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Short communication

Dideoxy fluoro-ketopyranosyl nucleosides as potent antiviral agents: Synthesis and biological evaluation of 2,3- and 3,4-dideoxy-3-fluoro-4- and -2-keto-β-D-glucopyranosyl derivatives of N^4-benzoyl cytosine

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ABSTRACT

The synthesis of the dideoxy fluoro ketopyranonucleoside analogues, 1-(2,3-dideoxy-3-fluoro-6-*O*-trityl-β-D-glycero-hexopyranosyl-4-ulose)-N^4-benzoyl cytosine (**7a**), 1-(3,4-dideoxy-3-fluoro-6-*O*-trityl-β-D-glycero-hexopyranosyl-2-ulose)-N^4-benzoyl cytosine (**13a**) and their detritylated analogues **8a** and **14a**, respectively, is described. Condensation of peracetylated 3-deoxy-3-fluoro-D-glucopyranose (**1**) with silylated N^4-benzoyl cytosine, followed by selective deprotection and isopropylidenation afforded compound **2**. Routine deoxygenation at position 2′, followed by a deprotection-selective reprotection sequence afforded the partially tritylated dideoxy nucleoside of cytosine **6**, which upon oxidation of the free hydroxyl group at the 4′-position, furnished the desired tritylated 2,3-dideoxy-3-fluoro ketonucleoside **7a** in equilibrium with its hydrated form **7b**. Compound **2** was the starting material for the synthesis of the dideoxy fluoro ketopyranonucleoside **13a**. Similarly, several subsequent protection and deprotection steps as well as routine deoxygenation at position 4′, followed by oxidation of the free hydroxyl group at the 2′-position of the partially tritylated dideoxy nucleoside **12**, yielded the desired carbonyl compound **13a** in equilibrium with its hydrated form **13b**. Finally, trityl removal from **7a**/**b** and **13a**/**b** provided the unprotected 2,3-dideoxy-3-fluoro-4-keto and 3,4-dideoxy-3-fluoro-2-ketopyranonucleoside analogues **8a** and **14a**, in equilibrium with their gem-diol forms **8b** and **14b**. None of the compounds showed inhibitory activity against a wide variety of DNA and RNA viruses at subtoxic concentrations, except **7a**/**b** that was highly efficient against rotavirus infection. Nucleoside **7a**/**b** also exhibited cytostatic activity against cells of various cancers. BrdU-cell cycle analysis revealed that the mechanism of cytostatic activity may be related to a delay in G1/S phase and initiation of programmed cell death.

1. Introduction

Nucleosides are structural modules of nucleic acids and therefore of fundamental importance in all living systems [1,2]. They have been playing a major role in treating tumor and virus either as selective inhibitors of certain obligatory enzymes for cancer or viral replication [3], or as nucleic acid chain terminators, which interrupt the replication of cancer cells or a virus [4–6]. Pyrimidine nucleosides have attracted increased attention as a result of their important physiological and biogenetic properties. Among them, nucleosides bearing cytosine as a base have been developed as potent antitumor agents, which are effective not only on leukemias and lymphomas, but also on a wide variety of solid tumors *in vitro* as well as *in vivo* [7–13].

The last decades, nucleosides with a six-membered carbohydrate moiety have been evaluated for their potential antiviral [14–18], antibiotic [19] and antioxidant [20] properties and as building blocks in nucleic acid synthesis [21,22]. A series of six-membered nucleoside analogues, the ketonucleosides, proved to be key intermediates in synthetic and biosynthetic processes and exhibited interesting antitumor and antiviral properties [23–27]. These nucleosides are known to inhibit DNA, RNA, and protein

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synthesis [28], to interact with sulfhydryl groups of cellular proteins and enzymes [29], while they were found to be highly cytotoxic *in vitro* [24,30,31] and to exert powerful inhibitory action against L1210 leukemia *in vivo* [32,33].

In view of these facts and taking into account that introduction of fluorine into the sugar moiety of nucleosides enhances activity [34–39], we have previously synthesized various unsaturated fluoro ketopyranonucleoside analogues, which proved to be efficient as tumor growth inhibitors and showed to have a promising potential in combating the rotaviral infections [40,41].

Thus, in continuation of our studies on the synthesis of sugar-modified pyranonucleosides and based on the interesting biological properties of deoxyhexopyranose nucleosides [14–16,42–45], we decided to design and synthesize a new class of fluoro ketopyranonucleoside analogues, the fluorinated 2,3-dideoxy-4-keto and 3,4-dideoxy-2-keto nucleosides of N^4-benzoyl cytosine, in order to assess their biological activity. Chemical synthesis and biological activity of these compounds are presented herein.

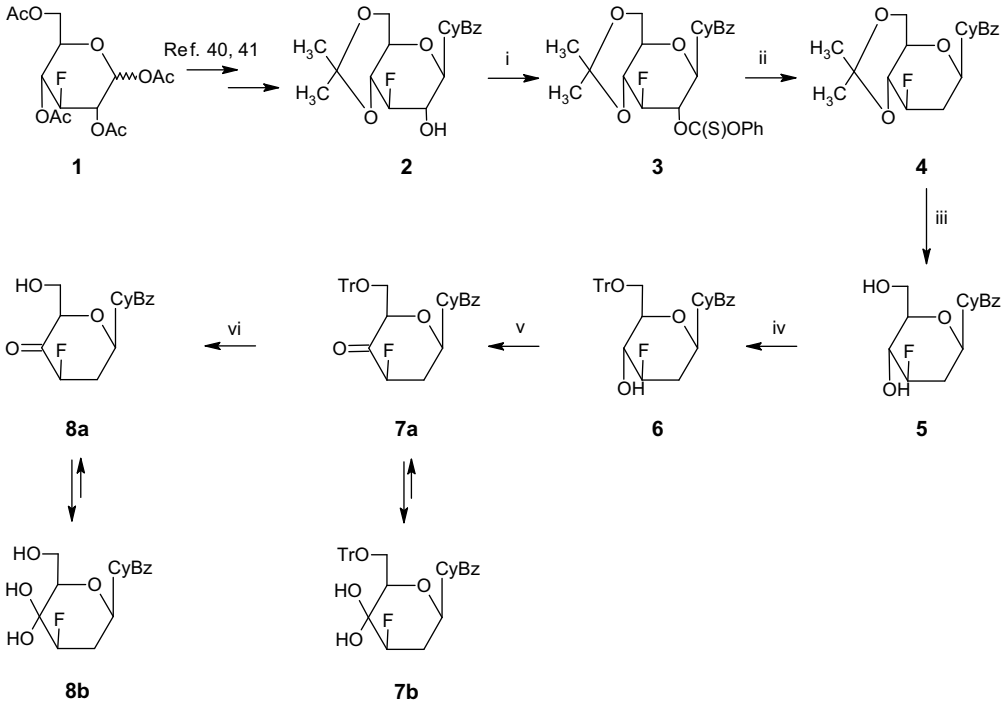
2. Results and discussion

2.1. Chemistry

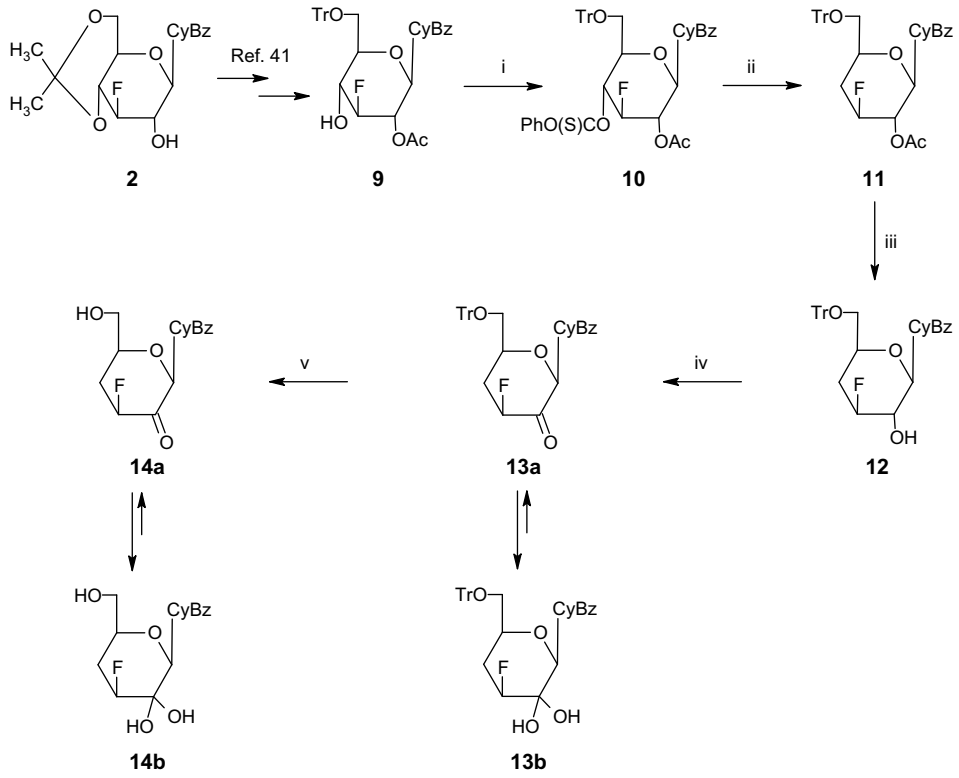
Two different synthetic routes were investigated for the synthesis of the title compounds, 1-(2,3-dideoxy-3-fluoro-β-D-glycero-hexopyranosyl-4-ulose)-N^4-benzoyl cytosine (**8a**) and 1-(3,4-dideoxy-3-fluoro-β-D-glycero-hexopyranosyl-2-ulose)-N^4-benzoyl cytosine (**14a**). In the first reaction scheme, condensation of 3-deoxy-3-fluoro-1,2,4,6-tetra-*O*-acetyl-glucopyranose (**1**) [46,47] (Scheme 1) with silylated N^4-benzoyl cytosine followed by selective deprotection and specific acetalation using 2,2-dimethoxypropane in *N*,*N*-dimethylformamide (DMF), gave the 4′,6′-isopropylidene derivative **2** [40,41].

Phenoxythiocarbonylation of **2** under a commonly used condition, phenyl chlorothionoformate (PhOC(S)Cl) and an excess amount of 4-dimethylaminopyridine (DMAP) in acetonitrile [48–50], afforded the 2′-*O*-phenoxythiocarbonyl derivative **3**, in 70% yield. The deoxygenation of compound **3** was carried out with tri-*n*-butyltin hydride (Bu_3SnH) to afford the 2′-deoxynucleoside **4**, in 60% yield [50,51]. The 4′,6′-*O*-isopropylidene group of **4** was removed upon treatment with aqueous trifluoroacetic acid (TFA) and selective protection of the primary 6′-hydroxyl group with a trityl group yielded the partially protected derivative **6**, in 65% yield. Oxidation of the fluoro tritylated dideoxy precursor **6** was performed with pyridinium dichromate (PDC)/acetic anhydride (Ac_2O) and afforded 4′-keto form, 1-(2,3-dideoxy-3-fluoro-6-*O*-trityl-β-D-glycero-hexopyranosyl-4-ulose)-N^4-benzoyl cytosine (**7a**), in equilibrium with its hydrated analogue **7b** in a 3:7 ratio, as it was clearly determined by integration of the related ^{1}H NMR signals. It appears that the presence of the electron-withdrawing fluorine atom α to the carbonyl, causes the easy hydration and gem-diol formation [52]. Finally, detritylation of **7a**/**b** in CH_2Cl_2/formic acid, 1:1, led to the corresponding dideoxy carbonyl compound, 1-(2,3-dideoxy-3-fluoro-β-D-glycero-hexopyranosyl-4-ulose)-N^4-benzoyl cytosine (**8a**) in equilibrium with its gem-diol **8b**.

Compound **2** [40,41] was the starting material for the synthesis of 1-(3,4-dideoxy-3-fluoro-β-D-glycero-hexopyranosyl-2-ulose)-N^4-benzoyl cytosine (**14a**) (Scheme 2). Acetylation of the nucleoside **2**, followed by deisopropylidenation and finally by selective tritylation of the primary hydroxyl group gave compound **9** [41], which was then converted to the 4′-deoxy nucleoside analogue **11** *via* 4′-*O*-phenoxythiocarbonyl derivative **10** by the similar methodology as described above. Deacetylation of **11** was performed using NaOH/ethanol (EtOH)/pyridine [53] to afford compound **12**, in good yield (88%). Oxidation of the free hydroxyl group in 2′-position of the sugar moiety of nucleoside **12** with PDC/Ac_2O afforded the desired 2′-keto form, 1-(3,4-dideoxy-3-fluoro-6-*O*-trityl-β-D-glycero-hexopyranosyl-2-ulose)-N^4-benzoyl cytosine (**13a**) in equilibrium with its hydrated analogue **13b** in a 6:4 ratio, clearly determined by integration of the related ^{1}H



Scheme 1. Reagents and conditions: (i) PhOC(S)Cl, DMAP, CH_3CN, 20 °C, 90 min; (ii) Bu_3SnH, 1,1-azobis(cyclohexane carbonitrile), toluene, 80 °C, 3 h; (iii) TFA, THF/H_2O, 20 °C, 90 min; (iv) pyridine, triphenylmethyl chloride, DMAP, 100 °C, 1 h; (v) PDC, Ac_2O, CH_2Cl_2, 55 °C, 90 min; (vi) formic acid/CH_2Cl_2 (1:1), 20 °C, 20 min.



Scheme 2. Reagents and conditions: (i) PhOC(S)Cl, DMAP, CH_3CN, 20 °C, 90 min; (ii) Bu_3SnH, 1,1-azobis(cyclohexane carbonitrile), toluene, 80 °C, 3 h; (iii) EtOH, pyridine, NaOH, 0 °C, 1 h, Amberlite IR-120 (H^+) resin; (iv) PDC, Ac_2O, CH_2Cl_2, 55 °C, 90 min; (v) formic acid/CH_2Cl_2 (1:1), 20 °C, 20 min.

NMR signals. Finally, deprotection of the primary hydroxyl group of **13a**/**b** was performed using CH_2Cl_2/formic acid, 1:1, and furnished ketone **14a** in equilibrium with its gem-diol **14b**. It should be mentioned that all attempts to remove the benzoyl group of the target nucleoside analogues, were unsuccessful and only degradation products were obtained [23,54,55].

All compounds were well characterized by NMR and UV spectroscopies, mass spectrometry and elemental analysis. The ^{1}H NMR spectrum of **7a**/**b** showed a doublet at 6.35 ppm ($J_{1',2'} = 10.1$ Hz) and a doublet of doublet of doublets at 5.22 ppm ($J_{3',F} = 47.0$ Hz, $J_{2'a,3'} = J_{2'b,3'} = 6.9$ Hz), which are assigned to H-1′ and H-3′ protons, respectively, of keto compound **7a**, as well as a doublet at 5.94 ppm ($J_{1',2'} = 10.6$ Hz) and a doublet of doublet of doublets at 4.72 ppm ($J_{3',F} = 48.1$ Hz, $J_{2'a,3'} = 5.0$ Hz, $J_{2'b,3'} = 5.1$ Hz), assigned to H-1′ and H-3′ protons, respectively, of gem-diol **7b**. Similarly, in the ^{1}H NMR spectrum of **13a**/**b**, H-1′ signals of keto **13a** and gem-diol form **13b** appeared as two sharp singlets at 6.41 and 5.86 ppm, respectively and H-3′ signals of **13a** and **13b** appeared as two doublet of doublet of doublets at 5.41 ppm ($J_{3',F} = 47.5$ Hz, $J_{3',4'a} = J_{3',4'b} = 7.4$ Hz) and 4.82 ppm ($J_{3',F} = 48.3$ Hz, $J_{3',4'a} = 5.5$ Hz, $J_{3',4'b} = 5.3$ Hz), respectively. Thus, in both cases, ^{1}H NMR spectra confirmed the equilibrium between keto and gem-diol forms. However, ^{1}H NMR spectra of **8a**/**b** as well as **14a**/**b** in methanol-d_4 (CD_3OD) solution, showed a doublet at 5.94 ppm ($J_{1',2'} = 11.0$ Hz) assigned to H-1′ proton for **8b** and a sharp singlet at 5.85 ppm, assigned to H-1′ proton for **14b**, while H-3′ signals appeared as a doublet of doublet of doublets, for both compounds, at 4.72 ppm ($J_{3',F} = 48.4$ Hz, $J_{2'a,3'} = 4.8$ Hz, $J_{2'b,3'} = 4.9$ Hz) and 4.94 ppm ($J_{3',F} = 48.5$ Hz, $J_{3',4'a} = 5.5$ Hz, $J_{3',4'b} = 5.6$ Hz), respectively, indicating the presence of gem-diol forms exclusively. Finally, the target compounds **7a**/**b**, **8a**/**b** and **13a**/**b**, **14a**/**b** were characterized by the absence of the H-4′ proton and H-2′ proton respectively, in their ^{1}H NMR spectra.

2.2. Antiviral activity

Compounds **7a**/**b**, **8a**/**b**, **13a**/**b** and **14a**/**b** have been evaluated against a broad variety of DNA and RNA viruses, including herpes simplex virus type 1 [HSV-1 (KOS)], HSV-2 (G), vaccinia virus and vesicular stomatitis virus (VSV) in HEL cell cultures; VSV, Coxsackie virus B4 and respiratory syncytial virus (RSV) in HeLa cell cultures; parainfluenza-3 virus, reovirus-1, Sindbis virus, Coxsackie virus b4 and Punta Toro virus in Vero cell cultures; influenza virus A (H1N1, H3N2) and influenza virus B in MDCK cell cultures, feline corona virus (FIPV) and feline herpes virus in CRFK cell cultures and human immunodeficiency virus (HIV-1(III_B)) and HIV-2(ROD) in CEM cell cultures. None of the compounds showed inhibitory activity against any of these viruses at subtoxic concentrations [i.e. MCC (minimal cytotoxic concentration): 100, >100, 20 and >100 μM for **7a**/**b**, **8a**/**b**, **13a**/**b** and **14a**/**b** in HEL, HeLa and Vero cell cultures]; CC_{50} (50%-cytostatic concentration): 11, 40, 0.6 and 45 μM in MDCK cell cultures and 9.9, >100, 2.3 and >100 μM in CRFK cell cultures].

The results of the antiviral assays against rotavirus on the newly synthesized dideoxy fluoro-pyranonucleoside analogues **7a**/**b**, **13a**/**b** and their detritylated analogues **8a**/**b** and **14a**/**b**, respectively, are summarized and compared with AZT in Table 1.

In the neutralization assay, **8a**/**b** was found to neutralize rotavirus before its attachment to Caco-2 cells at the concentration of 2.1 μM (*vide* IC_{50}) as compared to AZT (75 μM). Similar results were observed for **7a**/**b** in inhibiting infectivity, following virus attachment (Table 1); analogue **7a**/**b** was actually more potent than its congener **8a**/**b** as it produced the same effect at a much lower concentration (0.63 μM) (treatment B). Interestingly, both **7a**/**b** and its detritylated analogue **8a**/**b**, although active in the same antiviral assay, seem to exert their action by a different mechanism.

Table 1
Antiviral activity of compounds **7a**/**b**, **8a**/**b**, **13a**/**b**, **14a**/**b** and AZT against rotavirus RF strain on Caco-2 cells (IC_{50}). CC_{50}/IC_{50} ratios were calculated from CC_{50} values given in Table 2.

Compound	Treatment A[a]			Treatment B[a]		
	IC_{50}		CC_{50}/IC_{50}[b]	IC_{50}		CC_{50}/IC_{50}[b]
	mg/mL	μM		mg/mL	μM	
7a/**b**	n.e.	n.e.	–	0.0004	0.63	32
8a/**b**	0.0008	2.1	320	n.e.	n.e.	–
13a/**b**	n.e.	n.e.	–	n.e.	n.e.	–
14a/**b**	n.e.	n.e.	–	n.e.	n.e.	–
AZT	0.02	75	0.75[c]	0.006	22	2.5[c]

n.e. = no effect.
[a] Treatment A: neutralization of the virus in the solution before its attachment. Treatment B: inhibition of infectivity following virus attachment.
[b] CC_{50}/IC_{50} values were calculated using CC_{50} values in Table 2.
[c] CC_{50} value for AZT on Caco-2 cells = 56.1 μM.

In contrast to compounds **7a**/**b** and **8a**/**b**, analogues **13a**/**b** and **14a**/**b** did not show any antiviral potency. We speculate that the position of the keto group in the ring skeletons of compounds **7a**/**b** and **8a**/**b** is critical in inhibiting the rotavirus replication in the cell as well as for neutralizing rotavirus before its attachment. According to the antiviral assay used, it can be suggested that the tested compounds **7a**/**b** and **8a**/**b** showed specific activity against rotavirus, and were capable of inhibiting the rotavirus infection in cells at significantly lower concentrations than AZT.

Compared to the recently reported unsaturated fluoro ketopyranonucleoside analogues [40,41], the dideoxy fluoro ketopyranonucleosides presented herein, were found to be more potent and thus more suitable candidates in anti-rotavirus therapy. Thus, it can be suggested that the incorporation of the carbon–carbon double bond into the ring system of the dideoxy fluoro ketopyranonucleosides has a marked reducing effect on their antiviral activity. It seems that this structural feature of the carbohydrate moiety influences the ability of the nucleoside analogue to participate in the antiviral mechanism.

2.3. Cytotoxic and growth inhibition activity

The cytotoxic potential of compounds **7a**/**b**, **8a**/**b**, **13a**/**b** and **14a**/**b** was studied on H4 normal human intestinal cells and HEL human lung fibroblast cells, and on a series of various other human tumor cells, such as human colonic adenocarcinoma derived Caco-2 cells, gastric cancer derived AGS cells, and epithelial breast cancer derived MCF-7 cells, Vero African green monkey cells and HeLa human cervix carcinoma cells. The growth inhibition of Caco-2 cells, caused by the new compounds, was measured by determining the 50% growth inhibitory concentration (IC_{50}). The results are summarized in Table 2 and compared with the values obtained for 5-fluorouracil (5FU).

Table 2
Cytotoxic effect (CC_{50}, μM) of compounds **7a**/**b**, **8a**/**b**, **13a**/**b**, **14a**/**b** and 5-fluorouracil (5FU) on Caco-2, H4, AGS and MCF-7 cells, and growth inhibition (IC_{50}, μM) on Caco-2 cells.

Compound	Cytotoxic effect (CC_{50}, μM)				TSI*			Growth inhibition (IC_{50}, μM)
	H4	Caco-2	AGS	MCF-7	Caco-2	AGS	MCF-7	Caco-2
7a/**b**	100	20	10	40	5.0	10.0	2.5	5.1
8a/**b**	659	659	132	659	1.0	5.0	1.0	237
13a/**b**	50	20	10	20	2.5	5.0	2.5	5.1
14a/**b**	659	264	132	264	2.5	5.0	2.5	33
5FU	3844	390	769	641	9.8	5.0	6.0	1.5

*TSI: Tumor selectivity index (CC_{50} on H4 cells/CC_{50} on the specified host cells).

All of the tested compounds were cytotoxic on malignant cells of different origin and had a substantial tumor selectivity index (Table 2).

From the tested dideoxy fluoro-pyranonucleoside analogues, compounds **7a**/**b** and **13a**/**b** exhibited a noteworthy cytotoxicity, with good selectivity indices, particularly on AGS cells.

In comparison to the control compound, 5FU, compounds **7a**/**b**, **13a**/**b** and **14a**/**b** were more effective on MCF-7, AGS and Caco-2 cells; compound **8a**/**b** was more effective than 5FU only on AGS cells.

From the results obtained, it is clear that some of the new compounds show selectivity toward specific tumor cell lines. This is not unprecedented as there are examples in the literature, which demonstrate that the mode of inhibitory action especially on the target enzymes in carcinogenic cells is not always similar even among nucleoside antimetabolites which have the same nucleoside base [56].

In the growth inhibition assay compounds **7a**/**b** and **13a**/**b** were capable of inhibiting the growth of malignant cells in a range comparable to 5FU (IC_{50}, Table 2). In spite of the fact that differences were observed among the tested compounds in the growth inhibition activity in colon carcinoma cells, all analogues were strong inhibitors of malignant cell growth of other cancer types.

Conversely to the previously reported unsaturated fluoro ketopyranonucleoside analogues [40,41], the new dideoxy fluoro-pyranonucleosides, especially compounds **7a**/**b** and **13a**/**b**, showed satisfactory, but similar, cytotoxicity against Caco-2 cells and MCF-7 cells. It appears that the existence of a carbon–carbon double bond in the sugar residue does not seem to be a structural prerequisite for cytotoxic and growth inhibition activity. On the contrary, the presence of the keto-fluoro system is indispensable.

2.4. BrdU-cell cycle analysis

Many mechanisms have been implicated in 5FU resistance and/or sensitivity, such as pharmacokinetic resistance, decreased accumulation of activated metabolites, and altered effects on thymidylate synthase (TS) [57,58]. Because fluoropyrimidine treatment leads to cell cycle arrest [59,60], other parameters, such as cell cycle distribution can also be used to predict ketonucleosides sensitivity. In order to find out, whether the mechanism of antitumor activity and/or resistance to dideoxy fluoro ketopyranonucleoside analogues was similar to the mechanism of action of 5FU, the influences on cell cycle distribution and induction of apoptosis were studied by BrdU-cell cycle analysis. Our results clearly showed that in Caco-2 cell line, 5FU equitoxic treatment resulted in an increase of the percentage of cells in G0/G1 and in % decrease of cells in the G2/M phase (Table 3, compounds **7a**/**b** and **13a**/**b**, Fig. 1). However, BrdU analysis showed that a cell cycle delay took place specifically at the G1/S interface as previously reported for 5FU (Table 3, Fig. 1) [59,60]. Exposure of cancer cells to compounds **7a**/**b**, **13a**/**b** and 5FU led to a % BrdU-positive cells or labeling index (LI) increase. However, LI modifications were only

Table 3
Cell cycle distribution of Caco-2 cells treated with compounds **7a**/**b**, **13a**/**b** and 5-fluorouracil (5FU) as compared to untreated cells.

Compound	Cell cycle distribution (%)				%Apoptotic cells (sub G1)
	G0/G1	S (LI)	G2/M	G1/S	
7a/**b**	62.0	21.2	16.0	13.8	28.4
13a/**b**	62.0	19.8	17.1	14.0	25.6
5FU	65.0	17.5	16.4	12.4	29.2
Untreated	63.1	13.7	22.3	9.9	25.6

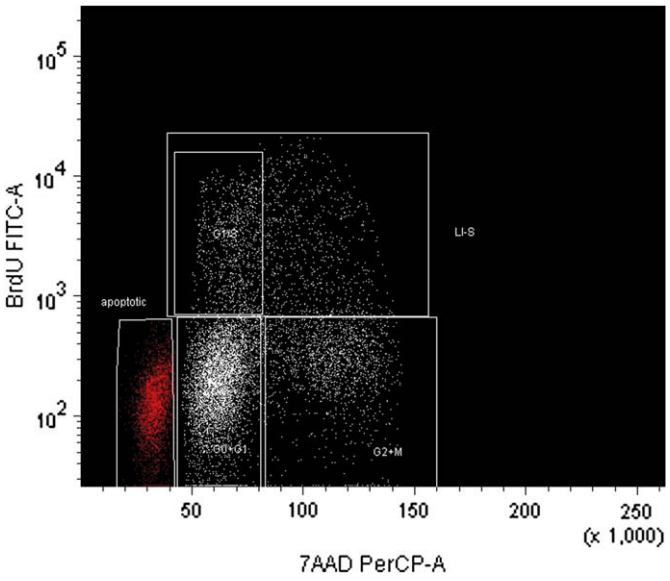


Fig. 1. Flow cytometry results of cell cycle analysis using bivariate BrdU versus 7-AAD labeling. Caco-2 cells were cultured as described in experimental procedures. Cells were treated with analyzed compounds at concentrations yielding 50% growth inhibition (IC_{50}) or left untreated. During the last hour of incubation, cells were pulsed with 10 μM BrdU solution. Samples for flow cytometry were processed using FITC-BrdU flow kit including 7-AAD labeling. Representative dot-plot of treated cells, gated according to their (FSC versus SSC) scatter properties is shown. Bivariate distributions of BrdU content (FITC) versus DNA content (7-AAD) were analyzed. Cell cycle distribution in G0/G1, S, G2/M phases and % of apoptotic (sub G1) cells was determined. The G1/S subpopulation, corresponding to BrdU-positive cells containing G1 DNA, was determined. Labeling index (LI) corresponds to S phase or percentage of BrdU-positive cells.

related to the G1/S phase accumulation, and not to a real enhanced S-phase fraction. In fact, BrdU analysis showed that G1 cells, as determined by 7-AAD analysis, were composed of BrdU-positive and BrdU-negative cell subpopulations (Fig. 1). These subpopulations cannot be distinguished using 7-AAD analysis alone.

This is viewed as a consequence of the cell fraction that was able to incorporate BrdU and maintain its G1 DNA content. This fraction was considered to be in phase G1, by 7-AAD analysis, whereas it was included in the labeled subpopulation by BrdU analysis. Thus, cells performing repair synthesis of DNA, with no net DNA increase, would be included in the LI score, but could still have a DNA content that was indistinguishable from G1 cells.

Synchronization of cells, caused by a delay in G1/S, may be related to the programmed cell death initiation. Induced delay in G1/S was representative of apoptosis involvement and of 5FU treatment sensibility. Actually, sub-G1 peak, representative of apoptotic cells was detected using flow cytometry already in untreated cells but was enhanced in cells treated with compounds **7a**/**b** and 5FU.

The apoptotic activity of the tested compounds was observed in the p53-mutated (Caco-2) cell line, suggesting that ketonucleoside-induced apoptosis is mediated by a p53-independent mechanism. This finding could have important implications in a clinical setting, since many tumors have p53 alterations and mutated p53 has been shown to be associated with a poor response to chemotherapy.

3. Conclusion

In summary, the newly synthesized fluorinated 2,3-dideoxy-4-keto and 3,4-dideoxy-2-keto nucleosides of N^4-benzoyl cytosine showed good antiviral activity toward rotavirus in very low concentrations as compared to AZT or/and previously synthesized corresponding unsaturated fluoro ketopyranonucleoside analogues [40,41]. We speculate that modification in sugar component has a very important role in this case. Furthermore, all newly synthesized compounds showed antitumor activity against tested cell lines, especially gastric cancer derived AGS cells. Compounds **7a**/**b**, **13a**/**b** and **14a**/**b** were even more cytotoxic to all cancer cell lines tested than control compound 5FU. Compounds **7a**/**b** and **13a**/**b** also showed to be as effective in their antitumor activity and in growth suppression of colon carcinoma cells as previously synthesized corresponding unsaturated fluoro ketopyranonucleoside analogues [40,41].

We have also shown, using flow cytometric BrdU-cell cycle analysis, that the mechanism of antitumor activity of the newly synthesized compounds may be related to delay in G1/S and programmed cell death initiation. Moreover, our results indicated that compound **7a**/**b** may have strong implication in clinical settings, especially for treatment of those tumors poorly responsive to the existing chemotherapy agents.

In view of these considerations, there has been increasing evidence suggesting a close correlation between the biological activity and the presence of the α-fluorocarbonyl moiety. However, the mechanism of action of the newly synthesized nucleosides is thus far unclear. It would therefore be interesting to further explore the structure–activity relationship by modifying the base part (keeping or removing the benzyl part) as well as replacing the fluorine group by different chemical entities.

4. Experimental

4.1. Chemistry

Melting points were recorded in a Mel-Temp apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed on Merck precoated $60F_{254}$ plates. Reactions were monitored by TLC on silica gel, with detection by UV light (254 nm) or by charring with sulfuric acid. Flash column chromatography was performed using silica gel (240–400 mesh, Merck). ^1H, ^{19}F and ^{13}C NMR spectra were obtained at room temperature with a Bruker 400 spectrometer at 400, 376 and 100 MHz, respectively using $CDCl_3$ and CD_3OD with internal tetramethylsilane (TMS) for ^1H and ^{13}C and internal trifluorotoluene (TFT) for ^{19}F.

The chemical shifts are expressed in parts per million (δ) and following abbreviations were used: s = singlet, br s = broad singlet, d = doublet, ddd = doublet doublet doublet, dtr = doublet triplet, dq = doublet quartet and m = multiplet. Mass spectra were obtained with a Micromass Platform LC (ESI-MS). Optical rotations were measured using Autopol I polarimeter. All reactions were carried out in dry solvents. CH_2Cl_2 was distilled from phosphorous pentoxide and stored over 4 Å molecular sieves. CH_3CN and toluene were distilled from calcium hydride and stored over 3 Å molecular sieves. Pyridine was stored over pellets of potassium hydroxide. All reactions sensitive to oxygen or moisture were carried out under nitrogen atmosphere.

4.2. Synthesis of 1-(2,3-dideoxy-3-fluoro-β-D-glycero-hexopyranosyl-4-ulose)-N^4-benzoyl cytosine (**8a**) and hydrated analogue **8b**

4.2.1. 1-(3-Deoxy-3-fluoro-4,6-O-isopropylidene-2-O-phenoxythiocarbonyl-β-D-glucopyranosyl)-N^4-benzoyl cytosine (**3**)

Compound **2** [40,41] (2.93 g, 6.99 mmol) was dissolved in anhydrous CH_3CN (46 mL). DMAP (1.71 g, 13.98 mmol) was added and the mixture was stirred for 15 min. Then, PhOC(S)Cl (1.04 mL, 7.69 mmol) was added dropwise, and the resulting solution was

stirred for 90 min at room temperature. The reaction was quenched by adding water (1.1 mL). The solvent was removed under reduced pressure, the obtained residue was dissolved in EtOAc (500 mL), washed with water (1 × 30 mL), dried over anhydrous magnesium sulfate ($MgSO_4$), evaporated under reduced pressure, and purified by flash column chromatography using ethyl acetate–*n*-hexane (4:6) as eluant to afford **3** as a yellow syrup. Yield: 2.72 g (70%); $R_f = 0.52$ in ethyl acetate–*n*-hexane (6:4); $[\alpha]_D^{22} + 76.0$ (*c* 0.5, $CHCl_3$); UV ($CHCl_3$): λ_{max} 260 nm (ε 29,207); ^{1}H NMR ($CDCl_3$): δ 8.73 (br s, 1H, NH), 7.90–7.54 (m, 7H, Bz, H-5 and H-6), 7.41–7.09 (m, 5H, C_6H_5), 6.26 (d, 1H, $J_{1',2'} = 8.0$ Hz, H-1′), 5.90 (m, 1H, H-2′), 4.95 (dtr, 1H, $J_{3',F} = 53.0$ Hz, $J_{2',3'} = J_{3',4'} = 8.8$ Hz, H-3′), 4.06 (m, 2H, H-6a′,6b′), 3.89 (m, 1H, H-4′), 3.62 (m, 1H, H-5′), 1.61 and 1.52 (2s, 6H, 2 × CH_3); Anal. Calcd for $C_{27}H_{26}FN_3O_7S$: C, 58.37; H, 4.72; N, 7.56. Found: C, 58.19; H, 4.97; N, 7.45; ESI-MS (*m*/*z*): 556.59 ($M + H^+$).

4.2.2. 1-(2,3-Dideoxy-3-fluoro-4,6-O-isopropylidene-β-D-glycero-hexopyranosyl)-N^4-benzoyl cytosine (**4**)

Compound **3** (2.72 g, 4.9 mmol) was coevaporated three times with anhydrous toluene, dissolved in toluene (106 mL), and degassed with nitrogen for 30 min. To this solution were added Bu_3SnH (3.95 mL, 14.7 mmol) and 1,1-azobis(cyclohexane carbonitrile) (0.36 g, 1.47 mmol) and the mixture was heated to 80 °C for 3 h. After cooling to room temperature, the mixture was evaporated, the residue was purified by flash column chromatography using ethyl acetate–*n*-hexane (6:4) as eluant and compound **4** was obtained as a white solid. Yield: 1.19 g (60%); $R_f = 0.26$ in ethyl acetate–*n*-hexane (6:4); m.p. 188–190 °C; $[\alpha]_D^{22} + 43.0$ (*c* 0.5, $CHCl_3$); UV ($CHCl_3$): λ_{max} 260 nm (ε 19,352); ^{1}H NMR ($CDCl_3$): δ 8.68 (br s, 1H, NH), 7.89–7.46 (m, 7H, Bz, H-5 and H-6), 5.95 (d, 1H, $J_{1',2'} = 10.7$ Hz, H-1′), 4.78 (m, 1H, H-3′), 3.98 (m, 1H, H-4′), 3.87 (m, 2H, H-6a′,6b′), 3.47 (m, 1H, H-5′), 2.83 and 1.77 (2 m, 2H, H-2′), 1.58 and 1.48 (2s, 6H, 2 × CH_3); ^{19}F NMR: δ −65.0; Anal. Calcd for $C_{20}H_{22}FN_3O_5$: C, 59.55; H, 5.50; N, 10.42. Found: C, 59.33; H, 5.61; N, 10.56; ESI-MS (*m*/*z*): 404.41 ($M + H^+$).

4.2.3. 1-(2,3-Dideoxy-3-fluoro-β-D-glycero-hexopyranosyl)-N^4-benzoyl cytosine (**5**)

To a stirred solution of compound **4** (1.19 g, 2.94 mmol) in 4:1 tetrahydrofuran (THF)/H_2O (13.6 mL) at 0 °C was added TFA (1.1 mL). The resulting solution was allowed to warm to room temperature, stirred for 90 min and then was concentrated at 40 °C under high vacuum in order to remove traces of TFA. Purification by flash column chromatography using ethyl acetate–methanol (9.7:0.3) as eluant gave compound **5** as yellowish foam. Yield: 0.77 g (72%); $R_f = 0.2$ in ethyl acetate–methanol (9.7:0.3); $[\alpha]_D^{22} + 30.0$ (*c* 0.5, MeOH); UV (MeOH): λ_{max} 258 nm (ε 17,223); ^{1}H NMR (CD_3OD): δ 7.91–7.30 (m, 7H, Bz, H-5 and H-6), 5.81 (d, 1H, $J_{1',2'} = 10.8$ Hz, H-1′), 4.65 (m, 1H, H-3′), 3.89–3.75 (m, 2H, H-6a′,6b′), 3.63 (m, 1H, H-4′), 3.43 (m, 1H, H-5′), 2.43 and 1.88 (2 m, 2H, H-2′); Anal. Calcd for $C_{17}H_{18}FN_3O_5$: C, 56.20; H, 4.99; N, 11.56. Found: C, 56.38; H, 4.86; N, 11.67; ESI-MS (*m*/*z*): 364.32 ($M + H^+$).

4.2.4. 1-(2,3-Dideoxy-3-fluoro-6-O-trityl-β-D-glycero-hexopyranosyl)-N^4-benzoyl cytosine (**6**)

To a solution of compound **5** (0.77 g, 2.12 mmol) in dry pyridine (10.6 mL) was added triphenylmethyl chloride (0.89 g, 3.18 mmol) and a catalytic amount of DMAP. The mixture was stirred at 100 °C for 1 h and then concentrated. Purification by flash column chromatography using ethyl acetate–*n*-hexane (7:3) as eluant gave pure **6** as a white solid. Yield: 0.83 g (65%); $R_f = 0.52$ in ethyl acetate–methanol (9.8:0.2); m.p. 150–153 °C; $[\alpha]_D^{22} + 63.0$ (*c* 0.5, $CHCl_3$); UV ($CHCl_3$): λ_{max} 258 nm (ε 16,111); ^{1}H NMR ($CDCl_3$): δ 8.91 (br s, 1H, NH), 7.95–7.29 (m, 22H, $3C_6H_5$, Bz, H-5 and H-6), 5.86 (d, 1H, $J_{1',2'} = 10.5$ Hz, H-1′), 4.74 (m, 1H, H-3′), 3.90 (m, 1H, H-4′), 3.62 (m, 1H, H-5′), 3.53 (m, 2H, H-6a′,6b′), 2.80 and 1.74 (2 m, 2H, H-2′); ^{19}F NMR: δ −65.5; Anal. Calcd for $C_{36}H_{32}FN_3O_5$: C, 71.39; H, 5.33; N, 6.94. Found: C, 71.11; H, 5.17; N, 6.65; ESI-MS (*m*/*z*): 606.67 ($M + H^+$).

4.2.5. 1-(2,3-Dideoxy-3-fluoro-6-O-trityl-β-D-glycero-hexopyranosyl-4-ulose)-N^4-benzoyl cytosine (**7a**) and hydrated analogue **7b**

A mixture of **6** (0.83 g, 1.38 mmol; dried by co-evaporation with toluene), PDC (0.78 g, 2.07 mmol) and Ac_2O (0.65 mL, 6.9 mmol) was refluxed at 55 °C in dry CH_2Cl_2 (12 mL) for 90 min. After cooling, ethyl acetate (1.6 mL) was added and the resulting slurry was transferred on the top of a silica-gel column packed in ethyl acetate. The solution was filtered through the column and washed with ethyl acetate (30 mL) until the product was eluted completely. The solvent was evaporated and the residue was rendered free of Ac_2O and pyridine by co-evaporation with dry toluene (3×). Finally, purification by flash column chromatography using ethyl acetate–*n*-hexane (6:4) as eluant afforded **7a**/**b** as a white foam. Yield: 0.42 g (50%), $R_f = 0.4$ in ethyl acetate–*n*-hexane (8:2); ^{1}H NMR for **7a** ($CDCl_3$): δ 9.17 (br s, 1H, NH), 7.99–7.28 (m, 22H, $3C_6H_5$, Bz, H-5 and H-6), 6.35 (d, 1H, $J_{1',2'} = 10.1$ Hz, H-1′), 5.22 (ddd, 1H, $J_{3',F} = 47.0$ Hz, $J_{2'a,3'} = J_{2'b,3'} = 6.9$ Hz, H-3′), 3.82–3.72 (m, 3H, H-5′ and H-6a′,6b′), 3.20 and 2.25 (2 m, 2H, H-2′); ^{1}H NMR for **7b** ($CDCl_3$): δ 9.17 (br s, 1H, NH), 7.99–7.28 (m, 22H, $3C_6H_5$, Bz, H-5 and H-6), 5.94 (d, 1H, $J_{1',2'} = 10.6$ Hz, H-1′), 4.72 (ddd, 1H, $J_{3',F} = 48.1$ Hz, $J_{2'a,3'} = 5.0$ Hz, $J_{2'b,3'} = 5.1$ Hz, H-3′), 3.73–3.58 (m, 3H, H-5′ and H-6a′,6b′), 2.60 and 2.07 (2 m, 2H, H-2′); Anal. Calcd for $C_{36}H_{30}FN_3O_5 \cdot 0.5H_2O$: C, 70.59; H, 5.07; N, 6.86. Found: C, 70.71; H, 4.93; N, 7.04; ESI-MS (*m*/*z*): 604.64 ($M + H^+$) for **7a**; ESI-MS (*m*/*z*): 622.64 ($M + H^+$) for **7b**.

4.2.6. 1-(2,3-Dideoxy-3-fluoro-β-D-glycero-hexopyranosyl-4-ulose)-N^4-benzoyl cytosine (**8a**) and hydrated analogue **8b**

Compound **7a**/**b** (0.42 g, 0.69 mmol) was dissolved in a mixture of CH_2Cl_2 (2.45 mL) and formic acid (2.45 mL, 90%). The solution was stirred for 20 min at room temperature, diluted with toluene, and co-distilled several times with the same solvent to avoid ester formation [61]. The concentrated residue was purified by flash column chromatography using ethyl acetate–methanol (9.8:0.2) as eluant and compound **8a**/**b** was obtained as a white powder. Yield: 0.14 g (52%); $R_f = 0.15$ in ethyl acetate–methanol (9.8:0.2); $[\alpha]_D^{22} + 48.0$ (*c* 0.5, MeOH); UV (MeOH): λ_{max} 258 nm (ε 16,169); ^{1}H NMR (CD_3OD): δ 8.29–7.53 (m, 7H, Bz, H-5 and H-6), 5.94 (d, 1H, $J_{1',2'} = 11.0$ Hz, H-1′), 4.72 (ddd, 1H, $J_{3',F} = 48.4$ Hz, $J_{2'a,3'} = 4.8$ Hz, $J_{2'b,3'} = 4.9$ Hz, 1H, H-3′), 4.06–3.94 (m, 2H, H-6a′,6b′), 3.57 (m, 1H, H-5′), 2.50 and 2.09 (2 m, 2H, H-2′); ^{13}C NMR (CD_3OD): δ 169.23 (C═O); 165.05 (C-4); 157.18 (C-2); 146.41 (C-6); 134.71 (C_{arom}); 134.18 (CH_{arom}); 129.88 ($2CH_{arom}$); 129.24 ($2CH_{arom}$); 99.08 (C-4′); 94.05 (C-5); 92.54 (C-3′); 80.51 (C-5′); 78.46 (C-1′); 61.44 (C-6′); 30.79 (C-2′); ^{19}F NMR: δ −63.2; Anal. Calcd for $C_{17}H_{18}FN_3O_6$: C, 53.83; H, 4.78; N, 11.08. Found: C, 53.98; H, 4.66; N, 11.19; ESI-MS (*m*/*z*): 380.36 ($M + H^+$).

4.3. Synthesis of 1-(3,4-dideoxy-3-fluoro-β-D-glycero-hexopyranosyl-2-ulose)-N^4-benzoyl cytosine (**14a**) and hydrated analogue **14b**

4.3.1. 1-(2-O-Acetyl-3-deoxy-3-fluoro-4-O-phenoxythiocarbonyl-6-O-trityl-β-D-glucopyranosyl)-N^4-benzoyl cytosine (**10**)

Cytosine derivative **10** was synthesized from **9** [41] by the same methodology as described for the synthesis of **3**. Compound **10** was obtained as a white solid following purification by flash column chromatography using ethyl acetate–*n*-hexane (1:1) as eluant. Yield: 1.51 g (55%); $R_f = 0.2$ in ethyl acetate–*n*-hexane (1:1); m.p. 153–156 °C; $[\alpha]_D^{22} + 21.0$ (*c* 0.5, $CHCl_3$); UV ($CHCl_3$): λ_{max} 260 nm (ε 21,501); ^{1}H NMR ($CDCl_3$): δ 8.66 (br s, 1H, NH), 7.90–6.81 (m, 27H,

Bz, H-5, H-6 and 4C_6H_5), 6.15 (d, 1H, $J_{1',2'} = 9.1$ Hz, H-1′), 5.98 (m, 1H, H-4′), 5.32 (m, 1H, H-2′), 4.97 (dtr, 1H, $J_{3',F} = 51.9$ Hz, $J_{2',3'} = 9.0$ Hz, $J_{3',4'} = 9.1$ Hz, H-3′), 4.00 (m, 1H, H-5′), 3.42–3.30 (m, 2H, H-6a′,6b′), 2.09 (1s, 3H, OAc); Anal. Calcd for $C_{45}H_{38}FN_3O_8S$: C, 67.57; H, 4.79; N, 5.25. Found: C, 67.69; H, 4.64; N, 5.53; ESI-MS (*m*/*z*): Found 800.88 ($M + H^+$).

4.3.2. 1-(2-O-Acetyl-3,4-dideoxy-3-fluoro-6-O-trityl-β-D-glycero-hexopyranosyl)-N^4-benzoyl cytosine (**11**)

Cytosine derivative **11** was synthesized from **10** by the same methodology as described for the synthesis of **4**. Compound **11** was obtained as a white solid following purification by flash column chromatography using ethyl acetate–*n*-hexane (1:1) as eluant. Yield: 0.83 g (68%); $R_f = 0.18$ in ethyl acetate–*n*-hexane (1:1); m.p. 138–140 °C; $[\alpha]_D^{22} + 19.0$ (*c* 0.5, $CHCl_3$); UV ($CHCl_3$): λ_{max} 260 nm (ε 20,723); ^{1}H NMR ($CDCl_3$): δ 8.65 (br s, 1H, NH), 7.90–7.27 (m, 22H, 3C_6H_5, Bz, H-5 and H-6), 5.95 (d, 1H, $J_{1',2'} = 9.1$ Hz, H-1′), 5.04 (m, 1H, H-2′), 4.85 (m, 1H, H-3′), 3.85 (m, 1H, H-5′), 3.26 (dq, 2H, $J_{5',6'a} = 4.9$ Hz, $J_{5',6'b} = 4.7$ Hz, $J_{6'a,6'b} = 10.0$ Hz, H-6a′,6b′), 2.40 and 1.91 (2 m, 2H, H-4′), 2.06 (1s, 3H, OAc); ^{19}F NMR: δ −65.5; Anal. Calcd for $C_{38}H_{34}FN_3O_6$: C, 70.47; H, 5.29; N, 6.49. Found: C, 70.59; H, 5.11; N 6.74; ESI-MS (*m*/*z*): 648.70 ($M + H^+$).

4.3.3. 1-(3,4-Dideoxy-3-fluoro-6-O-trityl-β-D-glycero-hexopyranosyl)-N^4-benzoyl cytosine (**12**)

Compound **11** (0.83 g, 1.28 mmol) was dissolved in EtOH–pyridine (12.8 + 3.85 mL), 2 M NaOH (0.86 mL) was added and the mixture stirred for 1 h at 0 °C. Amberlite IR-120 (H^+) was added to neutralize the base. The suspension was filtered, the resin was washed with EtOH and pyridine (10 + 10 mL) and the filtrate was evaporated. The solid residue was purified by flash column chromatography using ethyl acetate as eluant and **12** was obtained as a yellowish foam. Yield: 0.682 g (88%); $R_f = 0.4$ in ethyl acetate; $[\alpha]_D^{22} + 14.0$ (*c* 0.5, $CHCl_3$); UV ($CHCl_3$): λ_{max} 260 nm (ε 21,804); ^{1}H NMR ($CDCl_3$): δ 9.01 (br s, 1H, NH), 7.90–7.28 (m, 22H, 3C_6H_5, Bz, H-5 and H-6), 5.82 (d, 1H, $J_{1',2'} = 9.1$ Hz, H-1′), 4.78 (m, 1H, H-3′), 3.89 (m, 1H, H-5′), 3.70 (m, 1H, H-2′), 3.25 (dq, 2H, $J_{5',6'a} = 5.3$ Hz, $J_{5',6'b} = 4.6$ Hz, $J_{6'a,6'b} = 9.9$ Hz, H-6a′,6b′), 2.33 and 1.82 (2 m, 2H, H-4′); ^{19}F NMR: δ −63.2; Anal. Calcd for $C_{36}H_{32}FN_3O_5$: C, 71.39; H, 5.33; N, 6.94. Found: C, 71.64; H, 4.97; N, 6.72; ESI-MS (*m*/*z*): 606.64 ($M + H^+$).

4.3.4. 1-(3,4-Dideoxy-3-fluoro-6-O-trityl-β-D-glycero-hexopyranosyl-2-ulose)-N^4-benzoyl cytosine (**13a**) and hydrated analogue **13b**

Cytosine derivative **13a**/**b** was synthesized from **12** by the same methodology as described for the synthesis of **7a**/**b**. Compound **13a**/**b** was obtained as a white foam following purification by flash column chromatography using ethyl acetate as eluant. Yield: 0.35 g (52%); $R_f = 0.39$ in ethyl acetate; ^{1}H NMR for **13a** ($CDCl_3$): δ 8.98 (br s, 1H, NH), 8.15–7.27 (m, 22H, 3C_6H_5, Bz, H-5 and H-6), 6.41 (s, 1H, H-1′), 5.41 (ddd, 1H, $J_{3',F} = 47.5$ Hz, $J_{3',4'a} = J_{3',4'b} = 7.4$ Hz, H-3′), 4.29 (m, 1H, H-5′), 3.43 (dq, 2H, $J_{5',6'a} = 5.1$ Hz, $J_{5',6'b} = 5.9$ Hz, $J_{6'a,6'b} = 10.0$ Hz, H-6a′,6b′), 2.82 and 2.29 (2 m, 2H, H-4′); ^{1}H NMR for **13b** ($CDCl_3$): δ 8.98 (br s, 1H, NH), 8.15–7.27 (m, 22H, 3C_6H_5, Bz, H-5 and H-6), 5.86 (s, 1H, H-1′), 4.82 (ddd, 1H, $J_{3',F} = 48.3$ Hz, $J_{3',4'a} = 5.5$ Hz, $J_{3',4'b} = 5.3$ Hz, H-3′), 3.89 (m, 1H, H-5′), 3.23 (dq, 2H, $J_{5',6'a} = 4.1$ Hz, $J_{5',6'b} = 4.7$ Hz, $J_{6'a,6'b} = 10.0$ Hz, H-6a′,6b′), 2.16–1.96 (m, 2H, H-4′); Anal. Calcd for $C_{36}H_{30}FN_3O_5 \cdot 0.5H_2O$: C, 70.59; H, 5.07; N, 6.86. Found: C, 70.33; H, 5.18; N, 7.07; ESI-MS (*m*/*z*): 604.66 ($M + H^+$) for **13a**; ESI-MS (*m*/*z*): 622.67 ($M + H^+$) for **13b**.

4.3.5. 1-(3,4-Dideoxy-3-fluoro-β-D-glycero-hexopyranosyl-2-ulose)-N^4-benzoyl cytosine (**14a**) and hydrated analogue **14b**

Cytosine derivative **14a**/**b** was synthesized from **13a**/**b** by the same methodology as described for the synthesis of **8a**/**b**. Compound **14a**/**b** was obtained as a white powder following purification by flash column chromatography using ethyl acetate–methanol (9.8:0.2) as eluant. Yield: 0.11 g (50%), $R_f = 0.15$ in ethyl acetate–methanol (9.8:0.2); $[\alpha]_D^{22} + 52.0$ (*c* 0.5, MeOH); UV (MeOH): λ_{max} 258 nm (ε 21,395); ^{1}H NMR (CD_3OD): δ 8.39–7.50 (m, 7H, Bz, H-5 and H-6), 5.85 (s, 1H, H-1′), 4.94 (ddd, 1H, $J_{3',F} = 48.5$ Hz, $J_{3',4'a} = 5.5$ Hz, $J_{3',4'b} = 5.6$ Hz, H-3′), 3.87 (m, 1H, H-5′), 3.67 (m, 2H, H-6a′,6b′), 2.12–1.96 (m, 2H, H-4′); ^{13}C NMR (CD_3OD): δ 169.27 (C=O); 164.88 (C-4); 150.06 (C-2); 141.63 (C-6); 134.23 (C_{arom}); 134.16 (CH_{arom}); 129.87 (2CH_{arom}); 129.22 (2CH_{arom}); 98.41 (C-2′); 94.38 (C-5); 92.88 (C-3′); 83.11 (C-1′); 70.62 (C-5′); 65.12 (C-6′); 30.79 (C-4′); ^{19}F NMR: δ −64.3; Anal. Calcd for $C_{17}H_{18}FN_3O_6$: C, 53.83; H, 4.78; N, 11.08. Found: C, 54.03; H, 4.89; N, 10.87; ESI-MS (*m*/*z*): 380.34 ($M + H^+$).

4.4. Methods for measurement of biological activity

4.4.1. Cells and culture conditions

The human colon adenocarcinoma cell line Caco-2 (a generous gift of Dr. René L'Harridon, INRA, VIM, Jouy-en-Josas, France), MCF-7 cell line derived from breast carcinoma, gastric cancer derived AGS cell line and non-tumorigenic human fetal small intestine cell line H4 (control) were used for experiments. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich, Grand Island, USA), supplemented with 5% fetal calf serum (Cambrex, Verviers, Belgium), L-glutamine (2 mmol/L, Sigma, St. Louis, USA), penicillin (100 units/mL, Sigma, St. Louis, USA) and streptomycin (1 mg/mL, Fluka, Buchs, Switzerland) at 37 °C in 5% CO_2 atmosphere in tissue culture flasks until confluent. Cell culture medium was regularly changed.

4.4.2. Nucleoside solutions

Dideoxy fluoro ketonucleosides were freshly prepared in sterile dimethyl sulfoxide (DMSO) at the concentration of 0.5 mg/mL. The final concentration of DMSO was below 0.1% of cell culture medium. All solutions were protected against light.

AZT (*Retrovir*®) GlaxoSmithKline, USA, a drug used for antiretroviral therapy (ART) was used as a standard compound in biological experiments, prepared in the same way as dideoxy fluoro ketonucleosides.

5FU (fluorouracilum), Lederle Arzeinmittel GmbH, BRD, a drug used for antitumor therapy was used as standard compound (control) in growth inhibition and cytotoxicity assessment of compounds, prepared in the same way as dideoxy fluoro ketonucleosides.

4.4.3. Virus propagation

The rotavirus RF strain was propagated in Caco-2 cells in the presence of trypsin (1 μg per mL of DMEM) as described previously [62]. Supernatant containing the virus was collected from the flasks when cytopathic effect (CPE) was observed (24–48 h at 37 °C) by microscopy and clarified by centrifugation. Virus was stored at −70 °C until used.

4.4.4. Antiviral assay

The potential antiviral activity of the newly synthesized compounds was tested against rotavirus by investigating:

a) *The inhibition of infectivity following virus attachment*: Washed monolayers of Caco-2 and H4 cells were first incubated with rotavirus for 1 h at 37 °C in the atmosphere of 5% CO_2 (time for virus to attach to cell membrane receptors). After incubation the remaining virus was washed off with DMEM without supplements and the monolayer was treated immediately with the tested compounds or AZT added in 3-fold serial dilutions (initial concentration of 0.5 mg/mL). After 72 h of incubation

for rotavirus, the plates were stained with Crystal Violet in EtOH, rinsed with water, and destained with 10% (v/v) acetic acid. The A_{590} was measured, and the results were expressed, for each dilution, by the mean ratios (%, ±SD) of absorbances in virus-infected wells ($n = 6$) to those in control (only virus infected) wells ($n = 6$). The 50% inhibitory concentration (IC_{50}) of the tested compounds was obtained from the concentration–effect curve.

b) *The neutralization of the virus in solution before attachment*: 3-fold dilutions of each of the tested compounds or AZT (initial concentration of 0.5 mg/mL) were first co-incubated with rotavirus in DMEM supplemented with trypsin for 12 h prior the infection of Caco-2 and H4 cell monolayer at 37 °C and 5% CO_2. Residual viral infectivity was measured after 72 h post infection. Rotavirus alone was treated in the same way as the control. After 72 h of incubation, the plates were stained with Crystal Violet in EtOH, rinsed with water, and destained with 10% (v/v) acetic acid. The A_{590} was measured, and the results were expressed, for each dilution, by the mean ratios (%, ±SD) of absorbencies in virus-infected wells ($n = 6$) to those in control (only virus infected) wells ($n = 6$). The 50% inhibitory concentration (IC_{50}) of the tested compounds was obtained from the concentration–effect curve.

4.4.5. Cytotoxicity assay

Caco-2 cells, H4 cells, MCF-7 (breast carcinoma) and gastric cancer derived AGS cells ($6X10^6$ cells per plate) were seeded in P96 plates and treated with the compounds or AZT at 3-fold serial dilutions of each compound (initial concentration of 0.5 mg/mL). Then, the cells were incubated at 37 °C in the humidified incubator for 72 h. The plates were stained with Crystal Violet in EtOH, rinsed with water, and destained with 10% (v/v) acetic acid. The A_{590} was measured, and the results were expressed, for each dilution, by the mean ratios (%, ±SD) of absorbances in treated wells ($n = 2$) to those in control wells ($n = 24$). The 50% cytotoxic concentration (CC_{50}) of the tested compounds was obtained from the concentration–effect curve.

4.4.6. Growth inhibition assay

It was performed on Caco-2 cell line by modified method described previously [63]. Briefly, in 96-well plates, six wells of 2-fold dilutions of each compound or 5FU (initial concentration of 0.5 mg/mL) were applied to 10 cells/well in Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum. Incubation was performed at 37 °C in the humidified incubator for 10 days. The colonies were counted in each well and the results were expressed, for each dilution, by the mean ratios (%, ±SD) of colony number in treated wells ($n = 2$) to those in control wells ($n = 24$). The 50% growth inhibitory concentration (IC_{50}) of the tested compounds was obtained from the concentration–effect curve.

4.4.7. Flow cytometric BrdU-cell cycle analysis

Caco-2 cells in Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum were seeded in P96 plates (1×10^6 cells per well) and treated with the compounds at concentrations yielding 50% growth inhibition (IC_{50}) or left untreated. Then, the cells were incubated at 37 °C in the humidified incubator for 24 h. During the last hour of incubation cells were pulsed with 10 μM BrdU solution.

Samples for flow cytometry were processed using FITC-BrdU flow kit (BD Biosciences) according to the manufacturer protocol. Flow cytometry was performed on BD LSR II (BD Biosciences) instrument. The data were analyzed using FACSDiva software (BD Biosciences) on cells gated according to their (FSC versus SSC) scatter properties. Bivariate distributions of BrdU content (FITC) versus DNA content (7-AAD) were analyzed. Cell cycle distribution in G0/G1, S, G2/M phases and % of apoptotic (sub G1) cells was determined according to gating represented in Fig. 1. Also the G1/S subpopulation, corresponding to BrdU-positive cells containing G1 DNA, was determined. Labeling index (LI) corresponded to S phase or percentage of BrdU-positive cells (Fig. 1).

4.4.8. Antiviral activity assays

The compounds were evaluated against the following viruses: herpes simplex virus type 1 (HSV-1) strain KOS, thymidine kinase-deficient (TK^-) HSV-1 KOS strain resistant to ACV (ACV^r), herpes simplex virus type 2 (HSV-2) strains Lyons and G, vaccinia virus Lederle strain, respiratory syncytial virus (RSV) strain Long, vesicular stomatitis virus (VSV), Coxsackie B4, Parainfluenza-3, Reovirus-1, Sindbis, Punta Toro, influenza virus type A (H1N1, H3N2) and type B and feline corona virus. The antiviral, other than anti-HIV, assays were based on inhibition of virus-induced cytopathicity in human embryonic lung (HEL) fibroblasts, African green monkey cells (Vero), human epithelial cervix carcinoma cells (HeLa), Crandel feline kidney cells (CFKC) and Madin-Darby canine kidney cells (MDCK). Briefly, confluent cell cultures in microtiter 96-well plates were inoculated with 100 CCID50 of virus (1 $CCID_{50}$ being the virus dose to infect 50% of the cell cultures). After a 1–2 h adsorption period, residual virus was removed, and the cell cultures were incubated in the presence of varying concentrations of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds. Antiviral activity was expressed as the EC_{50} or compound concentration required to reduce virus-induced cytopathicity by 50%.

4.4.9. Inhibition of HIV-induced cytopathicity in CEM cells

Human CEM cell cultures ($\sim 3 \times 10^5$ cells mL^{-1}) were infected with ~100 CCID50 HIV-1(III_B) or HIV-2(ROD) per mL and seeded in 96-well (200 μL/well) microtiter plates, containing appropriate dilutions of the test compounds. After 4 days of incubation at 37 °C, syncytia formation was examined microscopically in the CEM cell cultures.

4.4.10. Cytostatic and cytotoxicity assays

The cytostatic concentration (for MDCK and CRFK cells) was calculated as the CC_{50}, or the compound concentration required to reduce cell proliferation by 50% relative to the number of cells in the untreated controls. CC_{50} values were estimated from graphic plots of the number of cells (percentage of control) as a function of the concentration of the test compounds. Alternatively, cytotoxicity of the test compounds (for HEL, Vero and HeLa cells) was expressed as the minimum cytotoxic concentration (MCC) or the compound concentration that caused a microscopically detectable alteration of cell morphology.

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