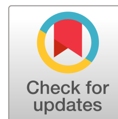

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Title: Synthesis and Cytotoxic and Antiviral Activity Profiling of All-Four Isomeric Series of Pyrido-Fused 7-Deazapurine Ribonucleosides

Authors: Lucia Veselovská, Natálie Kudlová, Soňa Gurská, Barbora Lišková, Martina Medvedíková, Ondřej Hodek, Eva Tloušťová, Nemanja Milisavljevic, Michal Tichý, Pavla Perlíková, Helena Mertlíková-Kaiserová, Jana Trylčová, Radek Pohl, Blanka Klepetářová, Petr Džubák, Marián Hajdúch, and Michal Hocek

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To be cited as: *Chem. Eur. J.* 10.1002/chem.202001124

Link to VoR: https://doi.org/10.1002/chem.202001124

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10.1002/chem.202001124

Synthesis and Cytotoxic and Antiviral Activity Profiling of All-Four Isomeric Series of Pyrido-Fused 7-Deazapurine Ribonucleosides

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Abstract: All-four isomeric series of novel 4-substituted pyrido-fused 7-deazapurine ribonucleosides possessing pyridine nitrogen at different positions were designed and synthesized. The total synthesis of each isomeric fused heterocycle through multistep heterocyclization was followed by glycosylation and derivatization at position 4 by cross-coupling reactions or nucleophilic substitutions. All compounds were tested for cytostatic and antiviral activity. The most active were pyrido[4',3':4,5]pyrimidine nucleosides bearing MeO, NH_2, MeS or CH_3 groups at position 4 which showed submicromolar cytotoxic effects and good selectivity for cancer cells. The mechanism involved activation by phosphorylation and incorporation to DNA where the presence of the modified ribonucleosides causes double-strand breaks and apoptosis.

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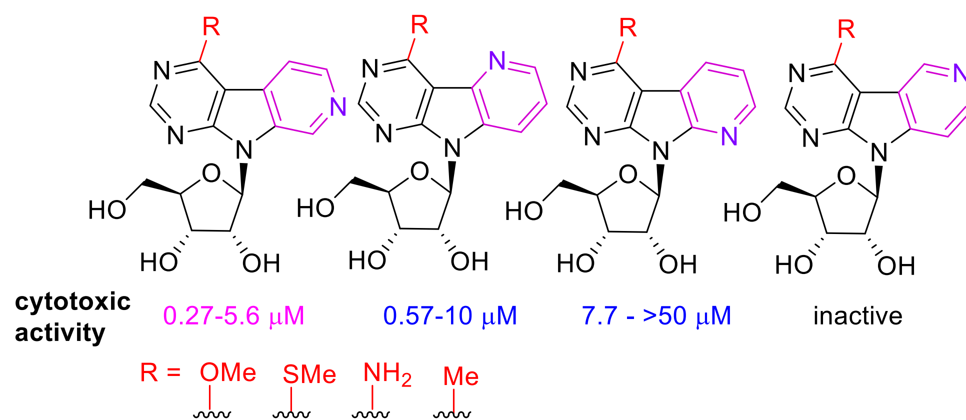

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Introduction

Nucleoside derivatives or analogues modified at the heterocyclic base are an important class of molecules with wide range of biological activities. Several clinical antiviral[1] and antineoplastic[2] drugs are based on base-modified nucleosides, either with or without additional modifications at the sugar part. The need for treatment of drug-resistant leukemia and cancers,[3] as well as of emerging new viruses,[4] justifies the continuous interest in the development of other novel nucleoside derivatives with potential antiviral or cytostatic effects. Particularly interesting are modified pyrrolo[2,3-*d*]pyrimidine (7-deazapurine) nucleosides.[5] Long-known natural products tubercidin,[6] toyocamycin[7] and sangivamycin[8] display significant biological activities and served as inspiration for further structural modifications at both the heterocyclic and ribose parts. Several types of 7-substituted 7-deazapurine nucleosides bearing 2'-methyl- or 2'-ethynyl-ribose (or 2'-fluororibose) displayed antiviral activity against HCV or other RNA viruses.[9] Some other modified 7-deazapurine nucleosides were potent inhibitors of human or pathogen adenosine kinases[10] or have exerted antiparasitic activities.[11] We have previously reported significant cytostatic activities of 7-hetaryl-7-deazaadenine (e.g. AB-61)[12] or other 6-substituted 7-deazapurine ribonucleosides,[13] which act through an unprecedented mechanism based on activation to (ribo)nucleoside triphosphates (NTPs) and incorporation to DNA, where they cause double-strand (ds) breaks and eventually apoptosis.[14]

More recently, we have designed and synthesized fused 7-deazapurine ribonucleosides. Benzo-fused (pyrimidoindole) nucleosides were found[15] to be non-cytotoxic, however they exerted moderate antiviral activities against HCV or Dengue virus, whereas the more bulky naphtho-fused nucleosides[16] were inactive. On the other hand, the less bulky heteroanalogues, i.e. thieno-,[17] furo-[18] or methylpyrrolo-fused[18] 7-deazapurine nucleosides exerted submicromolar in vitro cytotoxic effects and some derivatives were selective against cancer and leukemia cell lines. Also these compounds were found[18] to be phosphorylated in the cell, got incorporated to DNA and induced DNA damage and apoptosis. As a continuation of the systematic SAR study of this new interesting class of biologically active nucleosides, and to prove whether the activity of the hetero-analogues is based on their lower bulkiness or on the presence of the heteroatoms, we designed and present here the synthesis of all-four novel isomeric series of pyrido-fused 7-deazapurine ribonucleosides and their biological profiling.

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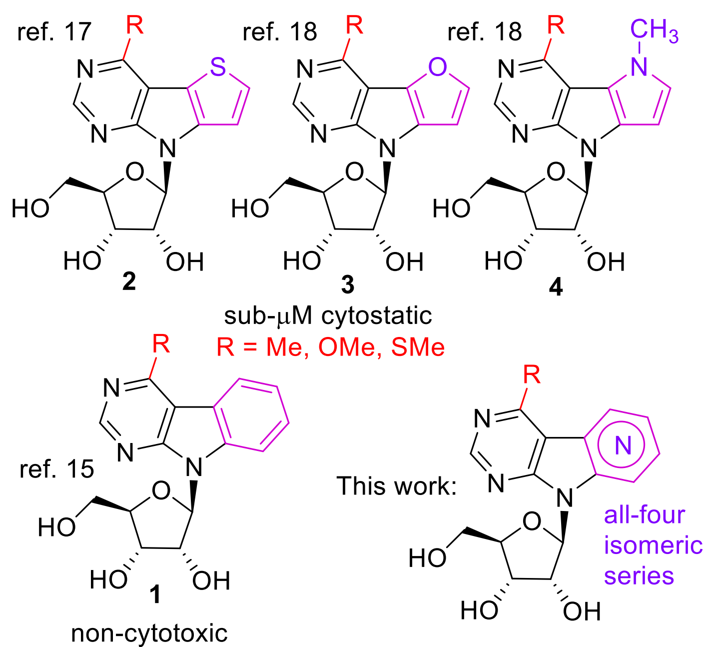

Chart 1. Structures of previously reported fused-deazapurine nucleosides

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Results and discussion

Chemistry.

There are four possible isomers of pyrido-fused 7-deazapurines: pyrido[2',3':4,5]- (series **A**), pyrido[3',4':4,5]- (series **B**), pyrido[4',3':4,5]- (series **C**) and pyrido[3',2':4,5]pyrrolo[2,3-*d*]pyrimidine (series **D**) (Scheme 1) and we aimed at the synthesis of all of them. Each individual heterocycle required independent total synthesis. In all four cases, the synthesis was designed in analogy to the previous syntheses of pyrimidoindoles (Scheme 1), but every step needed to be optimized for each isomer.

The key isomeric 4-chloropyridopyrrolopyrimidine intermediates **9A-D** were synthesized in 4 steps (Scheme 1), starting from corresponding isomeric chloronitropyridines **5A-D**. The synthesis started with a nucleophilic substitution of chlorine with ethyl cyanoacetate which in the case of electron-poor chloronitropyridines proceeded smoothly. In case of **5A** and **5C**, potassium *tert*-butoxide in *tert*-butyl alcohol was used as a base according to literature procedure.[19] In case of compounds **5B** and **5D**, a stronger base NaH in DMF was used to reach good conversions.[20] In each of the four series, formation of a different tautomer or mixture of tautomers of the arylcyanoacetate **6A-D** was observed (see Figure S1 in SI). Compounds **6A-D** were subsequently reduced by zinc dust in acetic acid followed by spontaneous cyclization to obtain isomeric pyrrolopyridine products **7A**-**7D** (94, 53, 73 and 81%). The next cyclocondensation step with formamide afforded the tricyclic heterocycles **8A**-**D** (86, 67, 79 and 92%).[20] The next step was the conversion of the pyrimidone derivatives to **8A**-**D** to fused chloropyrimidines **9A**-**D**. However, the literature procedure using $POCl_3$ with Et_3N reported for preparation of related chloropyrimidine nucleobases[20] did not give the desired chloropyrimidine, but diethylamino nucleobase **9Ax** (see SI). Therefore, these tricyclic pyrimidone derivatives were converted to chloropyrimidine using $POCl_3$ in presence of dimethylaniline and benzyltriethylammonium chloride (BTEACl) (in analogy to procedure developed previously in our group[13]). The desired key intermediates **9A**-**D** were isolated in yields of 65, 13, 52 and 59%, respectively. The synthesis of pyrido[3',4':4,5]pyrimidine **9B** was more problematic in each step due to lower stability, partial decomposition of the intermediates and due to partial hydrolysis back to **8B** in the last step. The total yield of synthesis of **9B** was only 4.5% and only ca. 400 mg of the material was prepared even after repeated attempts. The other isomers **9A**,**C**,**D** were prepared in reasonable overall yields of 52, 29 and 44%, respectively.

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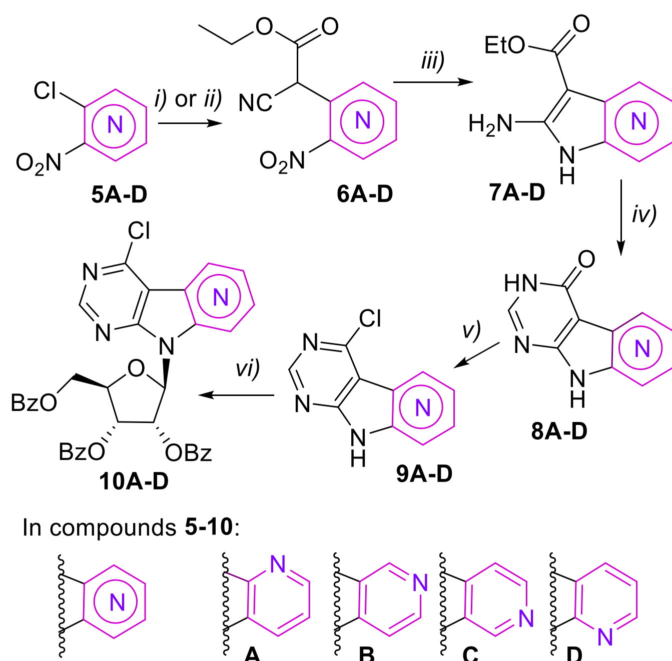

Scheme 1: Reagents and conditions: (i) *t*BuOK, ethyl cyanoacetate, *t*BuOH, 95 °C, 6 h; (ii) NaH, ethyl cyanoacetate, DMF, rt, 30 min or 6 h; (iii) Zn dust, AcOH, 95 °C, 75 min or 90 min; (iv) formamide, $HCOONH_4$, 170 °C, 12 h; (v) *N,N*-dimethylaniline, BTEACl, $POCl_3$, MeCN, 90 °C, 1 h; (vi) BSA, MeCN, 60 °C, 30 min; then 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-*β*-D-ribofuranose, TMSOTf, 60 °C, 12 h.

The pyridopyrrolopyrimidine nucleobases **9A-D** were then subjected to Vorbrüggen glycosylation with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-*β*-D-ribofuranose[15]. Nucleobases were dissolved in anhydrous MeCN and stirred with *N*,*O*-bis(trimethylsilyl)acetamide (BSA) for 30 minutes at 60 °C and then trimethylsilyl triflate (TMSOTf) and 1-*O*-acetyl-2,3,4-tri-*O*-benzoyl-*β*-D-ribofuranose were added and the mixture was stirred overnight at 60 °C. This procedure produced the key intermediates, protected 4-chloropyridopyrrolopyrimidine nucleosides **10A-D** (yields 72, 12, 58 and 57%, respectively). In case of the **B-**series, we also observed formation of bis-ribosylated by-products **10Bx** and **10By** bearing another sugar linked to the pyridine nitrogen (see Figure S2 in SI). These intermediates were characterized by NMR and MS and by-product **10Bx** was easily converted back to desired **10B** by increased temperature (to increase the yield to 25%, See SI). Interestingly, in case of series **D**, a separable mixture of *α* (yield 5%) and *β* anomers (57%) was formed after glycosylation. Because of overlapping signals, the anomeric configuration of the α-nucleoside **10Dα** side-product could not be determined directly and, therefore, it was transformed to methoxy-derivative **12Daα** which was fully characterized and the stereochemistry was confirmed by H,H-ROESY experiment (see SI).

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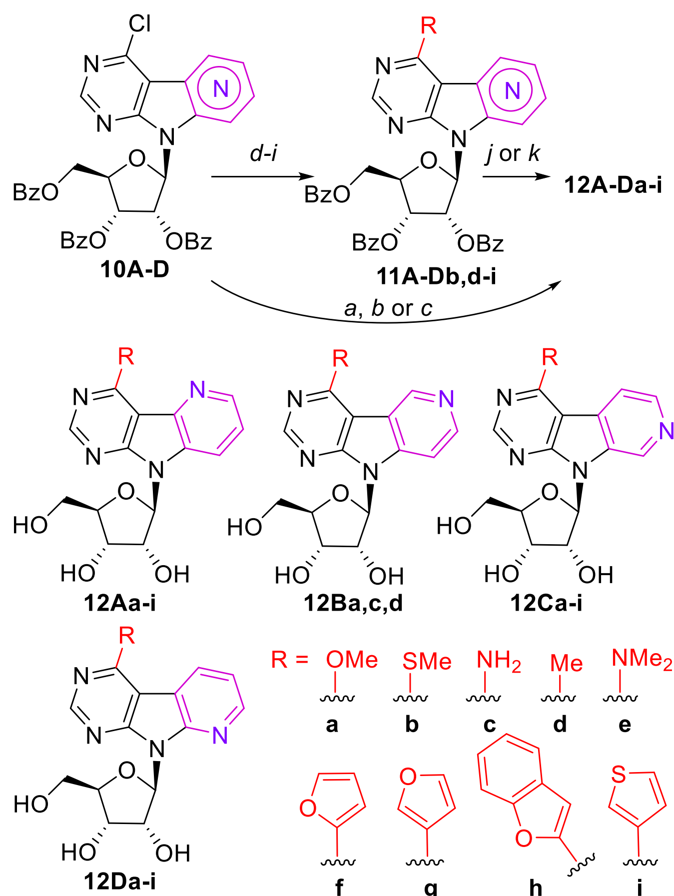

Scheme 2. Reagents and conditions: (a) MeONa, MeOH or MeOH:DMF, rt–90 °C, 16 h; (b) MeSNa, MeOH, rt–60 °C, 16 h; (c) NH_3(aq), 1,4-dioxane, 120 °C, 24 h; (d) Me_2NH in THF, *i*PrOH:DCM 1:1, rt, 16 h; (e) Me_3Al, $Pd(PPh_3)_4$, THF, 70 °C, 16 h; (f) 2-tributylstannylfuran, $PdCl_2(PPh_3)_2$, DMF, 100 °C, 4 h; (g) R-boronic acid, $Pd(PPh_3)_4$, K_2CO_3, toluene, 100 °C, 3–24 h; (h) R-boronic acid, $Pd(PPh_3)_2Cl_2$, K_2CO_3, Et_3N, toluene, 100 °C, 24 h; (i) MeSNa, DMF, rt, 16 h; (j) MeONa, MeOH:DMF, 90 °C, 16 h; (k) MeONa, MeOH, rt–70 °C, 16 h.

Table 1. Synthesis of 4-Substituted Nucleosides **12A–Da–h**

Entry	Method	R	Protected Nucleoside	Yield [%]	Final Nucleoside	Yield [%]
1	(a) MeONa	OMe			**12Aa**	62
2	(i) MeSNa	SMe	**11Ab**	52	**12Ab**	76
3	(c) NH_3(aq)	NH_2			**12Ac**	53
4	(e) Me_3Al	Me	**11Ad**	69	**12Ad**	87
5	(d) Me_2NH	NMe_2	**11Ae**	82	**12Ae**	81
6	(f) R-$SnBu_3$	2-furyl	**11Af**	90	**12Af**	75
7	(g) R-$B(OH)_2$	3-furyl	**11Ag**	73	**12Ag**	78
8	(g) R-$B(OH)_2$	3-thienyl	**11Ai**	70	**12Ai**	70
9	(h) R-$B(OH)_2$	2-benzofuryl	**11Ah**	56	**12Ah**	69
10	(a) MeONa	OMe			**12Ba**	58
11	(c) NH_3(aq)	NH_2			**12Bc**	41

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12	(e) Me_3Al	Me	[a]		**12Bd**	26[b]
13	(a) MeONa	OMe			**12Ca**	42
14	(b) MeSNa	SMe			**12Cb**	47
15	(c) NH_3(aq)	NH_2			**12Cc**	51
16	(e) Me_3Al	Me	**11Cd**	55	**12Cd**	63
17	(d) Me_2NH	NMe_2	**11Ce**	67	**12Ce**	91
18	(f) R-$SnBu_3$	2-furyl	**11Cf**	66	**12Cf**	71
19	(g) R-$B(OH)_2$	3-furyl	**11Cg**	84	**12Cg**	90
20	(g) R-$B(OH)_2$	3-thienyl	**11Ci**	57	**12Ci**	72
21	(g) R-$B(OH)_2$	2-benzofuryl	**11Ch**	64	**12Ch**	79
22	(a) MeONa	OMe			**12Da**	90
23	(b) MeSNa	SMe			**12Db**	80
24	(c) NH_3(aq)	NH_2			**12Dc**	85
25	(e) Me_3Al	Me	**11Dd**	82	**12Dd**	82
26	(d) Me_2NH	NMe_2	**11De**	81	**12De**	81
27	(f) R-$SnBu_3$	2-furyl	**11Df**	83	**12Df**	93
28	(g) R-$B(OH)_2$	3-furyl	**11Dg**	75	**12Dg**	80
29	(g) R-$B(OH)_2$	3-thienyl	**11Di**	81	**12Di**	91
30	(g) R-$B(OH)_2$	2-benzofuryl	**11Dh**	89	**12Dh**	90

[a] Crude compound was used directly in the next step. [b] Overall yield after two steps.

The key intermediates, benzoylated 4-chloropyridopyrrolopyrimidine nucleosides **10A**, **10B**, **10C** and **10D**, were subsequently modified at position 4 through nucleophilic substitutions or cross-coupling reactions to provide four sets of desired nucleosides bearing several substituents, such as different size heterocycles, as well as methoxy, methylsulfanyl, methyl, dimethylamino and amino groups. The selection of substituents was based on analogy and previous experience with other fused-deazapurine ribonucleosides.[13,17,18,21] In case of intermediate **10B**, which was very difficult to obtain due to instability of intermediates and only limited amount was available, we were able to prepare only 3 most important derivatives **12Ba,c,d**. Methoxy derivatives **12Aa**, **12Ba**, **12Ca** and **12Da** and methylsulfanyl derivatives **12Cb**, **12Db** were obtained through nucleophilic substitution with sodium methoxide or sodium thiomethoxide in MeOH. During mentioned substitution with sodium thiomethoxide in MeOH, in all three series, mixture of methoxy and methylsulfanyl nucleosides was formed. While in series **C** and **D** these two compounds were easily separated, in series **A**, inseparable mixture was formed, therefore at first methylsulfanyl protected intermediate **11Ab** was prepared and subsequently deprotected under Zemplén conditions. To obtain amino derivatives **12Ac**, **12Bc**, **12Cc**, and **12Dc**, the key 4-chloropyridopyrrolopyrimidine intermediates were subjected to nucleophilic substitution using aqueous ammonia in 1,4-dioxane. In all previously mentioned cases (except of compound **12Ab**), removal of the benzoyl protecting groups was achieved simultaneously under the reaction conditions. 2-Furyl derivatives **11Af**, **11Cf** and **11Df** were prepared in good yields

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from 4-chloropyridopyrrolopyrimidine nucleosides **10A**, **C, D** by the Stille cross-coupling reaction with the corresponding tributylstannane, catalyzed by $PdCl_2(PPh_3)_2$ in DMF at 100 °C (Scheme 2). 3-Furyl, 3-thienyl and 2-benzofuryl moieties were introduced by Suzuki−Miyaura cross-coupling reactions with the corresponding hetarylboronic acids in toluene using $Pd(PPh_3)_4$ as a catalyst in presence of potassium carbonate. Benzoylated intermediates **11Ag**, **11Cg**, **11Dg**, **11Ai**, **11Ci**, **11Di** and **11Ch**, **11Dh** were isolated in good yields (Scheme 2, Table 1). In the case of protected 2-benzofuryl derivative **11Ah**, low yield was obtained and therefore **11Ah** was synthesized using $PdCl_2(PPh_3)_2$ as a catalyst and potassium carbonate and triethylamine. 4-Methyl derivatives **11Ad**, **11Cd**, **11Dd** were synthesized by palladium-catalyzed alkylation of 4-chloropyridopyrrolopyrimidine derivatives **10A**, **10C**, **10D** with trimethylaluminium in THF at 70 °C. In case of series **B**, intermediate **11Bd** was prepared in the same way but it was directly deprotected to **12Bd** without further purification or characterization. Dimethylamino groups of compounds **11Ae**, **11Ce**, and **11De** were introduced by nucleophilic substitution with dimethylamine in the mixture of DCM and propan-2-ol at the room temperature. Zemplén deprotection of benzoylated nucleosides **11Ab** and **11A-Dd–h** in presence of MeONa in MeOH furnished desired unprotected nucleosides **12Ab** and **12A–Dd–h** in good yields (Scheme 2, Table 1). Because of poor solubility of several benzoylated intermediates in MeOH, deprotection of these derivatives were performed at increased temperatures of 40–70 °C or in the mixture of MeOH and DMF. Yields and reaction conditions are summarized in Table 2.

For the study of the intracellular metabolism and mechanism of action of this type of compounds, selected most active nucleosides **12Ac**, **12Ad**, **12Cc** and **12Cd** were converted into their 5'-*O*-monophosphates **13Ac**, **13Ad**, **13Cc** and **13Cd** by treatment with $POCl_3$ followed by addition of 2 M TEAB (aqueous triethylammonium bicarbonate). The triethylammonium salts of products were converted to sodium salts by ion exchange on Dowex (Scheme 3).[22]

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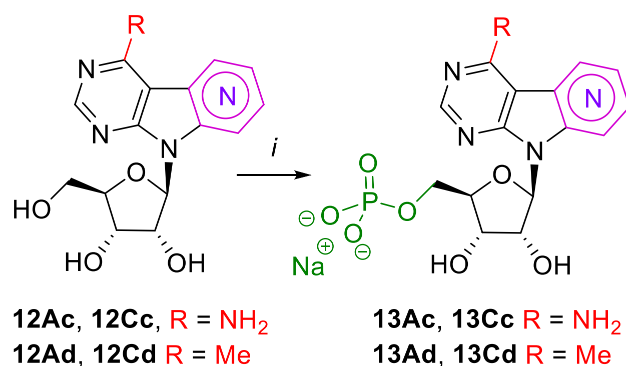
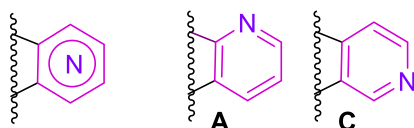


In compounds **12** and **13**:



Scheme 3. Reagents and conditions: (i) P(O)(OMe)3, $POCl_3$, 0 °C, 3 h, then aqueous 2 M TEAB, ion exchange on Dowex 50 (Na^+ form)

In order to obtain standards of selected 2'-deoxyribonucleotides for the study of intracellular incorporation to DNA and RNA,[14] it was required to prepare nucleoside 5'-*O*-monophosphates derived from 2'-deoxy analogues of amino **12Ac** and **12Cc** and methyl nucleosides **12Ad** and **12Cd**. To synthesize the key protected *β*-anomeric deoxyribonucleoside intermediates **14A** and **14C**, the corresponding pyrido-fused nucleobases **9A** and **9C** were deprotonated by potassium hydroxide or sodium hydride and then subjected to glycosylation using Hoffer's chlorosugar (1-chloro-2-deoxy-3,5-di-*O*-toluoyl-α-D-ribofuranose).[10] Palladium-catalyzed alkylation of **14A** and **14C** with trimethylaluminum provided protected methylated intermediates **15Ad** and **15Cd**, which were subsequently deprotected under Zemplén conditions to give the final free deoxyribonucleosides **16Ad** and **16Cd**. To obtain adenine-like 2'-deoxyribonucleosides **16Ac** and **16Cc**, the key intermediates were subjected to nucleophilic substitution using aqueous ammonia in 1,4-dioxane. Corresponding 2′-deoxy-5′-*O*-monophosphates **17Ad**, **17Cd**, **17Ac** and **17Cc** were synthesized in the same manner as their ribo analogues **13Ac**, **13Ad**, **13Cc** and **13Cd** (Scheme 4).

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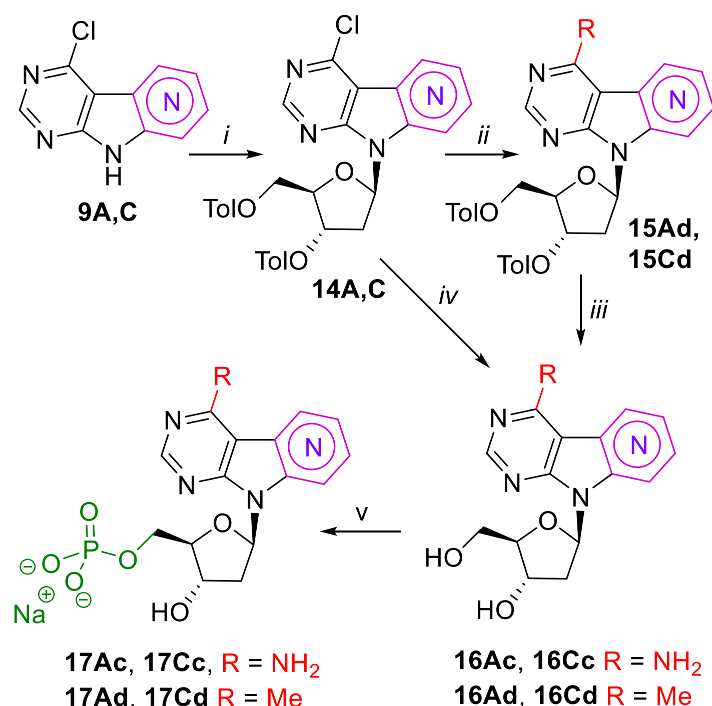
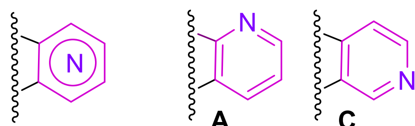


In compounds **9, 14-17**:



Scheme 4. Reagents and conditions: (i) 1) KOH, TDA-1, MeCN, rt, 30 min; or NaH, MeCN, rt, 30 min; 2) Hoffer's chlorosugar, rt, 30–120 min; (ii) Me_3Al, $Pd(PPh_3)_4$, THF, 70 ºC, 16 h; (iii) MeONa, MeOH, rt–60 °C, 16 h; (iv) NH_3 (aq), 1,4-dioxane, 120 °C, 16 h; (v) 1) $P(O)(OMe)_3$, $POCl_3$, 0 ºC, 3 h; 2) aqueous TEAB (2 M); 3) ion exchange on Dowex 50 (Na^+ form).

We were able to determine crystal structures of four final ribonucleosides **12Ae**, **12Ai**, **12Ag** and **12Ci** (Figure 1) and one deoxyribonucleoside **16Ac** by X-ray diffraction. In some cases, the crystal packing contained several conformers and the packing involved head-to-tail π-π stacking of the extended nucleobases. Conformers of each nucleoside and the crystal packings are given in Figures S4-S8 in Supporting Information.

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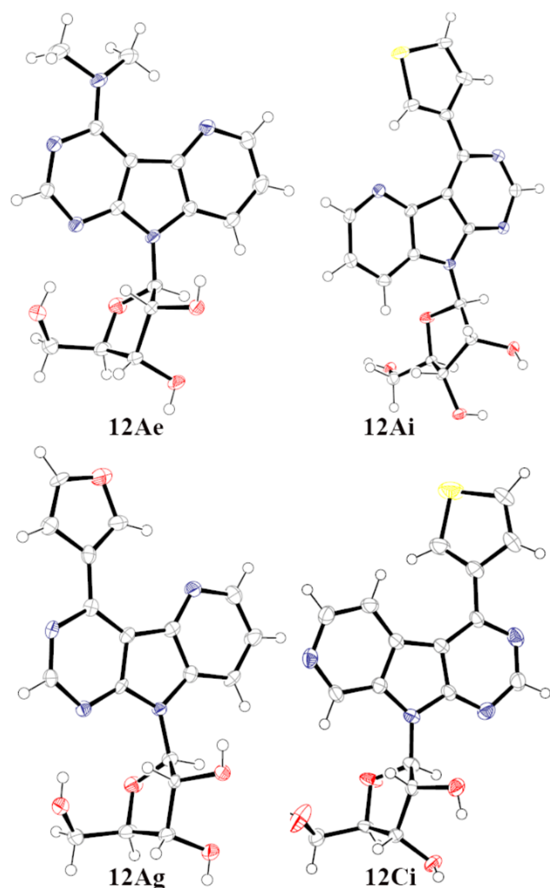

Figure 1. Crystal structures for compounds **12Ae**, **12Ai**, **12Ag** and **12Ci**.

In order to investigate if the pyridopyrrolopyrimidine ribonucleoside triphosphates are substrates for RNA polymerases, we synthesized amino and methyl triphosphates **18Ac**, **18Ad**, **18Cc**, **18Cd**, **18Dc** and **18Dd** by treatment with $POCl_3$ followed by addition of freshly prepared solution of bis(tri-*n*-butylammonium) pyrophosphate and tri-*n*-butylamine (Scheme 5). Using DMF as a solvent to dissolve the pyrophosphate turned out to be counterproductive in this case. It is known that polar solvent such as DMF reacts with phosphoryl oxychloride while Vilsmeier reagent is produced and by-product 5'-triphosphate-2', 3'-cyclic phosphate is formed.[23] In an attempt to reduce or eliminate the formation of cyclic by-product, MeCN was selected as more appropriate solvent. The cyclic by-products **18Cdx** and **18Ddx** were successfully separated by ion-exchange HPLC on Poros HQ and characterized by ^{1}HNMR and MS (see SI).

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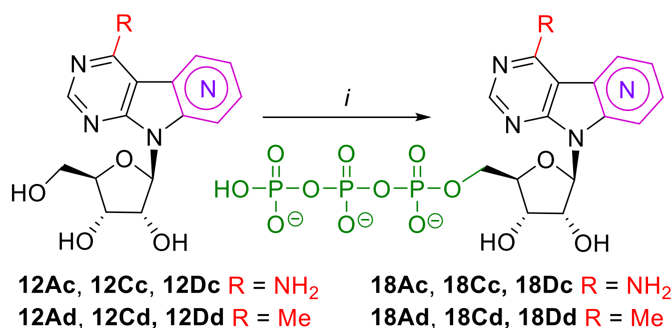
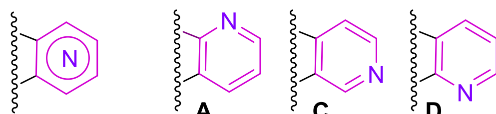


In compounds **12** and **18**:



Scheme 5. Reagents and conditions: (i) 1. $POCl_3$, $PO(OMe)_3$, 0 °C, 1.5 h; 2) $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF or MeCN, 0 °C, 1 h; 3) aqueous TEAB (2 M); 4) ion exchange on Dowex 50 (Na^+ form).

Fluorescence properties of pyrido-fused 7-deazapurine ribonucleosides

Many of the previously reported fused 7-deazapurine nucleosides have shown interesting fluorescence properties and some of them were used for the preparation of fluorescent DNA probes.[16,24,25] Therefore, we have studied also the photophysical properties of the title pyrido-fused 7-deazapurine ribonucleosides. Table 2 summarizes the results showing that the most compounds exerted at least some fluorescence in ethanol (except of dimethylamino derivative **12Ae**). Moderate to good fluorescence quantum yields (0.20–0.40) showed compounds **12Dg**, **12Aa**, **12Ah** and **12Cc**. Much brighter fluorescence with an excellent quantum yields in the range of 0.46–0.62 showed compounds **12Df**, **12Da**, **12Dc** and **12Dh** from the **D** series (2-furyl, methoxy, amino, 2-benzofuryl derivatives). These results indicate that at least some of pyrido-fused 7-deazapurine ribonucleosides have a potential in fluorescent labelling of nucleic acids when excited with UV lasers. On contrary, favourable excitation and emission wavelengths enables analysis of biological activity of compounds using routinely available reporters based on green or red fluorescent proteins.

Table 2. UV absorption and Fluorescence Spectra of Pyrido-fused Nucleosides **12A–Da–h**

Compound	Absorption						Emission	
	λ_{max} [nm] ($\varepsilon \cdot 10^{-4}$ [$L \cdot mol^{-1} \cdot cm^{-1}$])						φ	λ_{max} [nm]
12Bc	305	(0.53)	283	(0.82)			0.03	324
12Ba	275	(1.06)					0.03	464
12Da	315	(1.03)	279	(1.14)			0.50	331
12Db	325	(1.26)	298	(1.37)	253	(1.82)	0.02	345

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12Dc	320	(1.60)					0.56	339
12Dd	319	(0.45)	282	(1.33)	254	(1.93)	0.11	344
12Di	303	(0.67)	259	(2.67)			0.18	383
12De	332	(1.46)					0.02	364
12Dg	302	(1.18)	260	(2.19)			0.20	374
12Df	322	(1.81)	269	(2.83)			0.46	379
12Dh	365	(2.53)	348	(2.82)	275	(2.95)	0.62	399
12Aa	322	(0.92)	312	(0.92)	283	(0.80)	0.29	342
12Ab	336	(0.92)	324	(0.91)	256	(2.53)	0.01	343
12Ac	327	(1.65)					0.02	346
12Ad	287	(1.00)	254	(2.04)			0.11	362
12Ai	316	(1.30)	264	(3.29)			0.08	400
12Ae	340	(1.62)	250	(2.49)			0.00	364
12Ag	312	(1.08)	263	(2.66)			0.06	394
12Af	326	(2.03)	269	(3.23)			0.24	404
12Ah	356	(2.70)	274	(3.51)			0.36	414
17Ac	326	(1.60)					0.02	345
12Ca	310	(0.40)	280	(1.30)			0.16	349
12Cb	302	(1.63)					0.03	390
12Cc	319	(0.91)	290	(1.20)			0.31	339
12Cd	323	(0.28)	251	(2.19)			0.07	371
12Ci	301	(1.36)	257	(2.53)			0.08	412
12Ce	319	(1.31)	332	(1.39)			0.01	503
12Cg	298	(0.96)					0.07	405
12Cf	328	(2.04)	259	(2.30)			0.09	423
12Ch	348	(3.10)	266	(2.64)			0.13	445

UV spectra were measured in ethanol at 25 °C. Fluorescence quantum yields were measured in ethanol using quinine sulfate in 0.5 M H_2SO_4 ($\Phi_f = 0.55$) as a reference. Excitation wavelength was 296 nm.

Biological activity profiling

All the final ribonucleosides **12A–D** were subjected to screening of their biological activities.

Antiviral activity

The antiviral screening was performed against respiratory syncytial virus (RSV), hepatitis C virus (HCV), herpes simplex, influenza, coxsackie, human immunodeficiency virus (HIV) and dengue viruses (for detailed viruses strains and procedures see SI). All the final nucleosides were found to be inactive against RSV, influenza, coxsackie, herpes simplex, HIV and dengue viruses. The only exception were 2-benzofuryl derivatives from series **C** and **D**, which showed weak activities against dengue (**12Ch** EC_{50} = 20.1 μM and **12Dh** EC_{50} = 27.5 μM). The antiviral screening of anti-HCV activities (performed as described previously)[26] revealed activities against both HCV replicons 1B and 2A in sub-micromolar concentrations (Table 3) and some of them are even more active than Mericitabine, clinically developed nucleoside inhibitor of viral RNA polymerase NS5B.[27] The NS5B enzyme (non-structural protein 5B) builds a replicase complex with other cellular and viral proteins in the perinuclear part of infected cells.[28] It is remarkable that nucleoside or nucleotide analogues are the only direct-acting

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antivirals in clinical development displaying wide activity against all HCV genotypes, with non-nucleoside inhibitors being exclusively or mainly active against HCV genotype 1.[29] In our study, the anti-HCV activities differed in each series. The 2-furyl, 3-furyl and 3-thienyl derivatives **12Af**, **12Ag**, **12Ai**, **12Cf**, **12Cg** from **A** and **C** series and one methylsulfanyl derivative **12Db** from **D** series are active only against replicon 1B. Low micromolar activity against both HCV replicons showed 2-benzofuryl derivatives **12Dh**, **12Ah** and methyl derivative **12Ad**. The most potent and selective in both replicons were pyridopyrrolopyrimidine nucleosides from the **C** series **12Ca**, **12Cb**, **12Cc** and **12Cd**.

Table 3. Anti-HCV Activities of Pyrido-fused Nucleosides **12A–D**

Compd	HCV replicon 1B		HCV replicon 2A	
	EC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)
12Aa	>44.44	>44.44	>44.44	>44.44
12Ab	>44.44	>44.44	>44.44	>44.44
12Ac	0.63	>44.44	>44.44	>44.44
12Ad	0.64	32.33	1.57	>44.44
12Ae	>44.44	>44.44	>44.44	>44.44
12Af	0.16	>44.44	>44.44	>44.44
12Ag	0.36	>44.44	>44.44	>44.44
12Ai	0.84	>44.44	>44.44	>44.44
12Ah	5.27	>44.44	15.42	38.02
12Ba	>44.44	>44.44	>44.44	>44.44
12Bc	13.97	34.67	28.65	>44.44
12Bd	>44.44	>44.44	>44.44	>44.44
12Ca	0.46	>44.44	0.77	>44.44
12Cb	0.59	>44.44	0.29	>44.44
12Cc	0.09	21.66	0.66	40.13
12Cd	0.16	24.47	0.41	>44.44
12Ce	>44.44	>44.44	>44.44	>44.44
12Cf	10.0	>44.44	41.5	>44.44
12Cg	7.97	>44.44	>44.44	>44.44
12Ci	>44.44	>44.44	>44.44	>44.44
12Ch	3.40	15.50	6.31	20.29
12Da	>44.44	>44.44	>44.44	>44.44
12Db	14.26	>44.44	>44.44	>44.44
12Dc	>44.44	>44.44	>44.44	>44.44
12Dd	42.80	>44.44	>44.44	>44.44
12De	>44.44	>44.44	>44.44	>44.44
12Df	40.60	>44.44	>44.44	>44.44
12Dg	>44.44	>44.44	>44.44	>44.44
12Di	>44.44	>44.44	>44.44	>44.44
12Dh	3.04	22.73	7.47	27.5
12Daα	>44.44	>44.44	>44.44	>44.44
Mericitabine	1.20	>44.44	0.99	>44.44

Cytotoxic activity

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In vitro cytotoxic activity screening of nucleosides **12A–Da–h** was performed on several cancer cell lines (A549 – human lung adenocarcinoma, HCT116 and HCT116p53$^{-/-}$ – colon cancer cells with/without p53 gene, U2OS – human osteosarcoma, HeLa – human HPV positive cervical carcinoma, HepG2 – hepatocellular carcinoma) as well as leukemic cell lines (CCRF-CEM – acute lymphoblastic leukemia, CEM-DNR – CCRF-CEM cells daunorubicin resistant overexpressing multidrug resistance proteins and lacking topoisomerase 2-α gene, K562 – myelogenous leukemia bcr-abl positive, K562-TAX – K562 cells paclitaxel resistant overexpressing multidrug resistance proteins, HL60 – acute promyelocytic leukemia).[30] These cell lines were used for determination of concentration reducing cell viability by 50% (IC_{50}) by 3-days MTS (3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)[23] or XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay[31]. Non-malignant fibroblast cell lines MRC-5 and BJ were used in the MTS assay for comparison.

The cytotoxic activity screening (Table 4) showed that the presence and position of nitrogen atom is crucial for cytostatic and cytotoxic effect. Only pyridopyrrolopyrimidine ribonucleosides possessing nitrogen at the position 7 on the pyridine ring (series **C**) showed significant submicromolar *in vitro* cytotoxic activity, whilst some derivatives of pyridopyrrolopyrimidine ribonucleosides bearing nitrogen at the position 5 or 8 (series **A** and **D**) showed moderate to good activity and the three examples of the pyrido[3',4':4,5]pyrimidine nucleosides (series **B**) were entirely inactive. The most active compounds **12Ca**, **12Cb**, **12Cc** and **12Cd**, belonging to the **C** series, displayed submicromolar values of activities against leukemic and solid tumour cell lines, with low toxicity to BJ and no toxicity to MRC-5 fibroblasts, showing promising therapeutic index under *in vitro* conditions. It is interesting to point out that the amino derivative **12Cc** was one of the most active compounds in the study, which makes this class of compounds different from previously reported heteroaryl-fused 7-deazapurine nucleosides, where the 6-amino derivatives (analogues of adenine) were typically inactive[23,18]. Also interesting is the comparison of these cytotoxic pyridopyrrolopyrimidine nucleosides to previously reported benzo-fused (carba-analogues) nucleosides which did not show any cytotoxic activity. Apparently, it is not just the bulkiness of the fused (hetero)aromatic moiety, but also the presence and position of N atom (presumably as H-bond acceptor) are crucial factors for biological activity of this class of nucleosides. Cytotoxic activity of tested compounds was independent of p53 status, as cytotoxic activities were comparable in p53 proficient versus deficient HCT116 cells. However, biological activity was impaired by expression of multidrug resistance proteins across nucleoside series **12A-Da-h**, with exception

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of compounds **12Cd** and **12Cc**, where cytotoxic activities were comparable or only slightly different on parental versus multidrug resistant cell lines, respectively (Table 4).

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Table 4. Cytotoxic Activity of Nucleosides **12A–Da–h**

	MTS, IC_{50} (µM)										XTT, IC_{50} (µM)		
Compound	**BJ**	**MRC-5**	**A549**	**CCRF -CEM**	**CEM -DNR**	**HCT116**	**HCT116 p53-/-**	**K562**	**K562 -TAX**	**U2OS**	**Hep G2**	**HL 60**	**HeLa**
12Aa	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Ab	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Ac	9.1	>50	>50	0.57	>50	27	32	31	>50	1.5	8.4	0.3	>50
12Ad	>50	>50	>50	0.8	>50	>50	>50	10	>50	2.4	>50	>50	>50
12Ae	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Af	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Ag	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Ai	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Ah	>50	>50	>50	10	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Ba	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Bc	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Bd	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Ca	>50	>50	>50	0.56	5.1	1.5	1.51	1.0	5.6	1.6	1.53	0.71	1.38
12Cb	>50	>50	15	0.27	1.0	0.34	0.38	0.27	1.3	0.54	1.08	0.26	1.12
12Cc	3.0	>50	1.8	0.7	2.3	0.5	0.47	0.9	3.6	0.6	0.34	0.8	1.30
12Cd	7.7	>50	>50	0.4	0.9	2.7	2.7	1.5	1.8	1.3	2.9	0.35	2.9
12Ce	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Cf	>50	>50	>50	42	>50	32	>50	>50	>50	>50	>50	>50	>50
12Cg	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Ci	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Ch	46	17	19	7.4	22	7.1	13	19	19	16	>50	>50	>50
12Da	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Db	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Dc	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Dd	>50	>50	>50	7.7	>50	>50	>50	18	>50	>50	>50	>50	>50
12De	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Df	>50	>50	>50	38	>50	35	>50	>50	>50	>50	>50	>50	>50
12Dg	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Di	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
12Dh	>50	42	29	10	28	27	24	27	24	23	>50	>50	>50
12Daα	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50

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10.1002/chem.202001124

Cell cycle studies

Pyridopyrrolopyrimidine ribonucleosides which displayed the best cytotoxic activity against CCRF-CEM T-lymphoblastic leukaemia cells, were submitted also for cell cycle analysis. Cells were treated with 1 × IC_{50} and 5 × IC_{50} cytotoxic concentrations of nucleoside derivatives for 24 hours (Table 5). Most of the tested nucleoside analogues lead to a S-phase block with corresponding decrease of G0/G1 cellular population, except for two derivatives. The compound **12Cd**, induced accumulation of treated cells in G0/G1 phase with corresponding decrease of S-phase cells, while **12Ch** showed the G2/M-phase block accompanied with decrease of S-phase cells. Perturbations in DNA and RNA synthesis were observed for most of tested compounds and could be attributed to cell cycle alterations and/or apoptosis. Only analogues **12Cb**, **12Cc** and **12Ch** demonstrated significant (>90%) and dose dependent inhibition of RNA synthesis, which may contribute to their cytotoxic effect.

Table 5. Summary of Cell Cycle Analyses, Proportion of Apoptotic and Mitotic Cells ($pH3^{Ser10}$ Positive), DNA and RNA Synthesis in CCRF-CEM

Compound	Concentration	% of total cellular populations						
		sub-G1 (apoptosis)	G0/G1	S	G2/M	M $pH3^{Ser10}$	DNA synthesis	RNA synthesis
12Ac	0	2.8	38.7	42.8	18.5	1.61	52	51.1
	1 x IC_{50}	4.89	27.09	54.80	18.11	2.10	64.84	38.00
	5 x IC_{50}	9.88	18.37	65.41	16.22	1.77	67.70	32.88
12Ad	0	2.3	40	36.4	24	1.3	33	47
	1 x IC_{50}	3.54	36.84	41.28	21.87	1.13	36.65	17.93
	5 x IC_{50}	6.49	27.83	54.10	18.08	0.66	29.19	27.91
12Ca	0	2.8	38.7	42.8	18.5	1.61	52	51.1
	1 x IC_{50}	4.49	19.55	67.85	12.60	0.69	55.87	23.61
	5 x IC_{50}	7.17	14.13	69.93	15.94	0.61	49.58	27.56
12Cb	0	2.3	40	36.4	24	1.3	33	47
	1 x IC_{50}	3.16	20.85	64.25	14.89	0.30	22.71	9.72
	5 x IC_{50}	5.00	16.54	67.01	16.45	0.25	15.81	8.70
12Cc	0	2	42	45	13	1.5	37	46
	1 x IC_{50}	2.93	33.58	51.21	15.21	1.33	43.43	15.93
	5 x IC_{50}	4.37	9.66	78.46	11.88	1.06	43.35	4.67
12Cd	0	2.95	39	44	16.4	1.50	41	44
	1 x IC_{50}	4.20	46.01	39.33	14.66	1.62	32.12	32.72
	5 x IC_{50}	16.52	50.11	39.94	9.95	1.28	26.10	17.67
12Ch	0	4.1	45.7	36.4	18	2.0	36.3	37

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	1 x IC_{50}	14.82	49.60	11.28	39.12	1.56	25.92	16.42
	5 x IC_{50}	19.20	37.20	26.05	36.75	0.71	31.26	3.24

Intracellular phosphorylation

We anticipated that mechanism of action of pyridopyrrolopyrimidine ribonucleosides might be similar to the previous cases of thieno-, pyrrolo- and furo- fused deazapurine ribonucleosides in which the compound was first phosphorylated in cells and subsequently incorporated into DNA or RNA. To obtain experimental evidence to prove this hypothesis, we studied intracellular phosphorylation of methyl **12Ad**, **12Cd** and amino **12Ac**, **12Cc** derivatives from **A** and **C** series. The corresponding ribonucleoside monophosphates **13Ad**, **13Cd, 13Ac**, and **13Cc** were used as analytical standards. Intracellular phosphorylation was tested using BJ (normal fibroblasts) and HCT116 (malignant) cells at different concentrations of nucleosides (1 or 10 µmol/L) after 1 or 3 hours of treatment *in vitro*. The phosphorylation of all four nucleosides proceeded in all cases, however, in contrast with previously published thienopyrrolopyrimidine nucleosides[17] the levels of monophosphates were higher in malignant HCT116 cells than in BJ, what might explain higher selectivity of these nucleosides for cancer cells. In addition, the ratio of pyridopyrrolopyrimidine nucleoside monophosphate versus nucleoside was increased at higher concentration of corresponding nucleoside (10 µM), which insinuate rate limited phosphorylation in non-malignant cells. Different level of formed monophosphate in malignant and non-malignant cells after 1 and 3 h incubation in 10 µmol/L concentration is compared on the Figure 2. Table summarizing exact values of monophosphate in certain amount of cells (pmol/6 × 10^5 cells) in 1 or 10 µmol/L concentrations is attached in Supporting information.

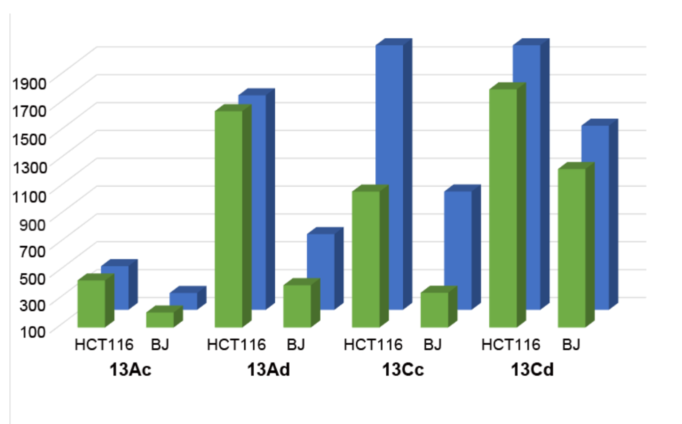

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Figure 2. Comparison of formed monophosphates (pmol/6 × 10^5 cells) in HCT116 and BJ cells after 1 h (green) and 3 h (blue), 10 μmol/L concentration of corresponding nucleoside.

Phosphorylation and inhibition by human adenosine kinase (ADK)

After we proved that the desired pyridopyrrolopyrimidine nucleosides are effectively phosphorylated in cells, we supposed that the enzyme responsible for phosphorylation could be a human adenosine kinase (ADK). Therefore, we studied the *in vitro* phosphorylation of the title nucleosides by human ADK[32] (Table 6).

Table 6. Phosphorylation of Nucleosides **12A–Da–h** by ADK and Inhibition of ADK

Compound	Phosphorylation (%)	Inhibition IC_{50}
Adenosine	86.5	–
12Aa	0	>10
12Ab	0	>10
12Ac	82.3	>10
12Ad	33.1	>10
12Ae	0	>10
12Af	9.4	245 ± 41 nM
12Ag	0	>10
12Ah	0	184 ± 21 nM
12Ai	0	20 ± 1.3 μM
12Ba	0	>10
12Bc	0	>10
12Ca	19.4	>10
12Cb	0	>10
12Cc	0	>10
12Cd	18.6	>10
12Ce	0	>10
12Cf	0	>10
12Cg	0	>10
12Ci	0	>10
12Ch	0	5.5 ± 0.9 μM
12Da	0	>10
12Db	0	>10
12Dc	0	>10
12Dd	0	>10
12De	0	>10
12Df	0	>10
12Dg	0	>10
12Di	0	>10
12Dh	0	2.9 ± 0.39 μM
12Daα	0	>10

Phosphorylation: 50 μM solution of compound, 243 ng of enzyme, 30 min, 37 °C
Inhibition: 50 μM solution of adenosine, 10 μM solution of compound, 97 ng of enzyme, 20 min, 37 °C.

Unlike in the previous case of thieno-, pyrrolo- and furo- fused deazapurine ribonucleosides where the rate of phosphorylation nicely correlated with cytotoxic activities, it was not the case

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for some pyridopyrrolopyrimidine ribonucleosides. The amino derivative **12Ac** is effectively phosphorylated by ADK comparably to adenosine. Methyl derivatives **12Ad** and **12Cd** and methoxy derivative **12Ca** are also phosphorylated but somewhat less efficiently. Surprisingly, two most active compounds **12Cb** and **12Cc** were not phosphorylated by ADK at all in our assay. However, because all the amino and methyl derivatives from series **A** and **C** were phosphorylated *in cellulo*, we can assume that these nucleosides might have been phosphorylated by other isoforms of ADK or by some other nucleoside kinase. All nucleosides were also tested for ADK inhibition. Generally, they are not effective inhibitors of ADK, only derivatives **12Ai**, **12Af**, **12Ah**, **12Ch** and **12Dh** displayed some moderate inhibition (Table 6).

Incorporation of nucleosides into nucleic acids of treated cells

After confirming that title nucleosides are phosphorylated preferentially in cancer cells, we studied the incorporation of selected nucleotides (derived from methyl **12Ad**, **12Cd** and amino **12Ac**, **12Cc** nucleosides from **A** and **C** series) to DNA and RNA in living cells. CCRF-CEM cells were incubated with 5 × IC_{50} concentrations of nucleosides for 2.5 h and immediately harvested. RNA and DNA were isolated and digested. The separation and detection of modified nucleosides and nucleotides and their concentration in nucleic acids were achieved by using HPLC chromatography with tandem mass spectrometry (LC-MS/MS). The corresponding ribonucleoside monophosphates **13Ad**, **13Cd, 13Ac**, and **13Cc** as well as their 2'-deoxy analogues (Scheme 4) were used as analytical standards.

Table 7. Incorporation of Selected Nucleosides into RNA and DNA in Treated CCRF-CEM Cells

sample	compd	NMP content (fmol/µg of nucleic acid)	dNMP content (fmol/µg of nucleic acid)
DNA	**12Ac**	471±69	nd[a]
	12Ad	539±101	5.5±1.8
	12Cc	494±96	nd[a]
	12Cd	407±232	nd[a]
RNA	**12Ac**	172±17	
	12Ad	nd[a]	
	12Cc	180±10	
	12Cd	nd[a]	

[a]Not detected. Incorporation analyzed after 2.5-hour treatment at 5 × IC_{50} and subsequent digestion with nuclease P1 (data from 2–4 independent experiments)

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The results show that all tested compounds are mainly incorporated into DNA as ribonucleosides, whereas only the methyl derivative **12Ad** is also incorporated into DNA as 2'-deoxyribonucleoside though in very minor quantities (2'-deoxyribonucleoside monophosphate **17Ad** found in residual quantities). All tested modified nucleosides are more efficiently incorporated into DNA (in conversion to fmol/µg of nucleic acid) than the previously reported AB61[14] or pyrrolo- and furo-fused nucleosides.[18] Interestingly, only amino derivatives **12Ac** and **12Cc** were detected in the RNA of treated cells. These results correlate with results of enzymatic incorporation by T7 RNA polymerase where just amino derivatives from **A** and **C** series are successfully incorporated into RNA (Figure 3).

Enzymatic incorporation of pyridopyrrolopyrimidine nucleotides into nucleic acids

Intracellular phosphorylation and subsequent incorporation into RNA that prevents its function is one of the parallel mechanisms of action of cytotoxic 7-hetaryl-7-deazaadenosines.[14] Therefore, selected ribonucleoside triphosphates **18Ac**, **18Ad**, **18Cc**, **18Cd**, **18Dc** and **18Dd**, were prepared and tested as substrates for model T7 RNA polymerase. We used DNA templates containing the promoter and three guanosines in the +1 to +3position according to our previous work.[33] The transcription has been performed using [α-^{32}P]-GTP and the RNA products were analyzed by PAGE (Figure 3) and quantified using QuantityOne software. From all six modified ribonucleoside triphosphates we tried, just amino derivatives **18Ac** and **18Cc** from **A** and **C** series were successfully incorporated (similarly as in case of incorporation by human polymerase). The RNA products were also characterized by MALDI-TOF, which confirmed the correct full-length modified RNA (see SI). These results suggest that only amino pyridopyrrolopyrimidine nucleosides with the specific position of N atom on pyridine ring are suitable substrates for RNA polymerases and are able to substitute for ATP during transcription and to be incorporated into RNA.

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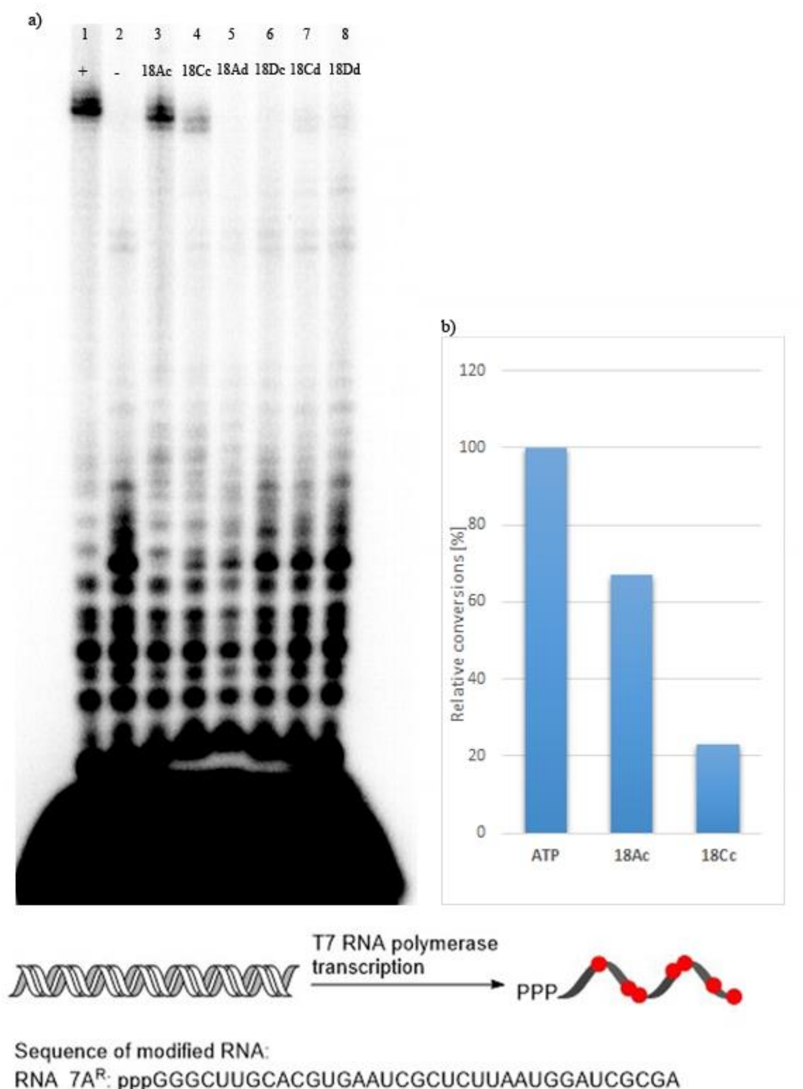

Figure 3. (a) In vitro transcription with amino and methyl triphosphates **18Ac**, **18Ad**, **18Cc**, **18Cd**, **18Dc** and **18Dd** from **A**, **C** and **D** series. Lanes 1, +: product of PAGE with natural ATP; lane 2, –: product of PAGE with H_2O; lane 3: **18Ac** incorporated; lane 4: **18Cc** incorporated; lane 5-9: **18Ad**, **18Dc**, **18Cd**, **18Dd** not incorporated; (b) Quantifications of the relative conversions (compared to transcription with natural ATP) of T7 RNA polymerase transcription experiments.

In vitro pharmacology of nucleoside analogues

Absorption, distribution, metabolism, and excretion (ADME) *in vitro* properties of a chemical compound are critical factors in predicting its pharmacokinetic behavior and drug-drug interaction potential *in vivo*. Therefore we have also studied ADME properties of the most active derivatives under *in vitro* conditions. Tested compounds showed high chemical stability in phosphate-buffered saline (PBS pH 7.4) after 120 minutes at 37 °C. Also, all nucleosides

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were found to be stable in plasma (all compounds showed more than 75% presence in plasma after 120 min). For microsomal stability, all compounds achieved the similar results (all compounds showed more than 73% presence in microsomes after 60 min) indicating low or medium clearance in human microsomal fraction. Correspondingly to chemical structure, more hydrophobic substitutions resulted in higher proportion of the compound bound to human serum proteins. For instance, it was higher for methoxy **12Ca** and methylsulfanyl **12Cb** derivative 78 and 81%, while amino derivative **12Cc** was bound only from 27%. All seven nucleosides showed moderate (– log Papp 5–6 cm/s) or low ability (– log Papp > 6 cm/s) to diffuse across an artificial cellular membrane in the Parallel artificial membrane permeability assay (PAMPA), suggesting alternative intracellular transport mechanism, probably involving nucleoside transporters.[34] The Caco-2 and MDCK-MDR1 permeability assays are established models of intestinal[35] and blood-brain barriers, respectively.[36] Studied compounds showed low ($Papp_{AB} < 5 \times 10^{-6}$ cm/s) or moderate ($Papp_{AB}$ 5–20 × 10^{-6} cm/s) probability of intestinal absorption and to cross blood-brain barriers PappAB < 10 × 10–6 cm/s CNS –; PappAB > 10 × 10–6 cm/s CNS +). Tested compounds are actively exported from the cells in both barrier models as indicated by efflux ratios > 2. Most probably, the test compounds are exported by MDR1 efflux pump present in both cell types, which is in good agreement with diminished cytotoxicity in cancer cells expressing MDR1 gene (Table 4). Results from all in vitro pharmacology testing are summarized in Table 8.

Table 8. Stability and PAMPA for Selected Cytotoxic Nucleosides

Metabolism in vitro	Time (min)	**12Ac**	**12Ad**	**12Ca**	**12Cb**	**12Cc**	**12Cd**	**12Dc**
		Compound remaining (%)						
Chemical stability	0	100	100	100	100	100	100	100
	15	98	100	100	100	100	100	100
	30	98	92	100	100	100	100	96
	60	95	92	100	96	94	100	94
	120	93	90	98	90	94	100	88
Plasma stability	0	100	100	100	100	100	100	100
	15	96	94	90	92	96	97	90
	30	89	96	89	91	86	90	90
	60	92	94	93	90	87	91	84
	120	89	93	88	88	75	88	83
Microsomal stability	0	100	100	100	100	100	100	100
	15	94	103	109	86	96	93	100
	30	90	93	93	87	86	89	97
	60	90	91	86	73	87	84	96

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Clearance		low	low	low	medium	low	low	low
Plasma protein binding	Fraction bound (%)							
		44	40	78	81	27	37	32
Permeability in vitro	PAMPA log Pe							
		–7.61	–6.63	–6.07	–5.67	–6.95	–7.17	–7.60
Category		low	low	low	medium	low	low	low
Permeability in vivo	MDR1-MDCK							
	CNS (–/+)	–	–	–	–	+	–	–
	Papp (×10e–6)	7.23	1.46	9.73	9.15	10.2	1.22	1.52
	Efflux ratio	2.22	14.2	3.11	3.35	1.07	7.70	9.11
	active efflux	yes	yes	yes	yes	no	yes	yes
	% recovery	80.1	59.6	78.3	100	88.4	88.4	91.2
	Caco-2							
	Category	low	low	mod.	low	mod.	mod.	low
	Papp (×10e–6)	0.90	2.67	5.68	0.84	10.9	12.3	1.42
	Efflux ratio	18.5	16.7	11.4	28.5	1.72	2.70	15.4
	active efflux	yes	yes	yes	yes	no	yes	yes
	% recovery	52.7	55.2	78.8	89.3	87.4	100.9	50

Induction of DNA damage in nucleoside analogues treated cells

To investigate potential involvement of the most active compounds **12Cc** and **12Cd** versus non-cytotoxic derivative **12Dc** in DNA repair machinery, the U2OS human osteosarcoma cell line was stably transfected with the 53BP1-GFP fusion gene as reported previously.[14] 53BP1 protein accumulates at DNA lesions, preferably at DNA double strand breaks, thus its GFP-conjugate enables visualization of DNA-damage sites in nucleus. Indeed, U2OS-53BP1-GFP cells treated with control DNA-damaging agent etoposide (topoisomerase 2α inhibitor) showed time-dependent accumulation of 53BP1 foci in the nuclei of treated cells (Figure 4). Out of three tested nucleosides, only **12Cc** induced a minor DNA damage with peak values 15 hours post exposure. Cytotoxic compound **12Cd** and also non-cytotoxic derivative **12Dc** showed no significant interference with formation of 53BP1 foci and thus indirectly with induction and/or repair of double-strand DNA breaks. Although the number of 53BP1 foci per nucleus in **12Cc**-treated cells (Figure 3a) was lower than in etoposide-exposed cells, the foci were larger and resembled those reported previously for 7-(2-thienyl)-7-deazaadenosine (AB-61)[14]. This corresponds well with preferential incorporation of compounds into DNA in the form of

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ribonucleosides. The presence of the modified ribonucleotides in the DNA causes ds-breaks and apoptosis.

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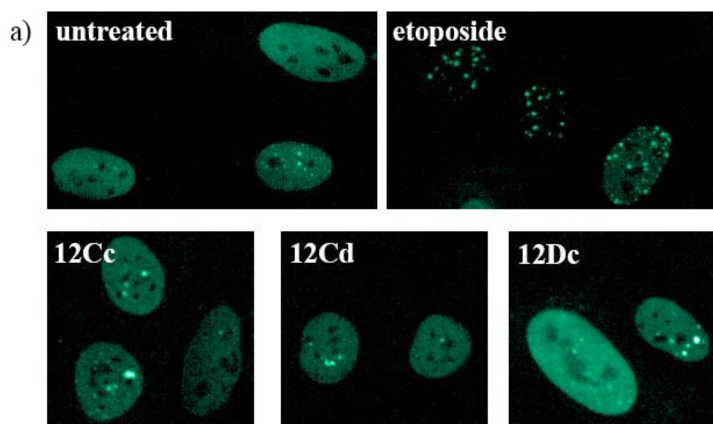
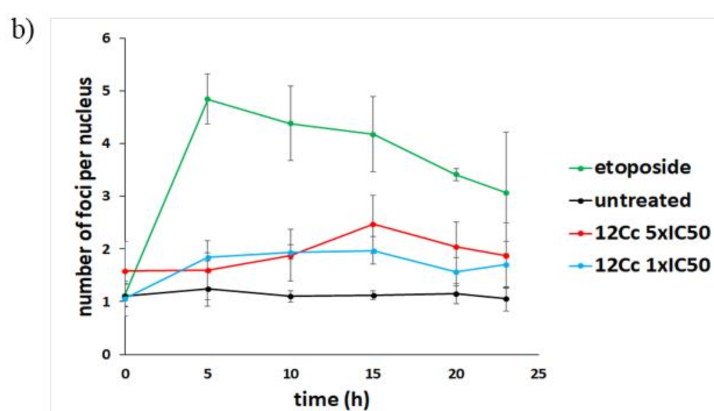
a)





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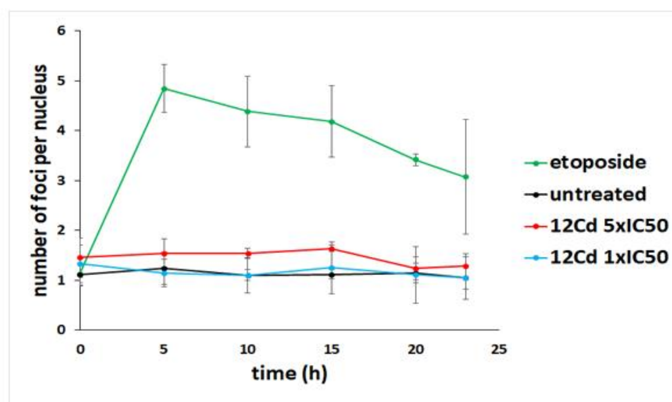

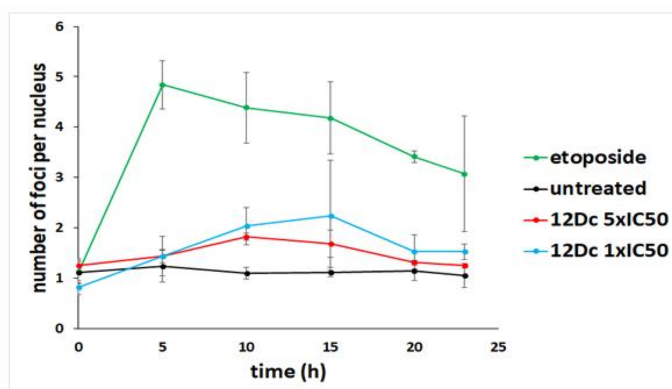

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Figure 4. 53BP1 foci in U2OS-53BP1-GFP cells exposed to a) vehicle, etoposide, cytotoxic nucleosides **12Cc**, **12Cd** and non-cytotoxic analogue **12Dc**. b) Time-dependence of 53BP1 foci formation during treatment with vehicle, etoposide and the compounds. All nucleosides were tested at 1 and 5 × IC_{50} concentrations, etoposide at 1 × IC_{50}.

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Conclusions

We have designed and synthesized all-four isomeric series of novel 4-substituted pyrido-fused 7-deazapurine ribonucleosides possessing pyridine nitrogen at different positions. The multistep synthesis of each isomeric heterocycle **9A-D** was followed by glycosylation and functionalization at position 4 through cross-coupling reactions or nucleophilic substitutions. All the new nucleosides were tested for antiviral and cytotoxic activities. The antiviral activity screening against a panel or RNA viruses revealed that the compounds were mostly inactive with few exceptions of compounds active against HCV. On the other hand, several derivatives of pyrido[4',3':4,5]pyrimidine nucleosides bearing MeO, NH_2, MeS or CH_3 groups at position 4 **12Ca**, **12Cb**, **12Cc** and **12Cd** exerted submicromolar cytotoxic effects and good selectivity toward cancer cells. Also several derivatives from the **A** and **D** series showed submicro- or micromolar cytotoxic effects. The mechanism of action involves activation by phosphorylation through adenosine kinase and then presumably other kinases to form NTPs and its incorporation to DNA and partially also to RNA. This activation is somewhat less selective compared to non-fused 7-deazapurine ribonucleosides (e.g. AB-61)[14] but still the phosphorylation in the cancer cells is more efficient than in non-proliferating cells giving the compounds reasonable selectivity and potential therapeutic window. Similarly to AB-61[14] and previously reported pyrrolo- or furo-fused deazapurine ribonucleosides,[18] the presence of the modified ribonucleotides in DNA causes genomic instability and double-strand breaks resulting in apoptosis. Since the corresponding benzo-fused (pydimidoindole) ribonucleosides did not exert any cytotoxic activity,[15] it is interesting to point out that the introduction of a nitrogen heteroatom in the right position of the fused benzene ring can restore the activity. On the other hand, since the mechanism involves activation by three consecutive kinases and polymerase incorporation into DNA, it is very difficult to determine at which step these minute structural changes play the crucial role. The most active and selective compounds **12Ca**, **12Cb**, **12Cc** and **12Cd** have a potential for further preclinical development as cytostatics.

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Experimental

Complete experimental part, characterization data for all compounds and methods for biological profiling are given in the Supporting Information. Only the most important synthetic procedures are given below.

General procedure A. Reduction with zinc dust and spontaneous cyclization

A solution of **6A**, **6B**, **6C** or **6D** (18.3 mmol) in AcOH (50 mL) was heated to 95 °C under argon. Zinc dust (91.5 mmol) was added, and then the reaction mixture was heated at 95 °C for 75 min or 90 min. Upon cooling, the insoluble material was filtered off through a pad of Celite and washed with fresh AcOH. The filtrate was concentrated and the residue was treated with saturated solution of $NaHCO_3$ to give a light brown solid. This was filtered, washed with water, and dried.

General procedure B. Cyclocondensation with formamide

A mixture of **7A, 7B, 7C** or **7D** (15.6 mmol) and ammonium formate (1.1 g, 17.5 mmol) in formamide (25 mL, 624 mmol) was heated at 170 °C for 12 h. Next day, 1 M HCl was added to the cooled reaction mixture, and the resulting suspension was filtered to remove insolubles. The filtrate was then adjusted to pH 7 with saturated solution of $NaHCO_3$. The resulting precipitate was collected by filtration, washed with water and dried.

General procedure C. Chlorination

A chloropyrimidine nucleobase was prepared according to a literature procedure.[13] $POCl_3$ (0.2 mL, 2.4 mmol) was added to a stirred solution of **8A, 8B, 8C** or **8D** (0.4 mmol), benzyltriethylammonium chloride (196 mg, 0.8 mmol) and *N,N*-dimethylaniline (62 μL, 0.5 mmol) in MeCN (1 mL), and stirring continued for 1 h at 90 °C.

General procedure D. Vorbrüggen glycosylation

A solution of tricyclic nucleobase **9A**, **9B**, **9C** or **9D** (1.1 mmol) in MeCN (20 mL), was treated with BSA (1.1 mmol) . The reaction mixture was heated at 60 °C for 30 min and then treated with TMSOTf (2.2 mmol) and 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-*β*-D-ribofuranose (2.2 mmol). The mixture was heated to 60 °C for 16 h. After that, the mixture was cooled and then extracted with DCM. Organic fraction was washed with saturated solution of $NaHCO_3$, water, dried over Na_2SO_4 and evaporated under reduced pressure.

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General procedure E. Amination

To a solution of protected 4-chloropyridopyrrolopyrimidine nucleoside **10A, 10B, 10C** or **10D** (0.72 mmol) in a dry 1,4-dioxane (2.8 mL) 30% aq. ammonia (9 mL) was added. The mixture was heated in pressure tube at 120 °C from 12 h to 24 h.

General procedure F. Cross-coupling with $AlMe_3$

$(Me)_3Al$ (2 M in toluene, 2.52 mmol) and $Pd(PPh_3)_4$ (97 mg, 0.08 mmol) were added to the solution of protected 4-chloropyridopyrrolopyrimidine nucleoside (0.84 mmol) in THF (25 mL); then the reaction mixture was stirred at 70 °C for 16 h.

General procedure G. Stille cross-coupling reaction

Protected nucleoside (0.62 mmol), 2-(tributylstannyl)furan (292 µl, 0.93 mmol) and $PdCl_2(PPh_3)_2$ (0.06 mmol) were dissolved in anhydrous DMF (4.5 mL) and heated to 100 °C for 4 h.

General procedure H. Suzuki-Miyaura cross-coupling reaction.

Protected nucleoside (0.62 mmol), R-boronic acid (1.86 mmol), K_2CO_3 (1.24 mmol) and $Pd(PPh_3)_4$ (0.03 mmol) were dissolved in toluene (13.5 mL) and heated to 100 °C from 3–24 h. Then, the reaction mixture was diluted with saturated solution of $NaHCO_3$ and extracted with EtOAc. Organic layer was dried over Na_2SO_4 and solvent was evaporated under reduced pressure.

General procedure I. Synthesis of nucleoside monophosphates

Free nucleoside (0.13 mmol) was dried in vacuo at 75 °C for 1 h (or at rt overnight) and then suspended in trimethyl phosphate (0.6 mL). The suspension was cooled to 0 °C and phosphorus oxychloride (16.1 µl, 0.17 mmol) was added dropwise to a stirred mixture and the solution was stirred at 0 °C for 3 h. Then the reaction mixture was treated with aq. TEAB (2 M, 0.7 mL). Volatiles were removed *in vacuo* and the rest was several times co-evaporated with water. The residue was purified by HPLC (0.1 M TEAB/MeOH 0 → 100%) and obtained triethylammonium salt of product was converted to sodium salt by passing through column of Dowex 50 (Na^+ form).

General procedure J. Synthesis of nucleoside triphosphates

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Free nucleoside (0.25 mmol) was dried in vacuo at 75 °C for 1 h (or at rt overnight) and then suspended in trimethyl phosphate (0.6 mL). The suspension was cooled to 0 °C, phosphorus oxychloride (30 µl, 0.32 mmol) was added dropwise to a stirred mixture and the solution was stirred at 0 °C for 3 h. A freshly prepared solution of bis(tri-*n*-butylammonium) pyrophosphate (604 mg, 1.1 mmol) and tri-*n*-butylamine (261 µl, 1 mmol) in dry DMF (2.3 mL) or dry MeCN was stirred at 0 °C for 30 min and then added to the stirred reaction mixture at 0 °C. The mixture was left at 0 °C for 1 h and treated with aq TEAB (2M, 2 mL). Volatiles were removed *in vacuo* and the rest was several times co-evaporated with water. The residue was purified by HPLC (0.1 M TEAB/MeOH 0 → 100%) and obtained triethylammonium salt of product was converted to sodium salt by passing through column of Dowex 50 (Na^+ form).

Statement of conflict of interest:

L. V., P. D., M. Ha. and M. Ho. are co-inventors of a patent application covering some compounds presented in this paper.

Acknowledgement.

This work was supported by the Academy of Sciences of the Czech Republic (RVO 61388963 and the Praemium Academiae award to M. Hocek), by the Czech Science Foundation (19-08124S to L. V., P. D., M. Ho.), Czech Health Research Council (15-31984A to M. T.), by Gilead Sciences, Inc. and by the Czech Ministry of Education, Youth and Sports (projects EATRIS-CZ No. LM2015064 and ENOCH No. CZ.02.1.01/0.0/0.0/16_019/0000868 to M. Ha.). The authors thank Drs. Joy Feng and Gina Bahador (Gilead Sciences, Inc.) for anti-HCV testing and Dr. Iva Pichová (IOCB) for cloning and expression of ADK.

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