simultaneously deprotected under the strongly basic conditions of the aqueous coupling (**3o**). It should be noted that in the case of *N*H containing boronic acids, we also observed the formation of the *N*-arylated product formed by coupling of the 5-membered heterocycle with chloro derivative **11**. Thus in the case of 4-pyrazolyl derivative **3r**, the concomitant *N*-arylation gave 1,4-bis{7-(β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl}-1*H*-pyrazole (**3rX**) as side-product in 18% yield.

The synthesis of analogous 7-fluoro-6-hetaryl(aryl)-7-deazapurine ribosides started with cross-coupling reactions of per-*O*-benzoylated 6-chloro-7-fluoro-7-deazapurine riboside **12**[30] (Scheme 4), under analogous conditions described for the coupling reactions on **8**. The resulting 6-substituted 7-fluoro-7-deazapurine nucleosides **13d**,**h**−**n** were then deprotected under Zemplén conditions to afford the unprotected 7-fluoro-6-substituted-7-deazapurine ribonucleosides **4d**,**h**−**n** in good yields.

Scheme 2[a]



10, 3	-R (-R')	M	Products (yields)	
a	-Et	$AlEt_2$	**10a** (82%)	**3a** (99%)
b	$-CH_2OH$	ZnI [a]	**10b** (77%) [d]	**3b** (92%) [e]
c	-Bn	ZnCl	**10c** (93%)	**3c** (85%)
d	-Ph	$B(OH)_2$	**10d** (95%)	**3d** (96%)
e		$B(OH)_2$	**10e** (99%)	**3e** (62%)
f		$B(OH)_2$	**10f** (77%)	**3f** (94%)
g		$B(OH)_2$	**10g** (95%)	**3g** (83%)
h		$SnBu_3$	**10h** (93%)	**3h** (63%)
i		$SnBu_3$	**10i** (95%)	**3i** (88%)
j		$SnBu_3$	**10j** (90%)	**3j** (85%)
k		$B(OH)_2$ [b]	**10k** (76%)	**3k** (83%)
l		ZnBr [c]	**10l** (66%)	**3l** (93%)

R'-M: [a] $BzOCH_2ZnI$ [b]  [c] 

[d] partly (23%) debenzoylated

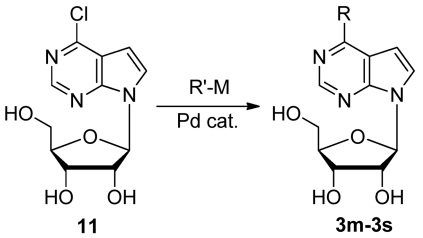
[e] additional treatment with NaOMe prior to TFA

[a] Conditions: (A) M = ZnX or $AlEt_2$:$Pd(PPh_3)_4$, THF, 70°C (rt for **10b**); (B) M = $B(OH)_2$:$Pd(PPh_3)_4$, K_2CO_3, toluene, or dimethoxyethane−water [3:1 for **10g** and 4:1 for **10k**], 100°C; (C) M = $SnBu_3$:$PdCl_2(PPh_3)_2$, DMF, 100°C.

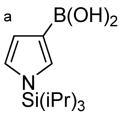
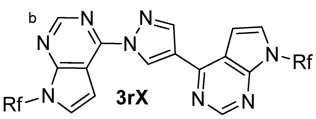
A different approach was used for the synthesis of 3-pyrrolyl derivative **4o** (Scheme 5). Glycosylation of α-chlorose **7** using the potassium salt of 4-chloro-5-fluoropyrrolo[2,3-*d*]-pyrimidine **15** provided protected nucleoside **16** in 43% yield (in two steps from **9**). Acidic deprotection afforded nucleoside **14** in 85% yield. Aqueous Suzuki reaction of 6-chloro-7-fluoro-7-deazapurine riboside **14** with TIPS-protected pyrrole-3-boronic acids gave the desired nucleoside **4o** in 73% yield.

Synthesis of compounds in the 7-chloro-7-deazapurine series consisted of palladium-catalyzed regioselective cross-coupling reactions on per-*O*-benzoyl-6,7-dichloro-7-deazapurine riboside **17**[31] (Scheme 6) to provide benzoylated 6-hetaryl(aryl) products **18d**,**h**,**i**,**m**,**n**. The cross-coupling reactions proceeded selectively at position 6 leaving the chlorine in position 7 intact. Deprotection of intermediates **18** with methanolic sodium methoxide afforded free ribonucleosides

Scheme 3[a]



3	-R (-R')	M	Products (yields)
m		$B(OH)_2$	**3m** (69%)
n		$B(OH)_2$	**3n** (67%)
o		$B(OH)_2$ [a]	**3o** (55%)
p		$B(OH)_2$	**3p** (64%)
q		$B(OH)_2$	**3q** (64%)
r		$B(OH)_2$	**3r** (12%) [b]
s		$SnBu_3$	**3s** (51%)

R'-M: [a]  [b] 

3rX

side-product, 18%

[a] Conditions: (A) M = $B(OH)_2$:$Pd(OAc)_2$, TPPTS, Na_2CO_3, water/MeCN (2:1), 100°C; (B) M = $SnBu_3$:$PdCl_2(PPh_3)_2$, DMF, 100 °C.

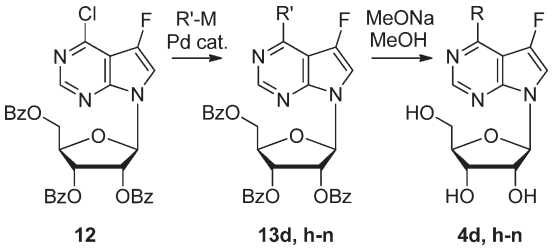
5d,**h**,**i**,**m**,**n** (again, no nucleophilic substitution of the 7-Cl was observed) in excellent yields.

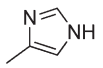
Biological Profiling

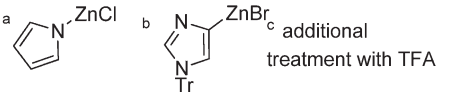
Cytostatic Activity. Cytostatic activity of all prepared compounds was initially evaluated against four different cell lines derived from various human solid tumors including lung (A-549 cells), prostate (Du-145 cells), colon (HCT-116 cells), and breast (HS-578 cells) carcinomas. Concentrations inhibiting the cell growth by 50% (GIC_{50}) were determined using a quantitative cellular staining with sulforhodamine B (SRB)[32] following a 5-day treatment. The SRB method allowed for determining a net effect on cell growth by subtracting background signal generated by the cell culture inoculum at the beginning of treatment.

In general, 6-substituted 7-deaza-nucleosides modified with a 6-member (het)aryl ring either with or without substitution (compounds **3c**−**f**,**3g**, and **3s**) showed minimal to no cytostatic activity against the tested cell lines (Table 1). In contrast, a number of nucleosides containing 5-member heterocyclic aryl in 6-position exhibited potent cytostatic effects, with those containing furan or thiophene being among the most active. In particular, compounds substituted with furan-2-yl (**3h**) and thiophen-2-yl (**3i**) showed the most potent cytostatic activity across all four tested cell lines with GIG_{50} values ranging from 7 to 100 nM. In comparison,

Scheme 4[a]

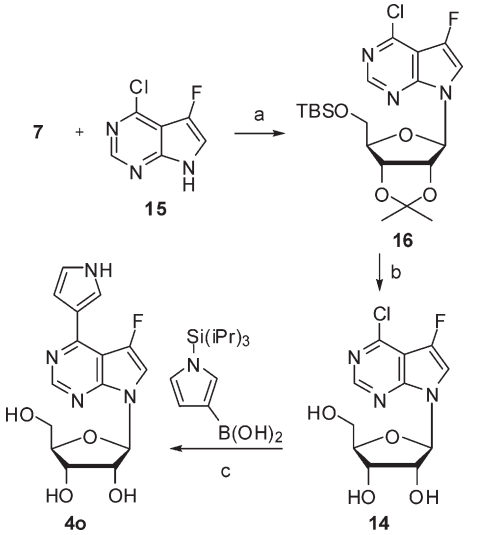


13, 4	-R (-R')	M	Products (yields)	
d	-Ph	$B(OH)_2$	**13d** (93%)	**4d** (79%)
h		$SnBu_3$	**13h** (99%)	**4h** (78%)
i		$SnBu_3$	**13i** (74%)	**4i** (74%)
j		$SnBu_3$	**13j** (86%)	**4j** (68%)
k		ZnCl [a]	**13k** (42%)	**4k** (89%)
l		ZnBr [b]	**13l** (54%)	**4l** (70%)[c]
m		$B(OH)_2$	**13m** (66%)	**4m** (78%)
n		$B(OH)_2$	**13n** (67%)	**4n** (81%)

R'-M:  additional treatment with TFA

[a] Conditions: (A) M = $B(OH)_2$:$Pd(PPh_3)_4$, K_2CO_3, toluene, 100°C; (B) M = $SnBu_3$:$PdCl_2(PPh_3)_2$, DMF, 100°C; (C) M = ZnX or $AlEt_2$: $Pd(PPh_3)_4$, THF, 70 °C.

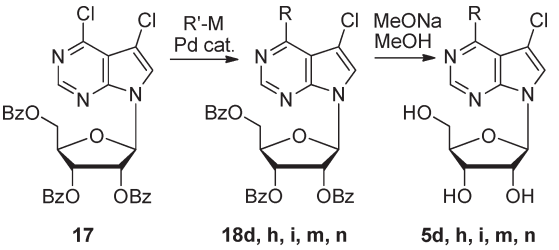
Scheme 5[a]



[a] Conditions: (a) KOH, TDA-1, toluene (43% from **9**); (b) 90% TFA (aq) (85%); (c) $Pd(OAc)_2$, TPPTS, Na_2CO_3, H_2O-MeCN (73%).

analogues substituted with furan-3-yl (**3m**) and thiophen-3-yl (**3n**) were slightly less active, exhibiting GIC_{50} values of approximately 30−700 nM. Most nucleosides containing

Scheme 6[a]



18, 5	-R (-R')	M	Products (yields)	
d	-Ph	$B(OH)_2$	**18d** (99%)	**5d** (91%)
h		$SnBu_3$	**18h** (99%)	**5h** (86%)
i		$SnBu_3$	**18i** (89%)	**5i** (94%)
m		$B(OH)_2$	**18m** (86%)	**5m** (80%)
n		$B(OH)_2$	**18n** (92%)	**5n** (87%)

[a] Conditions: (A) M = $B(OH)_2$:$Pd(PPh_3)_4$, K_2CO_3, toluene, 100°C; (B) M = $SnBu_3$:$PdCl_2(PPh_3)_2$, DMF, 100 °C.

5-member N-heterocycles (pyrrole, pyrazole, or imidazole; **3k**, **3l**, **3q**, and **3r**) with the exception of **3o** (pyrrol-3-yl) showed further reduced potency with some of them being inactive even at 10 μM concentration. A number of potent compounds were also identified among analogues further modified with 7-halogen atom; active compounds were identified both among 7-fluoro and 7-chloro derivatives. For example, compounds **4h** and **4i** exhibited similar cytostatic potency as the corresponding compounds without 7-substitution (**3h** and **3i**). In general, analogues with 7-fluoro substitution tended to be more active than corresponding compounds bearing 7-chloro modifications. Notably, examples of compounds were identified that demonstrated a profoundly negative effect of 7-chloro on the cytostatic potency (e.g., **5h**).

The synthesized nucleosides were also tested for their cytotoxic activity in human T-lymphoid (CCRF-CEM), promyelocytic leukemia (HL60), and cervical carcinoma (HeLa) cell lines using and XTT-based cell viability assay[33] following a shorter 72 h incubation. The potency of most tested compounds in the XTT-based assays was lower compared to the SRB-based cytostatic screening assays. In general, the compound potency ranking followed similar trends as those observed in the SRB assays, but the differences among individual nucleosides were substantially less pronounced compared to what has been observed in the SRB assay. Shorter duration of incubation during the XTT-based assay is likely the main reason for the lower activity and narrower potency differentiation observed in this assay, suggesting a delayed cytostatic and/or cytotoxic effect of these compounds.

Five nucleoside analogues (**3h**, **3i**, **3m**, **4h**, and **4i**) that were among the most potent compounds in the initial screening were further characterized against additional solid tumor cell lines and compared with the activity of two established anticancer nucleosides gemcitabine and clofarabine. Table 2 summarizes results from an independent set of tests conducted for all compounds in parallel against total of eight

Table 1. Cytostatic Activity (GIC_{50}) and Cytotoxic Activity (IC_{50}) of 6-(Het)aryl-7-deaza-purine Nucleosides

	GIC_{50} (μM)[a]				IC_{50} (μM)[a]		
compd	A549	Du145	HCT116	Hs578	HL-60	CCRF-CEM	HeLa S3
3a	4.4	1.2	2.6	4.7	5.6	1.25	3.6
3b	0.25	0.030	0.45	0.053	2.2	0.45	0.35
3c	> 10	> 10	> 10	> 10	> 10	3.8	> 7.5
3d	> 10	1.1	> 10	> 10	nd[b]	4.0	> 40
3e	> 10	1.3	> 10	> 10	> 10	10.9	> 10
3f	> 10	0.73	> 10	2.5	52.7	23.2	2.9
3g	> 10	> 10	> 10	> 10	> 10	> 10	> 10
3h	0.088	0.007	0.078	0.049	0.81	0.31	0.77
3i	0.045	0.009	0.049	0.10	1.2	0.29	8.8
3j	0.41	0.019	0.48	0.29	1.2	0.39	0.13
3k	> 10	0.63	> 10	1.3	n.d.	10.0	nd
3l	2.6	0.094	3.7	1.9	n.d.	0.71	3.3
3m	0.073	0.036	0.092	0.26	1.5	1.9	4.6
3n	0.63	0.032	0.22	0.71	> 40	4.0	n.d.
3o	0.36	0.030	0.50	0.44	1.2	0.28	0.26
3p	0.23	0.028	0.34	0.29	2.8	1.5	7.4
3q	3.3	0.41	7.3	3.9	10.1	0.91	1.1
3r	6.2	0.075	5.7	3.1	nd	1.0	4.1
3s	4.9	0.39	> 10	> 10	> 40	1.5	n.d.
4d	> 10	0.58	> 10	> 10	> 40	3.1	2.6
4h	0.11	0.005	0.039	0.18	1.2	1.4	8.0
4i	0.061	0.009	0.009	0.13	4.3	0.27	4.3
4j	0.90	0.024	1.3	0.57	1.0	0.55	3.1
4k	0.19	0.009	0.45	0.31	3.3	0.41	0.23
4l	> 10	0.89	> 10	2.1	n.d.	1.8	12.2
4m	0.060	0.005	0.018	0.18	2.0	1.0	0.17
4n	0.38	0.066	0.14	0.81	4.9	0.56	0.21
4o	0.13	0.010	0.25	0.36	1.9	0.93	8.9
5d	2.5	0.14	1.4	> 10	1.2	0.91	0.42
5h	> 10	> 10	> 10	> 10	21.6	3.8	> 40
5i	0.38	0.013	0.27	1.6	5.5	2.7	28.2
5m	1.1	0.054	2.0	1.6	2.5	1.1	2.7
5n	0.38	0.018	0.10	0.11	1.6	1.0	0.34

[a] Cytostatic and cytotoxic activities were determined by SRB (GIC_{50}) and XTT (IC_{50}) assay following a 5-day and 3-day incubation with tested compounds, respectively. Values represent means from 2 to 4 independent experiments. [b] nd, not determined.

Table 2. Cytostatic Activity of Selected 6-(Het)aryl-7-deaza-purine Nucleosides in Comparison with Clofarabine and Gemcitabine

	GIC_{50} (μM)[a]								
	lung		prostate		colon		breast		
compd	A549	NCI H23	Du145	PC3	HCT116	HCT15	HS578	BT549	geometric mean
3h	0.033	0.13	0.007	0.014	0.006	0.020	0.009	0.012	0.016
3i	0.11	0.43	0.009	0.033	0.007	0.078	0.024	0.023	0.038
3m	0.055	0.28	0.036	0.096	0.026	0.086	0.051	0.035	0.062
4h	0.11	4.3	0.005	0.075	0.039	0.057	0.18	0.11	0.096
4i	0.38	1.5	0.009	0.090	0.020	0.018	0.14	0.081	0.081
gemcitabine	0.007	0.002	0.003	0.006	0.002	0.003	0.001	0.004	0.003
clofarabine	0.086	0.040	0.125	0.063	0.106	0.180	1.241	0.065	0.12

[a] Cytostatic activity was determined by SRB assay following a 5-day incubation with tested compounds in parallel. Values represent means from three independent experiments.

human solid tumor cell lines. Results of these assays indicate that particularly the compounds **3h** and **3i** retain their potent cytostatic effect against all the tested cell lines. Although being less potent than gemcitabine, all five explored compounds showed comparable or better cytostatic effects than clofarabine either in the individual assays or as a composite geometric mean of all GIC_{50} values (Table 2).

Intracellular Metabolism. Similar to other nucleoside analogues, the 6-hetaryl-7-deaza purines are likely to undergo intracellular phosphorylation. Compounds **3h** and **5h** were selected to explore their intracellular metabolism; although

Table 3. Intracellular Metabolism of Compounds **3h** and **5h** in Du145 Prostate Cancer Cells

	intracellular concentration[a] [pmol/million cells]					
	6 h			24 h		
compd	nucleoside	NMP	NTP	nucleoside	NMP	NTP
3h	950	4124	88.4	207	6842	109
5h	20.1	164	19.2	45.0	332	21.2

[a] Intracellular levels of parent compound (nucleoside), monophosphate (NMP), and triphosphate (NTP) were determined following 6 and 24 h incubation of cells with 10 μM compounds.

Table 4. Summary of Cell Cycle, Apoptosis, Mitosis (pH3+), RNA (BrU+), and DNA (BrdU+) Synthesis Analysis in CCRF-CEM Cells Treated with Selected Nucleosides at GIC_{50} for 24 h[a]

	% of total cell populations						
compd	sub-G1	G1	S	G2/M	$pH3^{Ser10}$+	BrdU+	BrU+
control	10.6 ± 1.9	51.9 ± 4.1	36.7 ± 1.6	9.8 ± 2.5	1.96 ± 0.32	63.9 ± 4.8	51.8 ± 1.1
3h 1 × GIC_{50}	16.5 ± 7.2	44.9 ± 5.7	42.2 ± 7.1	12.9 ± 4.8	1.21 ± 0.07	54.3 ± 9.2	28.7 ± 4.3
3h 5 × GIC_{50}	60.2 ± 8.3	46.7 ± 6.7	44.6 ± 4.1	6.8 ± 2.6	0.48 ± 0.44	31.3 ± 2.2	4.7 ± 1.5
3i 1 × GIC_{50}	13.7 ± 3.8	45.5 ± 7.5	42.2 ± 7.7	12.2 ± 7.5	0.87 ± 0.33	55.5 ± 6.8	28.5 ± 5.2
3i 5 × GIC_{50}	55 ± 9.3	47.0 ± 7.7	47.0 ± 4.4	6.0 ± 3.5	0.44 ± 0.25	38.3 ± 9.4	3.5 ± 2.4
4i 1 × GIC_{50}	17.1 ± 7.2	43.9 ± 7.2	46.7 ± 9.8	9.3 ± 6.4	0.85 ± 0.48	56.2 ± 10.9	28.4 ± 6.8
4i 5 × GIC_{50}	43.4 ± 10.8	40.4 ± 4.9	50.5 ± 4.3	9.1 ± 6.2	0.47 ± 0.43	47 ± 12.12	5.7 ± 1.8
3m 1 × GIC_{50}	39.8 ± 8.9	55.6 ± 4.9	37.3 ± 3.9	7.1 ± 1.6	0.84 ± 0.29	44.6 ± 10.5	14.0 ± 7.3
3m 5 × GIC_{50}	61.2 ± 10.3	54.4 ± 8.4	39.7 ± 6.9	5.9 ± 2.1	0.39 ± 0.17	28.9 ± 7.5	7.2 ± 1.0
gemcitabine 1 × GIC_{50}	24.1 ± 1.2	27.1 ± 3.8	72.3 ± 4.2	0.6 ± 0.6	0.78 ± 0.59	56.7 ± 10.7	54.7 ± 7.2
gemcitabine 5 × GIC_{50}	54.0 ± 2.4	67.1 ± 5.2	30.5 ± 5.6	2.3 ± 0.7	0.53 ± 0.43	9.5 ± 6.6	44.8 ± 2.2
cladribine 1 × GIC_{50}	48.0 ± 4.0	73.2 ± 3.6	24.9 ± 3.5	1.9 ± 1.2	0.85 ± 0.71	32.1 ± 8.4	46.2 ± 1.8
cladribine 5 × GIC_{50}	61.5 ± 3.4	61.6 ± 6.6	30.8 ± 5.2	7.6 ± 3.6	0.44 ± 0.39	13.0 ± 3.6	41.3 ± 4.1

[a] Data are expressed as a percentage of total cellular population plus/minus standard deviation.

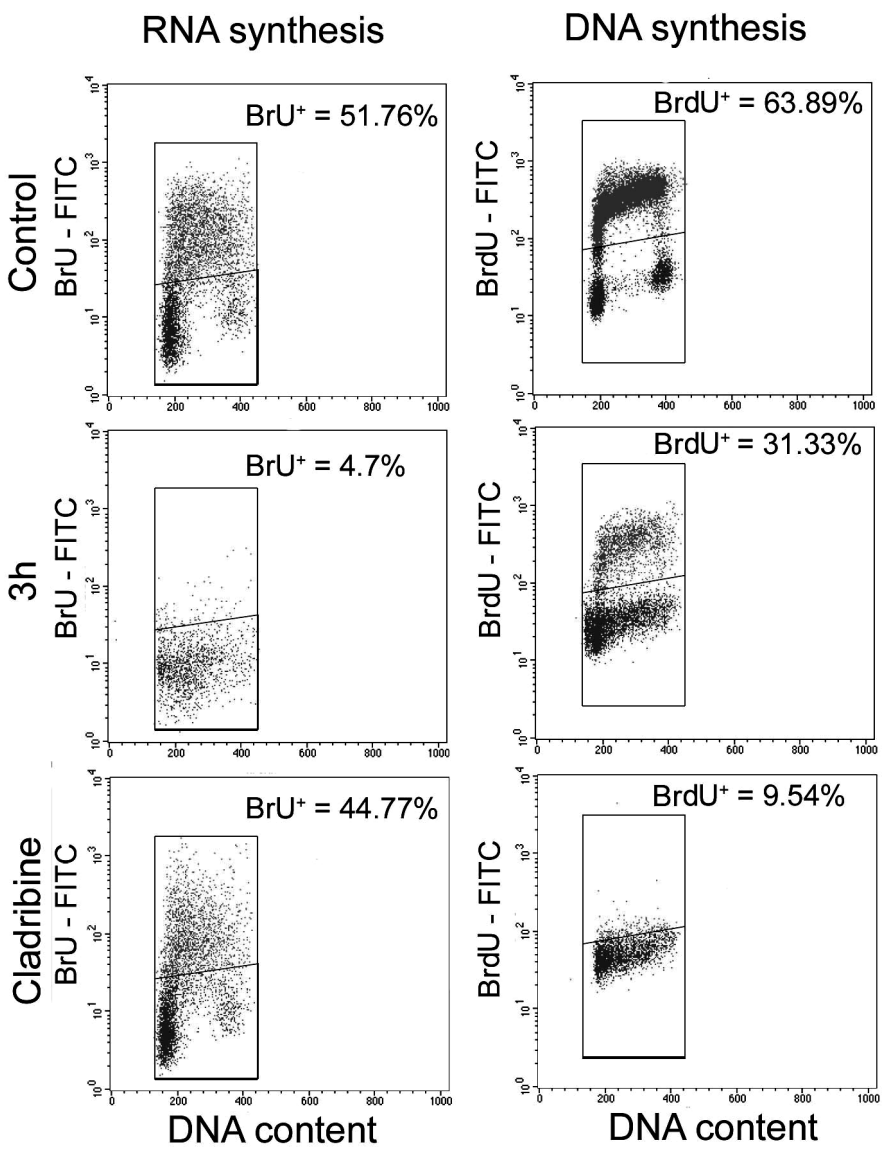


Figure 1. Specificity of RNA synthesis inhibition (BrU+ cells) in CCRF-CEM cells treated with compound **3h** in comparison with cladribine at 5× GIC_{50}.

they differ only in 7-substitution (7-H and 7-Cl, respectively), there is a striking difference in their cytostatic activity (Table 1). DU145 cells are among the most sensitive to **3h** but show no response to **5h**. Du145 cells were treated with the two compounds for 6 and 24 h and cellular extracts were analyzed using high-performance liquid chromatography

coupled to tandem mass spectrometery (LC/MS/MS). Results demonstrated the intracellular phosphorylation of both compounds as evidenced by the presence of increasing concentrations of monophosphates (MP) and triphophates (TP) in cell extracts over time (Table 3). Formation of diphosphate was not followed due to the lack of chemical standard required for the quantitative LC/MS/MS analysis. Intracellular levels of MP and TP of **3h** were approximately 20-fold and 5-fold higher, respectively, compared to those of **5h**. Although we have not formally established the role of intracellular metabolites in the cytostatic activity of these novel nucleosides, our data support a notion that, similar to many other cytostatic nucleosides, these compounds or one of their phosphorylated metabolites may act directly through incorporation of their TP by RNA polymerases, inhibiting the elongation of cellular RNAs and/or indirectly through the modulation of intracellular nucleotide pools.

Inhibition of RNA/DNA Synthesis and Cell Cycle Analysis. To better understand molecular mechanism of the cytostatic effect of the prepared nucleosides, we have performed the analysis of cell cycle and DNA and RNA synthesis in CCRF-CEM T-lymphoblastic leukemia cells treated for 24 h with selected active compounds at their 1× and 5× GIC_{50}. Consistently with data from intracellular metabolism showing formation of intracellular TP metabolites (Table 3), the 6-hetaryl-7-deaza purines exhibited a dose-dependent inhibition of RNA synthesis measured via the incorporation of bromouridine into the total pool of cellular RNA (Table 4, Figure 1). Specifically, the treatment of cells with concentrations corresponding to 1× and 5× GIC_{50} resulted in approximately 50 and 90% inhibition of total RNA synthesis, respectively. In contrast, the effect of nucleosides on total DNA synthesis and the DNA content in specific cell cycle phases was much less pronounced and likely nonspecific. Notably, profound inhibition of cellular RNA synthesis was accompanied by a decrease of mitotic phospho-histone H3 positive cells and rapid onset of apoptosis as evidenced by the high proportion of sub-G1 cells (Table 4, Figure 1).

Antiviral Activity. All synthesized compounds were tested in Huh-7 cells harboring subgenomic reporter replicon derived from HCV subtype 1B. Although a number of tested compounds inhibited the replicon reporter signal, the activity largely correlated with cytostatic effect in several different cell lines, suggesting that the activity detected in HCV replicon are likely a consequence of interference with host target(s). This conclusion is supported by observation that TP metabolites of the nucleosides active in HCV replicon do not inhibit the enzymatic activity of HCV RNA polymerase (data not shown).

Conclusions

6-Hetaryl-7-deazapurine and -7-fluoro-7-deazapurine ribonucleosides have been found to possess cytostatic effects at low nanomolar concentrations with the potency comparable to clofarabine. On the other hand, the corresponding 6-alkyl- and 6-phenyl-7-deazapurine and 6-hetaryl-7-chloro-7-deazapurine ribonucleosides were much less active. The highest activities were observed with 6-furyl and 6-thienyl derivatives, while the derivatives bearing N-heterocycles were less potent. The mechanism by which these compounds exert their cytostatic effect has not been fully elucidated yet, but intracellular triphosphate metabolites together with a potent inhibition of RNA synthesis (potentially through the direct inhibition of cellular RNA polymerases) have been detected in treated cells. Additional studies of the mechanism as well as in vivo cytostatic activity and pharmacokinetic testing are under way and will be reported in due course. The title 6-hetaryl-7-deazapurine ribonucleosides represent a new promising lead class for further development as potential cytostatics.

Experimental Section

General. NMR spectra were recorded on a 400 MHz (^{1}H at 400 MHz, ^{13}C at 100.6 MHz), a 500 MHz (500 MHz for ^{1}H and 125.7 MHz for ^{13}C), or a 600 MHz (600 MHz for ^{1}H and 151 MHz for ^{13}C) spectrometer. Melting points were determined on a Kofler block and are uncorrected. Optical rotations were measured at 25 °C, $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. The high resolution mass spectra were measured using electrospray ionization. High performance flash chromatography (HPFC) purifications were performed on C-18 columns using water−methanol gradient. The purity of final compounds (>95%) was confirmed by elemental analyses and clean NMR spectra. Only selected most important procedures and compound characterizations are given here, for complete experimental part including full characterization of all compounds including assigment of NMR signals; see Supporting Information.

4-Chloro-7-(2,3-*O*-isopropylidene-5-*O*-tert-butyldimethylsilyl-β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (8). Tris(dimethylamino)phosphine (9.7 mL, 53.5 mmol) was added dropwise to a stirred solution of 2,3-*O*-isopropylidene-5-*O*-*tert*-butyldimethylsilyl-β-D-ribofuranose **9** (12.5 g, 41 mmol) and carbon tetrachloride (6.4 mL, 61.5 mmol) in toluene (70 mL) over 35 min at −30 °C. The temperature of the reaction mixture was raised to 0 °C within 1 h. The cloudy toluene solution of halogenose **7** was then quickly washed with ice-cold brine (70 mL), dried over $MgSO_4$, and added to a vigorously stirred suspension of 4-chloropyrrolo[2,3-*d*]pyrimidine **6** (4.16 g, 27.1 mmol), powdered KOH (3.4 g, 60.7 mmol), and TDA-1 (4.3 mL, 13.4 mmol) in toluene (70 mL). The mixture was stirred for 24 h and then aqueous NH_4Cl (sat, 250 mL) was added and the mixture extracted with chloroform (400 mL, then 2 × 75 mL). The combined organic extracts were dried over $MgSO_4$, evaporated, and the residue chromatographed on silica (hexanes−AcOEt, 22:1) to afford **8** (7.57 g, 63%) as a colorless oil. It should be noted that the yield of **8** was variable and ranged from 39% to 63%. Handling of preprepared solution of unstable halogenose **7** should be as quick as possible. Analytical data of compound **8** are in agreement with published data.[25]

4-Ethyl-7-(2,3-*O*-isopropylidene-5-*O*-tert-butyldimethylsilyl-β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (10a). An argon purged mixture of compound **8** (200 mg, 0.454 mmol), triethylaluminum (1 M in hexanes, 910 μL 0.91 mmol), and $Pd(PPh_3)_4$ (26 mg, 0.022 mmol) in THF (5 mL) was stirred at 70 °C for 20 h. The mixture was diluted with hexane (30 mL) and washed with aqueous NH_4Cl (sat, 10 mL). The aqueous phase was re-extracted with hexane (2 × 10 mL). The combined organic extracts were dried over $MgSO_4$, concentrated in vacuo, and the residue chromatographed on silica (hexanes−AcOEt, 10:1 → 6:1) to afford **10a** (162 mg, 82%) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$): 0.046 and 0.053 (2 × s, 2 × 3H, CH_3Si), 0.90 (s, 9H, $(CH_3)_3C$), 1.39 (q, 3H, J = 0.6, $(CH_3)_2C$), 1.393 (t, 3H, J_{vic} = 7.7, CH_3CH_2), 1.65 (q, 3H, J = 0.6, $(CH_3)_2C$), 3.04 (q, 2H, J_{vic} = 7.7, CH_2CH_3), 3.79 (dd, 1H, J_{gem} = 11.2, $J_{5'b,4'}$ = 4.0, H-5′b), 3.87 (dd, 1H, J_{gem} = 11.2, $J_{5'a,4'}$ = 3.8, H-5′a), 4.33 (m, 1H, $J_{4',5'}$ = 4.0, 3.8, $J_{4',3'}$ = 3.1, $J_{4',2'}$ = 0.4, H-4′), 4.98 (ddd, 1H, $J_{3',2'}$ = 6.3, $J_{3',4'}$ = 3.1, $J_{3',1'}$ = 0.5, H-3′), 5.13 (ddd, 1H, $J_{2',3'}$ = 6.3, $J_{2',1'}$ = 3.1, $J_{2',4'}$ = 0.4, H-2′), 6.41 (d, 1H, $J_{1',2'}$ = 3.1, H-1′), 6.58 (d, 1H, $J_{5,6}$ = 3.7, H-5), 7.43 (d, 1H, $J_{6,5}$ = 3.7, H-6), 8.81 (s, 1H, H-2). ^{13}C NMR (151 MHz, $CDCl_3$): −5.50, −5.40, 12.87, 18.37, 25.47, 25.90, 27.34, 28.61, 63.37, 80.94, 84.80, 85.96, 90.17, 100.09, 114.11, 117.70, 125.60, 150.39, 151.64, 164.25. MS ESI, *m*/*z*:

434[M + H]. HR MS (ESI): calcd for $C_{22}H_{36}N_3O_4Si$ [M + H] 434.2475, found 434.2468.

4-Benzyl-7-(2,3-*O*-isopropylidene-5-*O*-*tert*-butyldimethylsilyl-*β*-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (10c). An argon purged mixture of compound **8** (191 mg, 0.43 mmol), benzylzinc bromide (0.5 M in THF, 1.75 mL, 0.875 mmol), and $Pd(PPh_3)_4$ (25 mg, 0.022 mmol) in THF (5 mL) was stirred at 70 °C for 24 h. The mixture was diluted with hexane (25 mL) and washed with aqueous NH_4Cl (satd, 10 mL). The aqueous phase was re-extracted with hexane (2 × 10 mL) and the combined organic extracts dried over $MgSO_4$, concentrated in vacuo, and the residue chromatographed on silica (hexanes−AcOEt, 6:1) to afford **10c** (201 mg, 93%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): 0.02 and 0.04 (2 × s, 2 × 3H, CH_3Si), 0.88 (s, 9H, $(CH_3)_3C$), 1.38 (q, 3H, $J = 0.6$, $(CH_3)_2C$), 1.64 (q, 3H, $J = 0.6$, $(CH_3)_2C$), 3.77 (dd, 1H, $J_{gem} = 11.2$, $J_{5'b,4'} = 4.0$, H-5′b), 3.86 (dd, 1H, $J_{gem} = 11.2$, $J_{5'a,4'} = 3.8$, H-5′a), 4.31 (q, 1H, $J_{4',5'} = 4.0$, 3.8, $J_{4',3'} = 3.1$, H-4′), 4.35 (s, 2H, CH_2Ph), 4.96 (ddd, 1H, $J_{3',2'} = 6.3$, $J_{3',4'} = 3.1$, $J_{3',1'} = 0.4$, H-3′), 5.10 (dd, 1H, $J_{2',3'} = 6.3$, $J_{2',1'} = 3.1$, H-2′), 6.39 (d, 1H, $J_{1',2'} = 3.1$, H-1′), 6.43 (d, 1H, $J_{5,6} = 3.7$, H-5), 7.21 (m, 1H, H-*p*-Ph), 7.25−7.33 (m, 4H, H-*o*,*m*-Ph),; 7.39 (d, 1H, $J_{6,5} = 3.7$, H-6), 8.83 (s, 1H, H-2). ^{13}C NMR (100.6 MHz, $CDCl_3$): −5.50, −5.40, 18.37, 25.47, 25.90, 27.34, 42.27, 63.38, 80.96, 84.79, 85.99, 90.21, 100.37, 114.15, 118.28, 126.00, 126.60, 128.57, 129.07, 138.11, 150.81, 151.65,161.14. MS FAB, *m*/*z* (rel %): 73 (100), 210 (30), 292 (10), 496 (95) [M + H]. HR MS (FAB): calcd for $C_{27}H_{38}N_3O_4Si$ [M + H] 496.2632, found 496.2636.

7-(2,3-*O*-Isopropylidene-5-*O*-*tert*-butyldimethylsilyl-*β*-D-ribofuranosyl)-4-phenyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (10d). An argon purged mixture of compound **8** (309 mg, 0.7 mmol), phenylboronic acid (128 mg, 1.05 mmol), K_2CO_3 (193 mg, 1.4 mmol), and $Pd(PPh_3)_4$ (41 mg, 0.035 mmol) in toluene (5 mL) was stirred at 100 °C for 5 h. The mixture was diluted with chloroform (20 mL) and washed with aqueous NH_4Cl (sat., 20 mL). The aqueous phase was re-extracted with chloroform (2 × 5 mL) and the combined organic extracts dried over $MgSO_4$, concentrated in vacuo, and the residue chromatographed on silica (hexanes−AcOEt, 10:1 → 7:1) to afford **10d** (320 mg, 95%) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$): 0.06 and 0.07 (2 × s, 2 × 3H, CH_3Si), 0.90 (s, 9H, $(CH_3)_3C$), 1.40 and 1.67 (2 × q, 2 × 3H, $J =$ 0.6, $(CH_3)_2C$), 3.82 (dd, 1H, $J_{gem} = 11.2$, $J_{5'b,4'} = 3.8$, H-5′b), 3.91 (dd, 1H, $J_{gem} = 11.2$, $J_{5'a,4'} = 3.7$, H-5′a), 4.36 (ddd, 1H, $J_{4',5'} = 3.8$, 3.7, $J_{4',3'} = 3.5$, H-4′), 5.00 (ddd, 1H, $J_{3',2'} = 6.3$, $J_{3',4'} = 3.5$, $J_{3',1'} =$ 0.4, H-3′), 5.15 (dd, 1H, $J_{2',3'} = 6.3$, $J_{2',1'} = 3.1$, H-2′), 6.49 (d, 1H, $J_{1',2'} = 3.1$, H-1′), 6.84 (d, 1H, $J_{5,6} = 3.8$, H-5), 7.51 (m, 1H, H-*p*-Ph), 7.55 (m, 2H, H-*m*-Ph), 7.56 (d, 1H, $J_{6,5} = 3.8$, H-6), 8.09 (m, 2H, H-*o*-Ph), 8.98 (s, 1H, H-2). ^{13}C NMR (151 MHz, $CDCl_3$): −5.49, −5.37, 18.39, 25.49,; 25.91, 27.37, 63.40, 80.92, 84.95, 86.03, 90.23, 101.44, 114.16, 116.43, 126.75, 128.77, 128.82, 130.02, 138.06, 151.65, 151.71, 157.63. MS FAB, *m*/*z* (rel %): 73 (100), 196 (40), 482 (90) [M + H]. HR MS (FAB): calcd for $C_{26}H_{36}N_3O_4Si$ [M + H] 482.2475, found 482.2479.

4-(Furan-2-yl)-7-(2,3-*O*-isopropylidene-5-*O*-*tert*-butyldimethylsilyl-*β*-d-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (10h). An argon purged mixture of compound **8** (294 mg, 0.67 mmol), 2-(tributylstannyl)furane (252 μL, 0.80 mmol), and $PdCl_2(PPh_3)_2$ (24 mg, 0.03 mmol) in DMF (3 mL) was stirred at 100 °C for 2 h. Volatiles were removed in vacuo and the residue coevaporated with toluene several times. Silica gel chromatography of the residue (hexanes−AcOEt, 20:1 → 10:1) afforded product **10h** (293 mg, 93%) as a colorless foam. ^{1}H NMR (600 MHz, $CDCl_3$): 0.069 and 0.074 (2 × s, 2 × 3H, CH_3Si), 0.91 (s, 9H, $(CH_3)_3C$), 1.40 and 1.67 (2 × q, 2 × 3H, $J = 0.6$, $(CH_3)_2C$), 3.81 (dd, 1H, $J_{gem} = 11.2$, $J_{5'b,4'} = 3.7$, H-5′b), 3.90 (dd, 1H, $J_{gem} =$ 11.2, $J_{5'a,4'} = 3.5$, H-5′a), 4.36 (ddd, 1H, $J_{4',5'} = 3.7$, 3.5, $J_{4',3'} =$ 3.1, H-4′), 4.99 (ddd, 1H, $J_{3',2'} = 6.3$, $J_{3',4'} = 3.1$, $J_{3',1'} = 0.4$, H-3′), 5.12 (dd, 1H, $J_{2',3'} = 6.3$, $J_{2',1'} = 3.1$, H-2′), 6.47 (d, 1H, $J_{1',2'} = 3.1$, H-1′), 6.64 (dd, 1H, $J_{4,3} = 3.5$, $J_{4,5} = 1.7$, H-4-furyl), 7.05 (d, 1H, $J_{5,6} = 3.7$, H-5), 7.41 (dd, 1H, $J_{3,4} = 3.5$, $J_{3,5} = 0.8$, H-3-furyl), 7.56 (d, 1H, $J_{6,5} = 3.7$, H-6), 7.72 (dd, 1H, $J_{5,4} = 1.7$, $J_{5,3} = 0.8$, H-5-furyl), 8.87 (s, 1H, H-2). ^{13}C NMR (151 MHz, $CDCl_3$): −5.50, −5.38, 18.38, 25.45, 25.90, 27.33, 63.36, 80.85, 84.92, 85.94, 90.04, 102.11, 112.36, 112.97, 113.55, 114.13, 126.80, 145.11, 147.12, 151.41, 151.82, 152.95. MS FAB, *m*/*z* (rel %): 73 (100), 186 (20), 472 (45) [M + H]. HR MS (FAB): calcd for $C_{24}H_{34}N_3O_5Si$ [M + H] 472.2268, found 472.2274.

4-Ethyl-7-(*β*-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (3a). Compound **10a** (149 mg, 0.34 mmol) was treated with aqueous TFA (90% v/v, 0.5 mL) for 1 h at RT. The volatiles were removed in vacuo, and the residue was several times coevaporated with MeOH. Chromatography on silica (3.5% MeOH in $CHCl_3$) afforded compound **3a** (100 mg, 99%) as a colorless glassy solid. After reverse phase chromatography, compound **3a** crystallized from water/MeOH as colorless needles; mp 120−121 °C; $[\alpha]_D$ −58.8 (*c* 0.473, DMSO). UV (MeOH): λ_{max} (ε) 271 (4407), 223 (25061). ^{1}H NMR (600 MHz, DMSO-d_6): 1.30 (t, 3H, $J_{vic} = 7.6$, $\mathbf{CH_3}CH_2$), 2.99 (q, 2H, $J_{vic} = 7.6$, $\mathbf{CH_2}CH_3$), 3.54 (ddd, 1H, $J_{gem} = 11.9$, $J_{5'b,OH} = 5.8$, $J_{5'b,4'} = 4.0$, H-5′b),; 3.63 (ddd, 1H, $J_{gem} = 11.9$, $J_{5'a,OH} = 5.3$, $J_{5'a,4'} = 4.0$, H-5′a), 3.91 (q, 1H, $J_{4',5'} = 4.0$, $J_{4',3'} = 3.3$, H-4′), 4.11 (td, 1H, $J_{3',2'} = 5.1$, $J_{3,OH} =$ 4.8, $J_{3',4'} = 3.3$, H-3′), 4.43 (td, 1H, $J_{2',OH} = 6.5$, $J_{2',1'} = 6.3$, $J_{2',3'} =$ 5.1, H-2′), 5.13 (t, 1H, $J_{OH,5'} = 5.8$, 5.3, OH-5′), 5.19 (d, 1H, $J_{OH,3'}$ = 4.8, OH-3′), 5.35 (d, 1H, $J_{OH,2'} = 6.5$, OH-2′), 6.18 (d, 1H, $J_{1',2'}$ = 6.3, H-1′), 6.77 (dd, 1H, $J_{5,6} = 3.7$, $J_{5,2} = 0.4$, H-5), 7.78 (d, 1H, $J_{6,5} = 3.7$, H-6), 8.69 (s, 1H, H-2). ^{13}C NMR (151 MHz, DMSO-d_6): 12.93, 27.97, 61.87, 70.87, 74.18, 85.38, 87.02, 100.09, 117.38, 126.78, 150.73, 151.15, 163.77. MS FAB, *m*/*z* (rel %): 149 (45), 280 (100) [M + H]. HR MS (FAB): calcd for $C_{13}H_{18}N_3O_4$ [M + H] 280.1297, found 280.1293. Anal. ($C_{13}H_{17}N_3O_4 \cdot {}^1/_2H_2O$): C, H, N.

4-(Furan-2-yl)-7-(*β*-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (3h). Deprotection of **10h** as described for **3a** gave compound **3h** as colorless needles after crystallization from 2-propanol (no silica column); yield 63%; mp 162−164 °C; $[\alpha]_D$ −72.0 (*c* 0.261, DMSO). UV (MeOH): λ_{max} (ε) 326 (15338), 234 (19556). ^{1}H NMR (400 MHz, DMSO-d_6): 3.57 and 3.66 (2 × dd, 2H, $J_{gem} = 11.9$, $J_{5',4'} = 4.0$, H-5′), 3.94 (q, 1H, $J_{4',5'} = 4.0$, $J_{4',3'} = 3.3$, H-4′), 4.13 (dd, 1H, $J_{3',2'} = 5.1$, $J_{3',4'} = 3.3$, H-3′), 4.45 (dd, 1H, $J_{2',1'} = 6.2$, $J_{2',3'} = 5.1$, H-2′), 6.25 (d, 1H, $J_{1',2'} = 6.2$, H-1′), 6.80 (dd, 1H, $J_{4,3} = 3.5$, $J_{4,5} = 1.7$, H-4-furyl), 7.08 (d, 1H, $J_{5,6} = 3.7$, H-5), 7.50 (dd, 1H, $J_{3,4} = 3.5$, $J_{3,5} = 0.7$, H-3-furyl), 7.95 (d, 1H, $J_{6,5} = 3.7$, H-6), 8.07 (dd, 1H, $J_{5,4} = 1.7$, $J_{5,3} = 0.7$, H-5-furyl), 8.78 (s, 1H, H-2). ^{13}C NMR (100.6 MHz, DMSO-d_6): 61.74, 70.76, 74.24, 85.40, 86.88, 101.41, 112.79, 112.89, 113.62, 128.32, 146.36, 146.60, 151.00, 152.24, 152.43. IR (KBr): ν = 1675, 1601, 1564, 1462, 1353, 1237, 1207, 1188, 1099, 1051, 1016 cm^{-1}. MS FAB, *m*/*z* (rel %): 318 (100) [M + H]. HR MS (FAB): calcd for $C_{15}H_{16}N_3O_5$ [M + H] 318.1090, found 318.1089. Anal. ($C_{15}H_{15}N_3O_5 \cdot {}^1/_2H_2O$): C, H, N.

4-(Furan-3-yl)-7-(*β*-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (3m). An argon purged mixture of compound **11** (226 mg, 0.79 mmol), furane-3-boronic acid (111 mg, 0.99 mmol), Na_2CO_3 (251 mg, 2.37 mmol), $Pd(OAc)_2$ (9 mg, 0.04 mmol), and TPPTS (57 mg, 0.1 mmol) in water/MeCN (2:1, 3 mL) was stirred at 100 °C for 3 h. After cooling, the mixture was neutralized by the addition of aqueous HCl (3M), volatiles were removed in vacuo, and the residue chromatographed on silica (3.5% MeOH in $CHCl_3$) to afford **3m** (172 mg, 69%) as a yellowish solid. Compound **3m** was recrystallized from 2-propanol to provide a white microcrystalline solid; mp 142−143 °C; $[\alpha]_D$ −64.3 (*c* 0.524, DMSO). UV (MeOH): λ_{max} (ε) 305 (8343), 214 (25297). ^{1}H NMR (500 MHz, DMSO-d_6): 3.57 (ddd, 1H, $J_{gem} = 11.9$, $J_{5'b,OH} = 5.8$, $J_{5'b,4'} = 4.0$, H-5′b), 3.66 (ddd, 1H, $J_{gem} = 11.9$, $J_{5'a,OH} = 5.3$, $J_{5'b,4'} = 4.0$, H-5′a), 3.94 (td, 1H, $J_{4',5'} =$ 4.0, $J_{4',3'} = 3.4$, H-4′), 4.14 (ddd, 1H, $J_{3',2'} = 5.1$, $J_{3',OH} = 4.9$, $J_{3',4'} = 3.4$, H-3′), 4.45 (ddd, 1H, $J_{2',OH} = 6.3$, $J_{2',1'} = 6.1$, $J_{2',3'} =$ 5.1, H-2′), 5.09 (dd, 1H, $J_{OH,5'} = 5.8$, 5.3, OH-5′), 5.18 (d, 1H, $J_{OH,3'} = 4.9$, OH-3′), 5.37 (d, 1H, $J_{OH,2'} = 6.3$, OH-2′), 6.24 (d, 1H, $J_{1',2'} = 6.1$, H-1′), 7.10 (d, 1H, $J_{5,6} = 3.8$, H-5), 7.26 (dd, 1H,

$J_{4,5}$ = 1.9, $J_{4,2}$ = 0.8, H-4-furyl), 7.90 (dd, 1H, $J_{5,4}$ = 1.9, $J_{5,2}$ = 1.5, H-5-furyl), 7.92 (d, 1H, $J_{6,5}$ = 3.8, H-6),; 8.74 (dd, 1H, $J_{2,5}$ = 1.5, $J_{2,4}$ = 0.8, H-2-furyl),; 8.78 (s, 1H, H-2). ^{13}C NMR (125.7 MHz, DMSO-d_6): 61.73, 70.73, 74.20, 85.32, 86.92, 100.86, 109.55, 114.65, 125.19, 127.77, 144.74, 145.01, 150.15, 151.12, 151.73. MS FAB, *m*/*z* (rel %): 73 (100), 217 (45), 318 (55) [M + H]. HR MS (FAB): calcd for $C_{15}H_{16}N_3O_5$ [M + H] 318.1090, found 318.1086. Anal. ($C_{15}H_{15}N_3O_5 \cdot 0.1C_3H_8O \cdot 0.2H_2O$): C, H, N.

5-Fluoro-4-(furan-2-yl)-7-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (13h). Compound **13h** was prepared as described for **10h** by coupling compound **12** and 2-(tributylstannyl)furane. A yellowish foam was obtained after chromatography on silica (hexanes−AcOEt, 20:1 → 6:1); yield 99%. ^{1}H NMR (600 MHz, $CDCl_3$): 4.69 (dd, 1H, J_{gem} = 12.2, $J_{5'b,4'}$ = 3.7, H-5′b), 4.80 (ddd, 1H, $J_{4',3'}$ = 4.1, $J_{4',5'}$ = 3.7, 3.1, H-4′), 4.88 (dd, 1H, J_{gem} = 12.2, $J_{5'a,4'}$ = 3.1, H-5′a), 6.09 (dd, 1H, $J_{3',2'}$ = 5.9, $J_{3',4'}$ = 4.1, H-3′), 6.14 (t, 1H, $J_{2',3'} = J_{2',1'}$ = 5.9, H-2′), 6.63 (dd, 1H, $J_{4,3}$ = 3.5, $J_{4,5}$ = 1.7, H-4-furyl), 6.84 (dd, 1H, $J_{1',2'}$ = 5.9, $J_{H,F}$ = 1.3, H-1′), 7.199 (d, 1H, $J_{H,F}$ = 2.4 H-6), 7.36 and 7.42 (2 × m, 2 × 2H, H-*m*-Bz), 7.50 (dd, 1H, $J_{3,4}$ = 3.5, $J_{3,5}$ = 0.7, H-3-furyl), 7.51 (m, 2H, H-*m*-Bz), 7.54, 7.60, and 7.62 (3 × m, 3 × 1H, H-*p*-Bz), 7.71 (dd, 1H, $J_{5,4}$ = 1.7, $J_{5,3}$ = 0.7, H-5-furyl), 7.93, 8.02, and 8.15 (3 × m, 3 × 2H, H-*o*-Bz), 8.85 (s, 1H, H-2). ^{13}C NMR (151 MHz, $CDCl_3$): 63.73, 71.40, 73.69, 80.26, 85.41, 103.47 (d, $J_{C,F}$ = 16), 108.46 (d, $J_{C,F}$ = 31), 112.66, 115.68 (d, $J_{C,F}$ = 11), 128.30, 128.48, 128.54, 128.60, 128.72, 129.23, 129.66, 129.81, 129.82, 133.56, 133.76, 142.79 (d, $J_{C,F}$ = 253), 145.84, 147.07 (d, $J_{C,F}$ = 4), 148.16 (d, $J_{C,F}$ = 3), 150.45, 152.25, 165.11, 165.42, 166.15. ^{19}F NMR (470.3 MHz, $CDCl_3$): −159.30. MS FAB, *m*/*z* (rel %): 648 (100) [M + H]. HR MS (FAB): calcd for $C_{36}H_{27}FN_3O_8$ [M + H] 648.1782, found 648.1775.

5-Fluoro-4-(furan-2-yl)-7-(β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (4h). Deprotection of **13h** as described for **4d** gave compound **4h** as a beige solid; yield 78%. Compound **4h** crystallized from MeOH as a beige microcrystalline solid; mp 177−179 °C; $[\alpha]_D$ −66.5 (*c* 0.409, DMSO). UV (MeOH): λ_{max} (ε) 333 (12452), 297 (12588), 235 (19787). ^{1}H NMR (600 MHz, DMSO-d_6): 3.56 (ddd, 1H, J_{gem} = 11.9, $J_{5'b,OH}$ = 5.5, $J_{5'b,4'}$ = 3.9, H-5′b), 3.64 (ddd, 1H, J_{gem} = 11.9, $J_{5'a,OH}$ = 5.5, $J_{5'a,4'}$ = 4.1, H-5′a), 3.92 (ddd, 1H, $J_{4',5'}$ = 4.1, 3.9, $J_{4',3'}$ = 3.3, H-4′), 4.11 (ddd, 1H, $J_{3',2'}$ = 5.1, $J_{3,OH}$ = 4.9, $J_{3',4'}$ = 3.3, H-3′), 4.36 (ddd, 1H, $J_{2',OH}$ = 6.3, $J_{2',1'}$ = 6.1, $J_{2',3'}$ = 5.1, H-2′), 5.10 (t, 1H, $J_{OH,5'}$ = 5.5, OH-5′), 5.22 (d, 1H, $J_{OH,3'}$ = 4.9, OH-3′), 5.43 (d, 1H, $J_{OH,2'}$ = 6.3, OH-2′), 6.31 (dd, 1H, $J_{1',2'}$ = 6.1, $J_{H,F}$ = 1.8, H-1′), 6.80 (dd, 1H, $J_{4,3}$ = 3.5, $J_{4,5}$ = 1.7, H-4-furyl), 7.48 (dd, 1H, $J_{3,4}$ = 3.5, $J_{3,5}$ = 0.8, H-3-furyl), 7.96 (d, 1H, $J_{H,F}$ = 1.9, H-6), 8.08 (dd, 1H, $J_{5,4}$ = 1.7, $J_{5,3}$ = 0.8, H-5-furyl), 8.81 (s, 1H, H-2). ^{13}C NMR (151 MHz, DMSO-d_6): 61.64, 70.68, 74.34, 85.47, 86.36, 102.12 (d, $J_{C,F}$ = 16), 110.75 (d, $J_{C,F}$ = 30), 113.15, 114.93 (d, $J_{C,F}$ = 6), 141.46 (d, $J_{C,F}$ = 249), 146.04 (d, $J_{C,F}$ = 4), 147.02, 147.80 (d, $J_{C,F}$ = 3), 151.12, 151.81. ^{19}F NMR (470.3 MHz, DMSO-d_6, ref (C_6F_6) = −163 ppm): −161.79. IR (KBr): ν = 1586, 1485, 1461, 1395, 1249, 1209, 1101, 1046, 1021 cm^{-1}. MS FAB, *m*/*z* (rel %): 204 (90), 336 (100) [M + H]. HR MS (FAB): calcd for $C_{15}H_{15}FN_3O_5$ [M + H] 336.0996, found 336.1003. Anal. ($C_{15}H_{14}FN_3O_5 \cdot 0.25H_2O$): C, H, N.

4-Chloro-5-fluoro-7-(2,3-*O*-isopropylidene-5-*O*-*tert*-butyldimethylsilyl-β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (16). Compound **16** was prepared from 4-chloro-5-fluoropyrrolo[2,3-*d*]pyrimidine **15** following the same procedure used for the synthesis of compound **8**; yield 43%; colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$): 0.10 and 0.11 (2 × s, 2 × 3H, CH_3Si), 0.92 (s, 9H, $(CH_3)_3C$), 1.38 (q, 3H, *J* = 0.5, $(CH_3)_2C$), 1.65 (q, 3H, *J* = 0.5, $(CH_3)_2C$), 3.81 (dd, 1H, J_{gem} = 11.4, $J_{5'b,4'}$ = 3.2, H-5′b), 3.91 (dd, 1H, J_{gem} = 11.4, $J_{5'a,4'}$ = 2.9, H-5′a), 4.38 (ddd, 1H, $J_{4',5'}$ = 3.2, 2.9, $J_{4',3'}$ = 2.4, H-4′), 4.91 (dd, 1H, $J_{3',2'}$ = 6.2, $J_{3',4'}$ = 2.4, H-3′), 4.93 (dd, 1H, $J_{2',3'}$ = 6.2, $J_{2',1'}$ = 2.6, H-2′), 6.47 (dd, 1H, $J_{1',2'}$ = 2.6, $J_{H,F}$ = 1.5, H-1′), 7.44 (d, 1H, $J_{H,F}$ = 2.5, H-6), 8.65 (s, 1H, H-2). ^{13}C NMR (151 MHz, $CDCl_3$): −5.33, −5.44, 18.38, 25.41, 25.87, 27.33, 63.53, 80.73, 85.32, 86.19, 90.16, 107.56 (d, $J_{C,F}$ = 14), 107.62 (d, $J_{C,F}$ = 27), 114.24, 141.49 (d, $J_{C,F}$ = 253), 146.50 (d, $J_{C,F}$ = 1), 150.54 (d, $J_{C,F}$ = 4), 151.66. ^{19}F NMR (470.3 MHz, $CDCl_3$, ref (C_6F_6) = −163 ppm): −168.82. MS ESI, *m*/*z*: 458 [M + H]. HR MS (ESI): calcd for $C_{20}H_{30}ClFN_3O_4Si$ [M + H] 458.1678, found 458.1669.

4-Chloro-5-fluoro-7-β-D-ribofuranosyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (14). Compound **16** was deprotected as described for **3a** to afford **14** as a white foam after chromatography on silica (4% MeOH in $CHCl_3$); yield 85%. ^{1}H NMR (600 MHz, DMSO-d_6): 3.56 (ddd, 1H, J_{gem} = 12.0, $J_{5'b,OH}$ = 5.4, $J_{5'b,4'}$ = 3.9, H-5′b), 3.64 (ddd, 1H, J_{gem} = 12.0, $J_{5'a,OH}$ = 5.4, $J_{5'a,4'}$ = 4.0, H-5′a), 3.93 (ddd, 1H, $J_{4',5'}$ = 4.0, 3.9, $J_{4',3'}$ = 3.3, H-4′), 4.10 (td, 1H, $J_{3',2'} = J_{3,OH}$ = 5.0, $J_{3',4'}$ = 3.3, H-3′), 4.33 (ddd, 1H, $J_{2',OH}$ = 6.2, $J_{2',1'}$ = 5.9, $J_{2',3'}$ = 5.0, H-2′), 5.09 (t, 1H, $J_{OH,5'}$ = 5.4, OH-5′), 5.22 (d, 1H, $J_{OH,3'}$ = 5.0, OH-3′), 5.44 (d, 1H, $J_{OH,2'}$ = 6.2, OH-2′), 6.25 (dd, 1H, $J_{1',2'}$ = 5.9, $J_{H,F}$ = 1.9, H-1′), 8.02 (d, 1H, $J_{H,F}$ = 2.0, H-6),; 8.70 (s, 1H, H-2). ^{13}C NMR (151 MHz, DMSO-d_6): 61.48, 70.55, 74.53, 85.66, 86.98, 106.55 (d, $J_{C,F}$ = 14), 111.42 (d, $J_{C,F}$ = 27), 140.45 (d, $J_{C,F}$ = 249), 146.97 (d, $J_{C,F}$ = 1), 149.09 (d, $J_{C,F}$ = 4), 151.65. ^{19}F NMR (470.3 MHz, DMSO-d_6, ref (C_6F_6) = −163 ppm): −169.72. MS ESI, *m*/*z*: 304 [M + H]. HR MS (ESI): calcd for $C_{11}H_{12}ClFN_3O_4$ [M + H] 304.0500, found 304.0506.

Biology. Cytostatic Activity Assays. All cell lines were obtained from ATCC (Manassas, VA). Colon (HCT116, HCT 15), breast (BT549, HS 578), and lung (A549, NCI-H23) 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 and 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.[32] Cells were distributed into the 96-well plates in 150 μL of media (HCT-116 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 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})/($OD_{negcontrol}$ − mean OD_{day0}). For GIC_{50} determination, plot a dose−response curves between the compound concentration and percent of growth inhibition. GIC_{50} values can be derived by fitting dose−response curves using a sigmoidal dose−response equation.

Intracellular Metabolism. Du145 cells were seeded into T25 flasks at 60% confluence in the MEM medium supplemented with 10% FBS. The next day, the medium was replaced with fresh media containing the tested compounds (**3h** or **5h** at 10uM). 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% MetOH and cell lysates were centrifuged, supernatants was collected, dried by vacuum, and resuspended in 10 μL of tetrabutyl ammonium acetate containing an internal standard. Transient ion-pairing high-performance liquid chromatography coupled to positive ion electrospray tandem mass spectrometery (LC/MS/MS) was used to quantitate intracellular nucleotides. 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.[34]

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 RMPI 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 point (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 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.[35]

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, 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).[36]

BrU Incorporation Analysis. Cells were cultured as for cell cycle analysis. Before harvesting, they were pulse-labeled with 1 mM 5-bromo-2′-uridine (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 fridge overnight. They were then washed in 1% glycin in PBS, washed in PBS, and stained with primary anti-BrdU antibody cross-reacting 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 are 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).[36]

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Supporting Information Available: Complete experimental section with characterization data and elemental analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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