Antiviral and cytostatic evaluation of the novel 6-acyclic chain substituted thymine derivatives

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A series of the novel 5-methyl pyrimidine derivatives with an acyclic side chain at the C-6 position were synthesized using lithiation of a 2,4-dimethoxy-5,6-dimethyl pyrimidine and subsequent nucleophilic addition or substitution reactions of the organolithium intermediate thus obtained with acetaldehyde, epichlorhydrine, fluorinated ketones and fluorinated ester. The novel compounds were evaluated for their cytostatic and antiviral activities. Among all the compounds evaluated, two fluorinated acyclic pyrimidine derivatives showed the highest cytostatic activities. The compound containing a 2-hydroxy-3,3,3-trifluoro-1-propenyl side chain exhibited a pronounced effect against breast carcinoma (MCF-7, IC_{50}=8.38 μg/ml), while the compound with a 2-fluoromethyl-2-acetoxypropyl chain exhibited moderate effect against cervical carcinoma (HeLa, IC_{50}=19.73 μg/ml).

Keywords: C-6 fluoroalkylated pyrimidine derivatives, conformational analysis, antiviral activity, cytostatic activity, apoptosis

Introduction

Pyrimidine derivatives and their nucleosides have great biological significance because they exhibit a wide range of antiviral and anticancer activities (Botta *et al*., 1999). In the last few years some uracil and pyrimidinone derivatives substituted at C-5 or C-6 position have emerged in the field of antiviral chemotherapy (Kim *et al*., 1997; Pontikis *et al*., 1997). Among the important 6-substituted uracil derivatives, 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)-thymine (HEPT) (Baba *et al*., 1991; Tanaka *et al*., 1992) and its analogues (Balzarini *et al*., 1993; Tanaka *et al*., 1995), as well as 3,4-dihydro-2-alkoxy-6-benzyl-4-oxo-pyrimidines (DABOs) (Mai *et al*., 1997) showed a potent and selective activity against human immunodeficiency virus type-1 (HIV-1). Among a series of 5- and 6-substituted uracil derivatives, the 6-amino-substituted analogue, 6-(2-aminoethyl)amino-5-chlorouracil, proved to be a selective and potent inhibitor of angiogenic action of thymidine phosphorylase (TP) (Klein *et al*., 2001). Furthermore, 6-(alkylamino)ethyl pyrimidine derivatives exhibited activity against rubella and Sindbis viruses (Saladino *et al*., 2002), whereas 6-oxiranyl-, 6-methyloxiranyluracils and pyrimidinone derivatives showed potent and selective antiviral activity against parainfluenza 1 virus (Sendai virus) (Saladino *et al*., 1998). Moreover, some of the 6-substituted uridines exhibited highly promising antileukaemic activities (Tanaka *et al*., 1983), while uracil derivatives containing an acylvinyl side chain at C-6 demonstrated cytostatic activity against mouse L1210 leukaemia cells (Kundu *et al*., 1997; Kundu *et al*., 1995).

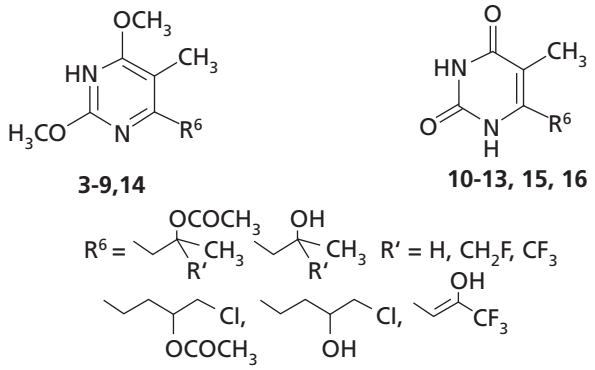
The pronounced biological activities exhibited by this class of compounds led us to synthesize the new type of C-6 alkylated pyrimidine derivatives **[3]**-**[16]** (Figure 1) and to evaluate their cytostatic and antiviral activities.

Materials and methods

Chemistry

Melting points (uncorrected) were determined with Kofler micro hot-stage (Reichert, Wien). Precoated Merck silica gel 60F-254 plates were used for thin layer chromatography (TLC) and the spots were detected under UV light (254 nm). Column chromatography (CLC) was performed using silica gel (0.063–0.2 mm) Kemika; glass column was slurry-packed under gravity. Solvent systems used for the

Figure 1. The C-6 alkylated pyrimidine derivatives **[3]**–**[16]**



TLC and CLC were petrolether:ethylacetate=1:1 (S_1), dichloromethane:methanol=20:1 (S_2), petrolether:ethylacetate=4:1 (S_3), petrolether:ethylacetate=5:1 (S_4).

The electron impact mass spectra were recorded with an EXTREL FTMS (Madison, WI, USA) 2001 instrument with ionizing energy 70 eV. Elemental analyses were performed in the Central Analytic Service, Ruđer Bošković Institute, Zagreb. ^{1}H and ^{13}C NMR spectra were recorded on Varian Gemini and Unity Inova 300 MHz NMR spectrometers (Varian, Palo Alto, CA, USA). The samples were dissolved in $CDCl_3$ or DMSO-d_6. The ^{1}H and ^{13}C NMR chemical shift values (δ) are expressed in ppm with respect to TMS and coupling constants (J) in Hz. Individual ^{1}H and ^{13}C NMR resonances were assigned on the basis of chemical shifts, signal intensities, magnitude and multiplicity of resonances, H-H, H-F and C-F coupling constants as well as a series of 2D NMR experiments including HSQC and HMBC. The assignments of ^{1}H NMR spectra are given in Table 1, whereas ^{13}C NMR chemical shifts are reported in the experimental part. Conformational properties were assessed with the use of 1D difference NOE enhancements and long range J_{HC} coupling constants.

6-(2-Hydroxypropyl)-2,4-dimethoxy-5-methylpyrimidine **[3]**

The solution of 2,4-dimethoxy-5,6-dimethylpyrimidine **[2]** (919 mg; 5.47 mmol) in tetrahydrofuran (THF) (15 ml) was cooled at -70°C and lithium diisopropylamide (LDA) (4.1 ml; 2 M LDA in THF/heptane/ethylbenzene) was added dropwise into the reaction mixture. The temperature was then raised to -55°C and the reaction mixture was stirred for 30 min. Acetaldehyde (0.37 ml; 6.56 mmol) was added and the mixture was additionally stirred for 3 h and then neutralized with glacial acetic acid. The temperature was raised to room temperature and the reaction mixture was stirred further for 15 min. The solvent was evaporated and the residual yellow oily product was extracted with CH_2Cl_2 and water. The organic layer was dried over Na_2SO_4 and purified on silica gel CLC using S_1 as eluent, and compound **[3]** (765 mg; 65 %) was isolated as yellow oil.

[3]: MS *m/z* 213 $[MH]^+$; ^{13}C NMR (DMSO) δ: 169.07 (C-4), 166.79 (C-6), 162.14 (C-2), 108.13 (C-5), 65.81 (C-2'), 53.94 (2-OCH_3), 53.78 (4-OCH_3), 43.45 (C-1′), 23.35 (C-3′), 9.80 (CH_3). Anal. calcd for $C_{10}H_{16}N_2O_3$: C, 56.59; H, 7.60; N, 13.20. Found: C, 56.42; H, 7.62; N, 13.23.

6-(2-Fluoromethyl-2-hydroxypropyl)-2,4-dimethoxy-5-methylpyrimidine **[4]**

The synthesis of **[4]** was performed by a procedure analogous to that of **[3]** using compound **[2]** (448 mg; 3.2 mmol) dissolved in THF (7 ml), LDA (2.0 ml; 2 M in THF/heptane/ethylbenzene) and fluoroacetone (0.2 ml; 3.17 mmol) as reagents. After column chromatography with S_3, the compound **[4]** (337 mg; 43%) was isolated as yellow oil.

[4]: MS *m/z* 245 $[MH]^+$; ^{13}C NMR (DMSO) δ: 169.29 (C-4), 165.03 (C-6), 161.66 (C-2), 109.43 (C-5), 88.41 (d, J_{CF}=173 Hz, CH_2F), 71.49 (d, J_{CF}=18 Hz, C-2′), 53.97 (2-OCH_3), 53.88 (4-OCH_3), 40.88 (d, J_{CF}=3 Hz, C-1′), 23.52 (d, J_{CF}=4 Hz, C-3′), 10.19 (CH_3). Anal. calcd for $C_{11}H_{17}FN_2O_3$: C, 54.09; H, 7.01; N, 11.47. Found: C, 54.25; H, 6.99; N, 11.49.

6-(2-Trifluoromethyl-2-hydroxypropyl)-2,4-dimethoxy-5-methylpyrimidine **[5]**

A procedure analogous to that used for **[3]** was used for the synthesis of **[5]**. Used reagents were: **[2]** (514 mg; 3.06 mmol), LDA (2.3 ml; 2 M in THF/heptane/ethylbenzene), THF (7 ml) and trifluoroacetone (0.35 ml; 3.67 mmol). After column chromatography with system S_4, the compound **[5]** was isolated as yellow oil (67.7%).

[5]: MS *m/z* 281 $[MH]^+$; ^{13}C NMR (DMSO) δ: 169.40 (C-4), 163.06 (C-6), 161.64 (C-2), 126.63 (q, J_{CF}=287 Hz, CF_3), 110.13 (C-5), 73.15 (q, J_{CF}=27 Hz, C-2′), 54.07 (2-OCH_3), 54.00 (4-OCH_3), 38.03 (q, J_{CF}=1 Hz, C-1′), 20.67 (q, J_{CF}=2 Hz, C-3′), 10.19 (CH_3). Anal. calcd for $C_{11}H_{15}F_3N_2O_3$: C, 47.14; H, 5.39; N, 10.00. Found: C, 47.23; H, 5.41; N, 10.01.

6-(2-Hydroxy-3,3,3-trifluoro-1-propenyl)-2,4-dimethoxy-5-methylpyrimidine **[6]**

The synthesis of **[6]** was performed by a procedure analogous to that used for **[3]**, now starting from compound **[2]**

Table 1. ^{1}H NMR chemical shifts (δ/ppm)* and H-H coupling constants (J/Hz)† in ^{1}H NMR spectra for compounds **[3]–[16]** (*c.f.* Figure 2)

Compound	NH, NH	4-OCH_3 2-OCH_3	H-3′	H-4′	OH	H-1′	$COCH_3$	CH_3	H-2′
[3]	-	3.84, s, 3H 3.89, s, 3H	1.10, d, 3H, J=6.3	-	4.65, d, 1H, J=4.9	2.58, dd, 1H, J_1=6.0, J_2=13.3 2.77, dd, 1H, J_1=6.98, J_2=13.3	-	2.02, s, 3H	4.02–4.10, m, 1H
[4]‡	-	3.89, s, 3H 3.83, s, 3H	1.14, d, 3H, J_{HF}=2.1	-	5.10, s, 1H	2.75, dd, 1H, J=13.4, J_{HF}=1.4 2.83, dd, 1H, J=13.4, J_{HF}=1.4	-	2.04, s, 3H	-
[5]	-	3.90, s, 3H 3.85, s, 3H	1.33, s, 3H	-	6.16, s, 1H	2.74, d, 1H, J=13.3 3.12, d, 1H, J=13.3	-	2.04, s, 3H	-
[6]	-	3.99, s, 3H 4.00, s, 3H	-	-	-	5.94, s, 1H	-	1.92, s, 3H	-
[7]	-	3.81, s, 3H 3.85, s, 3H	3.65–3.67, m, 1H	3.53-3.56, m, 2H	5.19, d, 1H, J=5.32	2.63–2.71, m, 2H	-	1.98, s, 3H	1.82–1.89, m, 2H
[8]	-	3.81, s, 3H 3.88, s, 3H	1.23, d, 3H, J=6.3	-	-	2.78, dd, 1H, J_1=5.7, J_2=14.6 2.97, dd, 1H, J=7.6, J=14.5	2.01, s, 3H	1.91, s, 3H	5.25–5.31, m 1H
[9]§	-	3.93, s, 3H 3.97, s, 3H	1.26,s, 3H	-	-	3.16,d,1H,J=13.7 3.95,d,1H,J=12.4	2.10, s, 3H	2.02, s, 3H	-
[10]	10.60, s, 1H 10.97, s, 1H	-	1.22, d, 3H, J=6.3	-	-	2.46–2.51, m, 2H	1.94, s, 3H	1.76, s, 3H	5.04–5.10, m, 1H
[11]	10.43, s, 1H 10.92, s, 1H	-	1.10, d, 3H, J=6.2	-	4.82, s, 1H	2.38,dd,1H, J_1=5.1, J_2=13.3 2.48-2.51, m, 1H	-	1.75, s, 3H	3.88–3.94, m, 1H
[12]¶	10.36, s, 1H 11.04, s, 1H	-	1.43, d, 3H, J_{HF}=2.3	-	-	2.74, d, 1H, J=14.2 2.98, d, 1H, J=14.2	1.98,s, 3H	1.77,s, 3H	-
[13]#	-	-	1.25, s, 1H 1.55, s, 1H 1.84, s, 1H	-	-	2.95, d, 1H, J=17.1 3.89, d, 1H, J=12.0	-	2.01, s, 3H	-
[14]	-	3.84, s, 3H 3.88, s, 3H	5.02–5.06, m, 1H	3.78–3.86, m, 2H	-	2.65, t, 2H, J=7.65	2.03, s, 3H	1.98, s, 3H	1.96–2.06,m,2H
[15]	10.58, s, 1H 10.92, s, 1H	-	4.93–4.97, m,1H	3.72–3.84, m, 2H	-	2.40, t, 2H, J=7.85	2.04, s, 3H	1.72, s, 3H	1.77–1.90,m,2H
[16]	10.57, s, 1H 10.94, s, 1H	-	3.51–3.59, m, 3H	-	5.25, s, 1H	2.35–2.40, m, 1H 2.45–2.46, m, 1H	-	1.72–1.73, m, 4H	1.54–1.55, m, 1H

*DMSO-d_6 as a solvent for all compounds except for **[6]**, **[9]** and **[13]** which were recorded in $CDCl_3$; chemical shifts are referred to TMS. Signal multiplicities (d, doublet; m, multiplet; s, singlet; t, triplet) and number of protons are reported; †Digital resolution ±0.28 Hz. ‡Signal for $–CH_2F$: 4.27 ppm, dd, 1H, J_{HF}=24.4 Hz, J_{HH}=9.0 Hz; 4.20 ppm, dd, 1H, J_{HF}=24.4 Hz, J_{HH}=8.94 Hz. §Signal for $–CH_2F$: 4.79 ppm, dd, 1H, J_{HF}=26.2 Hz, J_{HH}=9.6 Hz; 4.63 ppm, dd, 1H, J_{HF}=26.0 Hz, J_{HH}=9.6 Hz. ¶Signal for $–CH_2F$: 4.76 ppm, dd, 1H, J_{HF}=52.3 Hz, J_{HH}=9.8 Hz; 4.60 ppm, dd, 1H, J_{HF}=52.3 Hz, J_{HH}=9.8 Hz. #Signal for $–CH_2F$: 5.86 ppm, b, 1H; 6.05 ppm, b, 1H.

(498 mg; 2.96 mmol) dissolved in THF (7 ml), LDA (2.2 ml; 2 M in THF/heptane/ethybenzene) and methyltrifluoroacetate (0.36 ml; 3.53 mmol) as reagents. After column chromatography with S_4, colourless crystals of **[6]** (267 mg; 34.2%) were isolated.

[6]: m.p.=79–80°C; MS *m/z* 265 $[MH]^+$; ^{13}C NMR (DMSO) δ: 168.87 (C-4), 158.82 (q, J_{CF}=34 Hz, C-2′), 158.51 (C-6), 158.19 (C-2), 119.01 (q, J_{CF}=280 Hz, CF_3), 102.63 (C-5), 88.83 (q, J_{CF}=4 Hz, C-1′), 55.29 (2-OCH_3), 54.80 (4-OCH_3), 8.61 (CH_3). Anal. calcd for $C_{10}H_{11}F_3N_2O_3$: C, 45.46; H, 4.20; N, 10.60. Found: C, 45.41; H, 4.21; N, 10.61.

6-(4-Chloro-3-hydroxybutyl)-2,4-dimethoxy-5-methylpyrimidine **[7]**

The synthesis of **[7]** was performed by a procedure analogous to that described for **[3]**. Used reagents: **[2]** (500 mg; 2.94 mmol), THF (7 ml), LDA (2.2 ml; 2 M in THF/heptane/ethylbenzene) and epychlorhydrine (326 mg; 3.53 mmol). After CLC with S_1, **[7]** (501 mg; 66%) was isolated as white crystals.

[7]: m.p.=205–208°C; MS *m/z* 261 $[MH]^+$; ^{13}C NMR (DMSO) δ: 169.05 (C-4), 168.73 (C-6), 162.41 (C-2), 107.18 (C-5), 69.61 (C-4′), 54.06 (2-OCH_3), 53.91 (4-OCH_3), 49.78 (C-3′), 32.14 (C-2′), 29.88 (C-1′), 9.47 (CH_3). Anal. calcd for $C_{11}H_{17}ClN_2O_3$: C, 50.67; H, 6.57; N, 10.74. Found: C, 50.77; H, 6.56; N, 10.72.

6-(2-Acetoxypropyl)-2,4-dimethoxy-5-methylpyrimidine **[8]**, 6-(2-acetoxypropyl) 2,4-dihydroxy-5-methylpyrimidine **[10]** and 6-(2-hydroxypropyl)-2,4-dihydroxy-5-methylpyrimidine **[11]**

Compound **[4]** (756 mg; 3.57 mmol) was dissolved in acetyl chloride (9 ml). The reaction mixture was refluxed for 5 h. Water (1 ml) was then added and the reaction mixture stirred overnight at room temperature. The solvent was evaporated at the reduced pressure and the remaining yellow oil was chromatographed on a silica gel column using S_2 as eluent. The following compounds were isolated: **[8]** (108 mg; 11.9%) as yellow oil, **[10]** (288 mg, 35.7%) and **[11]** as colourless crystals (209 mg, 31.8%).

[8]: MS *m/z* 255 $[MH]^+$; ^{13}C NMR (DMSO) δ: 169.58 (C=O), 169.07 (C-2), 164.72 (C-4), 162.14 (C-6), 108.20 (C-5), 68.92 (C-2′), 53.90 (2-OCH_3), 53.80 (4-OCH_3), 39.5 (C-1′), 20.87 ($CO\underline{C}H_3$), 19.27 (C-3′), 9.47 (CH_3). Anal. calcd for $C_{12}H_{18}N_2O_4$: C, 56.68; H, 7.13; N, 11.02. Found: C, 56.14; H, 7.12; N, 11.03.

[10]: m.p.=195–198°C; MS *m/z* 227 $[MH]^+$; ^{13}C NMR (DMSO) δ: 170.10 (C=O), 165.22 (C-2), 151.23 (C-4), 147.21 (C-6), 106.49 (C-5), 68.85 (C-2′), 36.45 (C-1′), 21.28 (C-3′), 20.19 ($CO\underline{C}H_3$), 10.26 (CH_3). Anal. calcd for $C_{10}H_{14}N_2O_4$: C, 53.09; H, 6.24; N, 12.38. Found: C, 52.98; H, 6.26; N, 12.39.

[11]: m.p.=199–202°C; MS *m/z* 185 $[MH^+]$; ^{13}C NMR (DMSO) δ: 164.80 (C-2), 150.69 (C-4), 148.56 (C-6), 105.21 (C-5), 64.86 (C-2′), 39.5 (C-1′), 23.25 (C-3′), 9.76 (CH_3). Anal. calcd for $C_8H_{12}N_2O_3$: C, 52.17; H, 6.57; N, 15.21. Found: C, 52.22; H, 6.56; N, 15.24.

6-(2-Fluoromethyl-2-acetoxypropyl)-2,4-dimethoxy-5-methylpyrimidine **[9]**, 6-(2-fluoromethyl-2-acetoxypropyl)-2,4-dihydroxy-5methylpyrimidine **[12]** and 6-(2-fluoromethyl-2-hydroxypropyl)-2,4-dihydroxy-5-methylpyrimidine **[13]**

The procedure analogous to that used for deprotection of **[4]** was used for the synthesis of **[9]**, **[12]** and **[13]**. Used reagents were: compound **[4]** (300 mg; 1.23 mmol), acetyl chloride (4.5 ml) and water (0.5 ml). The following compounds were obtained: **[9]** (24 mg, 6.8%) as yellow oil, **[12]** (68 mg, 21.5%) and **[13]** (85 mg, 32.0%) as colourless crystals.

[9]: MS *m/z* 287 $[MH]^+$; ^{13}C NMR ($CDCl_3$) δ: 170.27 (C=O), 169.99 (C-2), 163.02 (C-4), 162.13 (C-6), 110.21 (C-5), 86.29 (d, J_{CF}=176.7 Hz, $\underline{C}H_2F$), 82.25 (d, J_{CF}=17.0 Hz, C-2′), 54.28 (2-OCH_3), 54.98 (4-OCH_3), 38.16 (d, J_{CF} = 6.4 Hz, C-1′), 29.58 ($CO\underline{C}H_3$), 19.97 (d, J_{CF}=4.7 Hz, C-3′), 10.30 (CH_3). Anal. calcd for $C_{13}H_{19}FN_2O_4$: C, 54.54; H, 6.69; N, 9.78. Found: C, 54.43; H, 6.71; N, 9.81.

[12]: m.p.=195–198°C; MS *m/z* 259 $[MH]^+$; ^{13}C NMR (DMSO) δ: 169.68 (C=O), 164.80 (C-4), 150.57 (C-2), 145.01 (C-6), 107.75 (C-5), 84.61 (d, J_{CF}=175 Hz, CH_2F), 81.42 (d, J_{CF} =17 Hz, C-2′), 36.14 (d, J_{CF}=4 Hz, C-1′), 21.89 ($CO\underline{C}H_3$), 19.32 (d, J_{CF}=4 Hz, C-3′), 10.71 (CH_3). Anal. calcd for $C_{11}H_{15}FN_2O_4$: C, 51.16; H, 5.85; N, 10.85. Found: C, 51.06; H, 5.86; N, 10.82.

[13]: m.p.=203–206°C; MS *m/z* 215 [M-H]; ^{13}C NMR (DMSO) δ: 164.91 (C-4), 150.49 (C-2), 146.87 (C-6), 106.85 (C-5), 88.83 (d, J_{CF}=174.00 Hz, CH_2F), 71.53 (d, J_{CF}=5.5 Hz, C-2′), 22.82 (d, J_{CF}=3.7 Hz, C-1′), 16.22 (C-3′), 10.56 (CH_3). Anal. calcd for $C_9H_{13}FN_2O_3$: C, 50.00; H, 6.06; N, 12.96. Found: C, 49.95; H, 6.07; N, 12.99.

6-(3-Acetoxy-4-chlorobutyl)-2,4-dimethoxy-5-methylpyrimidine **[14]**, 6-(3-acetoxy-4-chlorobutyl)-2,4-dihydroxy-5-methylpyrimidine **[15]** and 6-(3-hydroxy-4-chlorobutyl)-2,4-dihydroxy-5-methylpyrimidine **[16]**

The procedure analogous to that used for deprotection of **[4]** was used for the synthesis of **[14]**, **[15]** and **[16]**. Used reagents were: **[7]** (1.16 g; 4.44 mmol), acetyl chloride (16 ml) and water (2 ml). After CLC with S_2 as eluent, the

following compounds were isolated: **[14]** (32 mg; 2.4%) as yellow oil, **[15]** (187 mg, 22.2%) and **[16]** (20 mg, 1.9%) as colourless crystals.

[14]: MS *m/z* 303 $[MH]^+$; ^{13}C NMR (DMSO) δ: 169.86 (C=O), 169.01 (C-2), 167.44 (C-4), 162.30 (C-6), 107.20 (C-5), 71.97 (C-4′), 53.95 (2-OCH_3), 53.81 (4-OCH_3), 46.07 (C-3′), 29.05 (C-1′), 28.90 (C-2′), 20.66 ($CO\underline{C}H_3$), 9.27 (CH_3). Anal. calcd for $C_{13}H_{19}ClN_2O_4$: C, 51.57; H, 6.33; N, 9.25. Found: C, 51.62; H, 6.34; N, 9.24.

[15]: m.p.=217–219°C; MS *m/z* 276 $[MH]^+$; ^{13}C NMR (DMSO) δ: 169.90 (C=O), 164.83 (C-2), 150.92 (C-4), 149.85 (C-6), 104.36 (C-5), 71.87 (C-4′), 45.80 (C-3′), 29.15 (C-1′), 25.68 (C-2′), 20.72 ($CO\underline{C}H_3$), 9.28 (CH_3). Anal. calcd for $C_{11}H_{15}ClN_2O_4$: C, 48.10; H, 5.50; N, 10.20. Found: C, 48.00; H, 5.51; N, 10.16.

[16]: m.p.=235–238°C; MS *m/z* 233 $[MH]^+$ ^{13}C NMR (DMSO) δ: 165.36 (C-2), 151.42 (C-4), 151.29 (C-6), 104.56 (C-5), 69.80 (C-4′), 49.95 (C-3′), 32.46 (C-1′), 26.60 (C-2′), 9.78 (CH_3). Anal. calcd for $C_9H_{13}ClN_2O_3$: C, 46.46; H, 5.63; N, 12.04. Found: C, 46.41; H, 5.64; N, 12.08.

7-Hydroxy-3-methoxy-4-methylpiperido[1,2-c]-pyrimidine-1-one **[17]**

The mixture of **[7]** (250 mg; 0.95 mmol) in methanol (5 ml) was saturated with gaseous NH_3. The flask was firmly stopped and the reaction stirred overnight at 60°C. The solvent was evaporated under reduced pressure and the product was purified on the silica gel column using S_1 system. The crystals of the bicyclic compound **[17]** were isolated (144 mg; 72%).

[17]: m.p.=209–211°C; MS *m/z* 210 M^+; ^1H NMR (DMSO) δ: 5.12 (OH, J=3.4 Hz, d, 1H), 4.07–4.12 (H-3′, m, 1H), 3.81 (4-OCH_3, s, 3H), 3.65–3.80 (H-4′, m, 2H), 2.63–2.86 (H-1′, m, 2H), 1.84 (CH_3, s, 3H), 1.66–1.75 (H-2′, m, 2H); ^{13}C NMR (DMSO) δ: 168.50 (C-2), 155.80 (C-4), 154.32 (C-6), 98.35 (C-5), 61.72 (C-4′), 53.57 (OCH_3), 49.62 (C-3′), 25.79 (C-1′), 22.24 (C-2′), 9.02 (CH_3). Anal. calcd for $C_{10}H_{14}N_2O_3$: C, 57.13; H, 6.71; N, 13.33. Found: C, 56.96; H, 6.72; N, 13.31.

Antiviral activity assays

Antiviral activity against VZV, CMV, HIV-1, HIV-2, vaccinia virus, vesicular stomatitis virus, Coxsackie virus B4, respiratory syncytial virus, parainfluenza-3 virus, reovirus-1, Sindbis virus and Punta Toro virus was determined essentially as described previously (De Clercq *et al.*, 1986; Balzarini *et al.*, 1991). Confluent human embryonic lung (HEL) fibroblasts were grown in 96-well microtitre plates and infected with the human cytomegalovirus (HCMV) strains Davis and AD-169 at 100 PFU per well. After a 2 h incubation period, residual virus was removed and the infected cells were further incubated with the medium containing different concentrations of the tested compounds (in duplicate). After incubation for 7 days at 37°C, virus-induced cytopathogenicity was monitored microscopically after ethanol fixation and staining with Giemsa (Merck, Darmstadt, Germany). Antiviral activity was expressed as the concentration required to reduce virus-induced cytopathogenicy by 50% (EC_{50}). EC_{50} values were calculated from graphic plots of the percentage of cytopathogenicity as a function of concentration of the compounds.

Cytotoxicity assays

Cytotoxicity measurements were based on the inhibition of HEL cell growth. HEL cells were seeded at a rate of 5×10^3 cells/well into 96-well microtitre plates and allowed to proliferate for 24 h. Then, medium containing different concentrations of the test compounds was added. After 3 days of incubation at 37°C, the cell number was determined with a Coulter counter (Coulter Electronics, Luton, England). The cytostatic concentration was calculated as the compound concentration required to reduce cell growth by 50% relative to the number of cells in the untreated controls (CC_{50}). 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. Cytotoxicity was expressed as minimum cytotoxic concentration (MCC) or the compound concentration that causes a microscopically detectable alteration of cell morphology.

Antitumour activity assays

The HeLa (cervical carcinoma), MCF-7 (breast carcinoma), SW 620 (colon carcinoma), MiaPaCa-2 (pancreatic carcinoma), Hep-2 (laryngeal carcinoma) and WI 38 (diploid fibroblasts) cells were cultured as monolayers and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37°C.

The HeLa, MCF-7, SW 620, MiaPaCa-2, Hep-2 and WI 38 cells were seeded into a series of standard 96-well microtitre plates on day 0. Test agents were then added in five, 10-fold dilutions (10^{-8}–10^{-4} M) and incubated for a further 72 h. Working dilutions were freshly prepared on the day of testing. The solvent was also tested for eventual inhibitory activity by adjusting its concentration to be the same as in working concentrations. After 72 h of incubation the cell growth rate was evaluated by MTT assay (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, USA. Mossman *et al.*, 1983), which detects dehydrogenase activity in viable cells. The absorbancy (optical density, OD) was measured by a microplate reader at 570 nm. The percentage of growth

(PG) of the cell lines was calculated according to one or the other of the following two expressions:

If (mean OD_{test}−mean OD_{tzero})≥0 then:
PG=100×(mean OD_{test}−mean OD_{tzero})/
(mean OD_{ctrl}−meanOD_{tzero}).

If (mean OD_{test}−mean OD_{tzero})<0 then:
PG=100×(mean OD_{test}−mean OD_{tzero})/OD_{tzero}.

Where:
Mean OD_{tzero}=the average of optical density measurements before exposure of cells to the test compound.
Mean OD_{test}=the average of optical density measurements after the desired period of time.
Mean OD_{ctrl}=the average of optical density measurements after the desired period of time with no exposure of cells to the test compound.

Each test point was performed in quadruplicate in three individual experiments. The results are expressed as IC_{50}, a concentration necessary for 50% of inhibition. Each result is a mean value from three separate experiments. The IC_{50} values for each compound were calculated from dose-response curves using linear regression analysis by fitting the test concentrations that gave PG values above and below the reference value (that is 50%).

Cell cycle analysis.

A total of $1x10^6$ cells were seeded per 100 mm plate. After 24 h the compounds **[6]** and **[9]** were added at a concentration of $5x10^6$ mol/l. After 72 h the attached cells were trypsinized, combined with floating cells, washed with phosphate-buffered saline (PBS), fixed with 70% ethanol and stored at -20°C. Immediately before the analysis, the cells were washed with PBS and stained with 1 μg/ml of propidium iodide (PI) with the addition of 0.2 μg/μl of RNAse A. The stained cells were then analysed with a FACSCalibur flow cytometer (Becton Dickinson, USA) – 20 000 counts were measured. The percentage of the cells in each cell cycle phase was determined using the WinMDI software (Verity Software House, Inc., USA) based on the DNA histograms. Statistical analysis was performed in Microsoft Excel (2002, Microsoft Corporation, USA) using the ANOVA single factor test.

Annexin-V test

Detection and quantification of apoptotic cells at single cell level, was performed using Annexin-V-FLUOS staining kit (Roche, Switzerland), according to the manufacturer's recommendations. After 72 h, both floating and attached cells were collected. The cells were washed with PBS, pelleted and resuspended in staining-solution (annexin-V-fluorescein labelling reagent and propidium iodide (PI) in Hepes buffer). The cells were then analysed under a fluorescence microscope. Annexin-V (green fluorescent) cells were determined to be apoptotic and Annexin-V and PI cells were determined to be necrotic. Percentage of apoptotic cells was expressed as a number of fluorescent cells in relation to the total cell number (fluorescent and non-fluorescent cells), which was set at 100%.

Results

Chemistry

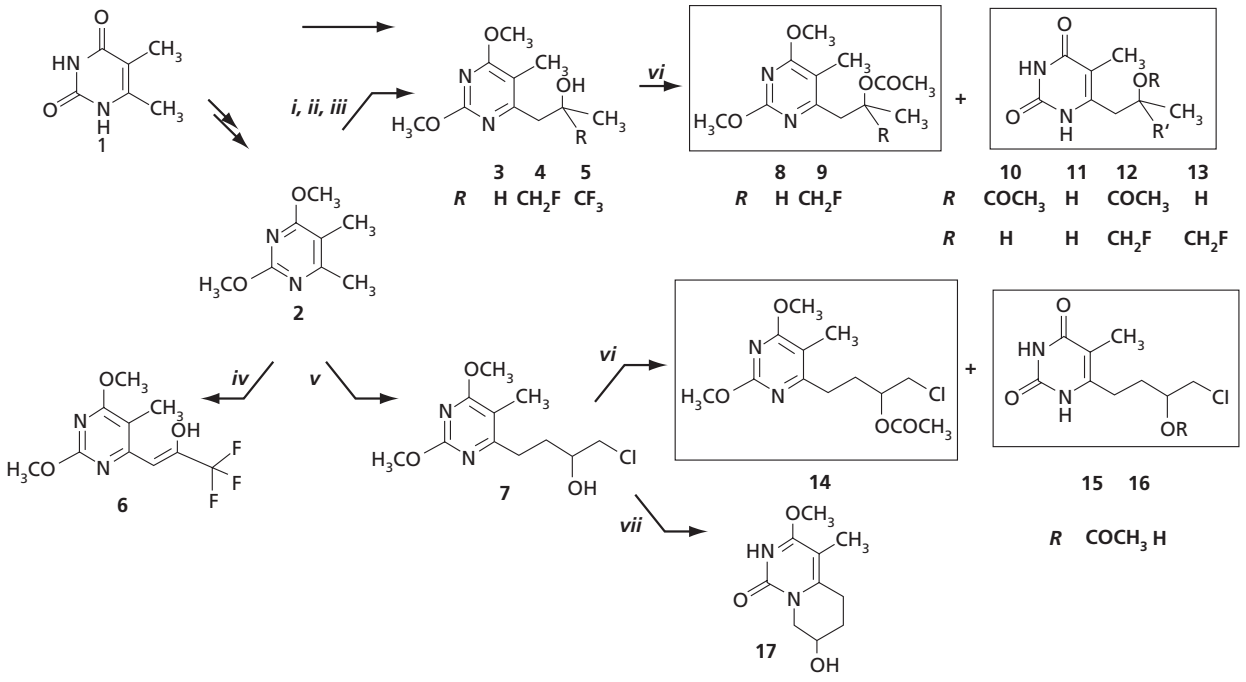
2,4-Dimethoxy-5,6-dimethylpyrimidine **[2]**, a key precursor for C-6 alkylation of the pyrimidine ring, was prepared by chlorination of 5,6-dimethylpyrimidin-2,4-dione **[1]** and methoxylation of the chlorinated pyrimidine derivative thus obtained using the procedure given in the literature by Schlenker (1901) (Figure 2). The lithiation reaction has been recognized as a useful method for introducing various functionalities into the pyrimidine moiety (Hsu & Lin, 1996; Hsu *et al*., 1992; Middleton, 1975). Using this method, the new 6-acyclic chain substituted 2,4-dimethoxy-5-methylpyrimidine derivatives (**[3]**-**[5]** and **[7]**) were synthesized (Figure 2). The synthetic pathway involved lithiation of the 6-methyl group of the pyrimidine derivative **[2]** and addition of the organolithium intermediate thus obtained to acetaldehyde, fluorinated acetones and epichlorhydrine (Figure 2). Nucleophilic substitution reaction of the 6-methyl lithiated derivative **[2]** and methyltrifluoroacetate afforded 2,4-dimethoxy pyrimidine derivative **[6]** containing trifluoromethylated 2-hydroxy-1-propenyl side chain.

The enol form of **[6]** was generated by keto-enol tautomerism of the initially formed propan-2-one moiety. Reaction of **[3]** and **[4]** with acetyl chloride and water gave 2,4-dimethoxy pyrimidine derivatives **[8]** and **[9]** acetylated in the side chain, the acetylated pyrimidine-2,4-dione derivatives **[10]** and **[12]** and pyrimidine-2,4-dione derivatives **[11]** and **[13]** containing a free hydroxy group. In the reaction with acetyl chloride and water, compound **[7]** gave acetylated 2,4-dimethoxy pyrimidine derivative **[14],** the pyrimidine-2,4-dione derivative **[15]** acetylated in the side chain**,** and pyrimidine-2,4-dione derivative **[16]** with a free hydroxy group in the alkyl side chain. We found that ring closure of **[7]** afforded the bicyclic compound **[17]** (Cetina *et al*., 2005) whose formation may be explained by the nucleophilic attack of the N_1 nitrogen of the pyrimidine ring on the C-4 methylene of the 4-chloro-3-hydroxybutyl side chain.

Structural determination

Conformational properties of compounds **[5]** and **[6]** as well as **[4]** and **[12]** were assessed with the use of 1D difference NOE enhancements and long range J_{HC} coupling

Figure 2. The synthesis of C-6 alkylated pyrimidine derivatives **[3]**–**[16]** and bicyclic compound **[17]**



i) LDA, THF, acetaldehyde; *ii*) LDA, THF, fluoroacetone; *iii*) LDA, THF, trifluoroacetone; *iv*) LDA, THF, methyltrifluoroacetate; *v*) LDA, THF, (±)-epichlorhydrine; *vi*) acetyl chloride, H_2O; *vii*) methanolic ammonia.

constants (Table 1). The saturation of of H-1′a (δ: 3.12 ppm) and H-1′b (δ: 2.74 ppm) protons in compound **[5]** resulted in different NOE enhancements at C5-Me group (9.2% and 1.6%, respectively, Figure 3, **[5]**). Proposed conformation is stabilized by the formation of hydrogen bond between hydroxyl proton and N1.

The long range proton-carbon $^3J_{H1'\underline{C}F3}$ coupling constant of 2.7 Hz in **[6]** clearly indicated *cis* geometry along the C1′=C2′ double bond (Figure 3, **[6]**) (Brandt *et al.*, 1999). The saturation of H-1′ proton showed strong NOE enhancement at C5-Me group of 14.0% which suggested that the propenyl group adopts a conformation in which the H-1′ proton is spatially close to C5-Me group. Such an orientation places the hydroxyl group in an orientation that is predisposed for the formation of hydrogen bond with N1 (Figure 3, **[6]**).

The saturation of H-1′a and H-1′b protons in **[4]** resulted in a strong and equal NOE enhancement at C5-Me group of 8.8%. Restricted rotation across the C-6-C-1′ bond is supported by N-1···H-O hydrogen bond. Saturation of H-1′a and H-1′b gave NOE enhancements at both CH_2F protons (3.1% and 2.9%, respectively) and C3′H_3 protons (2.6% and 3.7%, respectively), which indicated that H-1′a (δ: 2.83 ppm) is closer to the CH_2F group, while H-1′b (δ: 2.75 ppm) is spatially closer to the C3′H_3 protons. Individual saturations of H-1′a and H-1′b protons in **[12]** resulted in a comparable enhancement at the C5-Me group (5.0% and 3.8%, respectively), C-3′ methyl group (0.6% and 1.2%, respectively) and CH_2F group (0.2% and 1.8%, respectively), which led us to conclude that 2-fluoromethyl-propyl group predominantly adopts a conformation where it is turned away from the pyrimidine ring. The conformation along the single bonds of the 2-fluoromethyl-propyl group in compound **[12]** undergoes unrestricted rotation.

Biology

Antiviral activities

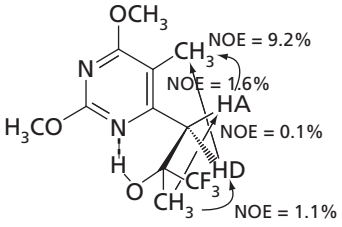
The compounds **[3]**-**[6]**, **[8]**-**[13]** and **[15]**-**[17]** were evaluated against HCMV in HEL cells and their activities were compared with those of ganciclovir (GCV) and (*S*)-1-[3-hydroxy-2-(phosphonylmethoxy)-propyl]cytosine (HPMPC, cidofovir, CDV) (Table 2). There was no significant activity of the studied compounds against both strains of HCMV. In addition, no specific antiviral effects were

Table 2. Activity of the C-6 alkylated pyrimidine derivatives of **[3]**–**[6]**, **[8]**–**[13]** and **[15]**–**[17]** against cytomegalovirus (CMV) in human embryonic lung (HEL) cells

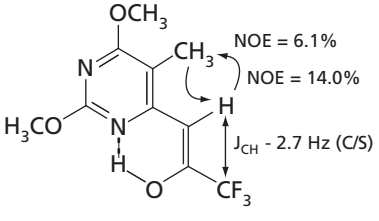
Compound	Antiviral activity, EC_{50} (μg/ml)*		Cytotoxicity (μg/ml)	
	AD-169 strain	Davis strain	Cell morphology (MCC)†	Cell growth (CC_{50})‡
[3]	>100	50	>100	>50
[4]	>20	>20	100	>50
[5]	>50	>50	>50	>50
[6]	50	32	>100	32
[8]	>100	37	>100	>50
[9]	>5	>5	20	48.5
[10]	>100	>100	>100	>50
[11]	>100	>100	>100	>50
[12]	ND§	ND	ND	ND
[13]	>100	>100	>100	>50
[15]	>100	>100	>100	>50
[16]	>100	>100	>100	>50
[17]	>100	>100	>100	>50
Ganciclovir	0.24	0.34	>400	67.3
Cidofovir	0.13	0.13	>400	27.5

*Effective concentration required to reduce virus plaque formation by 50%. Virus input was 100 plaque forming units (PFU). †Minimum cytotoxic concentration that causes a microscopically detectable alteration of cell morphology, MCC. ‡Cytotoxic concentration required to reduce cell growth by 50%, CC_{50}. §ND, not determined.

Figure 3. Predominant conformations of compounds **[5]** and **[6]** in solution as established by NOE and long range $^3J_{CH}$ coupling constants



[5]



[6]

The key NOE enhancements and long-range C-H coupling constant are shown. The proposed conformations are probably stabilised through the formation of N1···H-O hydrogen bond.

noted with the evaluated compounds against herpes simplex virus-1, (HSV-1 strain KOS), herpes simplex virus-2 (HSV-2 strain G), herpes simplex virus-1 (ACV-resistant KOS strain), vaccinia virus, vesicular stomatitis virus, Coxsackie virus B4, respiratory syncytial virus, parainfluenza-3 virus, reovirus-1, Sindbis virus, Punta Toro virus, HIV-1 and HIV-2.

Cytostatic activities

The compounds **[3]**-**[6]**, **[8]**-**[13]** and **[15]**-**[17]** were evaluated for their activities against human malignant tumour cell lines: cervical carcinoma (HeLa), breast carcinoma (MCF-7), pancreatic carcinoma (MiaPaCa-2), laryngeal carcinoma (Hep-2), colon carcinoma (SW 620) and normal fibroblasts (WI 38) (Table 3).

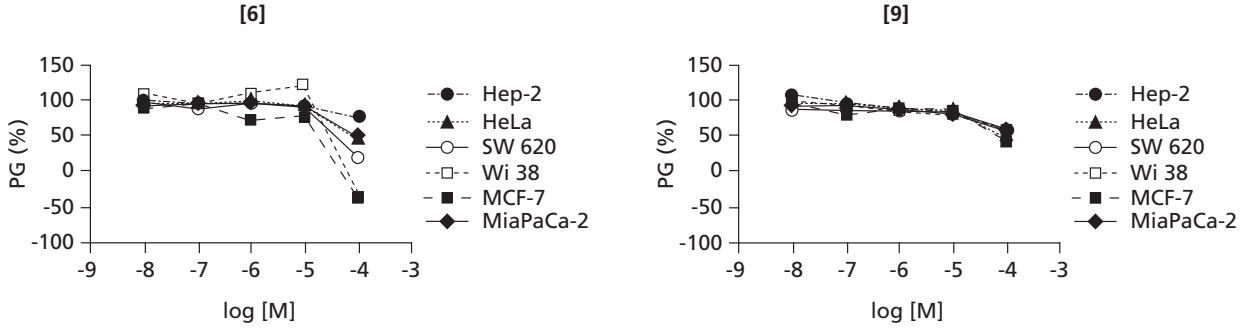
Amongst all evaluated compounds 2,4-dimethoxy pyrimidine derivatives **[6]** and **[9]** showed the highest cytostatic activity (Figure 4). Thus, compound **[6]** containing a trifluoro-1-propenyl side chain showed a rather marked inhibitory activity against MCF-7 cells (IC_{50}=8.38 μg/ml) and somewhat lower activity against HeLa (IC_{50}=22.11 μg/ml), MiaPaCa-2 (IC_{50}=25.42 μg/ml) and SW 620 (IC_{50}=16.01 μg/ml). However, compound **[6]** also exhibited cytostatic activity against normal human fibroblasts (WI 38) (IC_{50}=13.47 μg/ml). The 2,4-dimethoxy pyrimidine derivative **[9]** with a 2-fluoromethylpropyl side chain exhibited only moderate inhibitory effect against HeLa cells

Table 3. Inhibitory effects of the C-6 alkylated pyrimidine derivatives **[3]–[6]**, **[8]–[13]** and **[15]–[17]** on the growth of human malignant tumour cell lines and normal diploid fibroblasts (WI38)

	Tumour cell growth [IC_{50}* (μg/ml)]								
Compounds	L1210/0	MoltC4/C8	CEM/0	HeLa	MCF-7	MiaPaCa-2	Hep-2	SW 620	WI 38
[3]	≥200	≥200	≥200	>21.22	>100	>100	>21.22	>100	>100
[4]	>100	180 ±15	137 ±1	>24.42	>100	>24.42	>100	>24.42	>100
[5]	88 ±2	75 ±4	68 ±2	>28.02	>28.02	>100	>100	>100	>100
[6]	85 ±11	86 ±11	65 ±9	22.11	8.38	25.42	>25.42	16.01	13.47
[8]	146 ±47	124 ±16	105 ±0	>25.42	>25.42	>25.42	>25.42	>100	>100
[9]	30 ±1	23 ±1	22 ±2	19.73	≥28.63	>28.63	>28.63	>28.63	>28.63
[10]	>200	>200	>200	>200	>200	>22.62	>200	>200	>200
[11]	>200	>200	>200	>200	>18.42	>18.42	>200	>18.42	>200
[12]	>200	>200	>200	>200	>200	>200	>200	>200	>200
[13]	>200	>200	>200	>100	>21.62	>200	>21.62	>200	>200
[15]	>200	>200	>200	>200	>200	>200	>200	>200	>200
[16]	>200	>200	>200	>200	>200	>200	>200	>200	>23.26
[17]	>200	>200	>200	>21.02	>21.02	>21.02	>200	>200	>200

*50% inhibitory concentration, or compound concentration required to inhibit tumour cell proliferation by 50%, IC_{50}.

Figure 4. Dose-response profiles for compounds **[6]** and **[9]** on the panel 6-cell line tested *in vitro*.



The cells were treated with compounds **[6]** and **[9]** at different concentrations, and percentage of growth was calculated. Each point represents a mean value of four parallel samples in three individual experiments. PG, percentage of growth.

(IC_{50}=19.73 μg/ml) and minor cytostatic activity against normal fibroblasts.

Cell cycle analysis

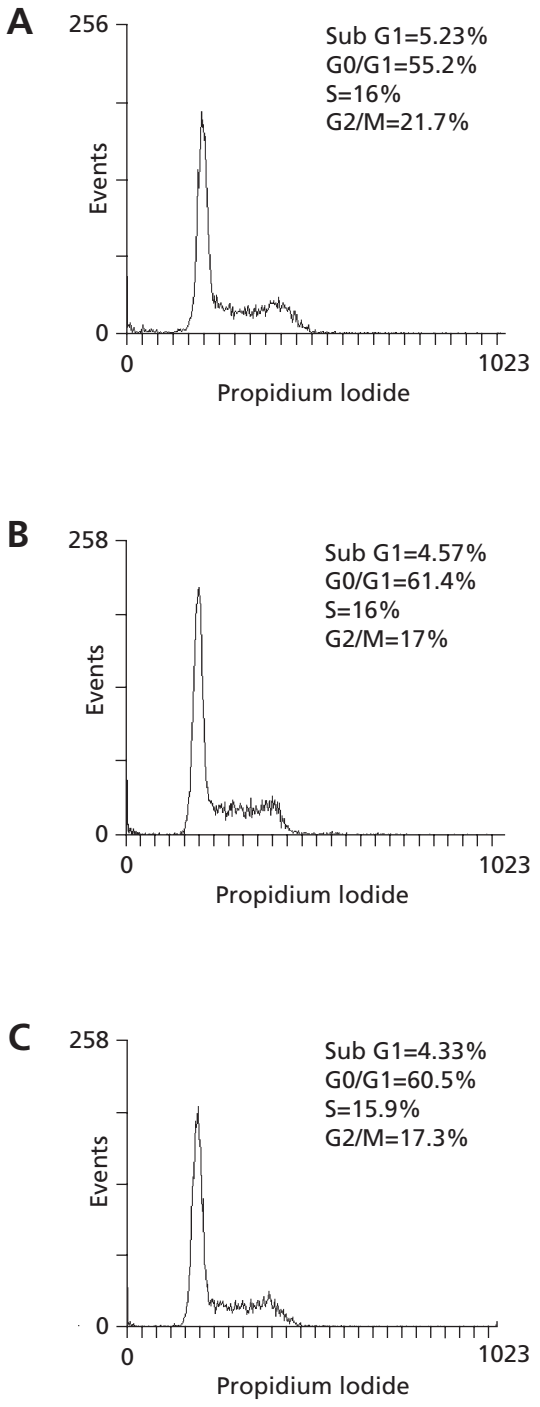
Flow cytometric analysis was performed after 72 h for compounds **[6]** and **[9]** at concentrations of $5x10^6$ and 10^5 mol/l, respectively, to identify whether the cell arrest was caused by a specific perturbation of cell cycle-related events. DNA contents of HeLa, MCF-7 (Figure 5) and WI 38 cells were measured.

The analysis showed that compound **[6]** increased the population of G0/G1 in MCF-7 cells and decreased the G0/G1 population in HeLa cells (Table 4). Similarly, compound **[9]** increased the G0/G1 population and decreased the G2/M phase in MCF-7 and caused no significant perturbations in HeLa cells (Table 4). However, perturbations in the cell cycle were also observed in WI 38 cells (Table 4).

Detection of apoptosis

Annexin V assay was performed on HeLa, MCF-7 and WI 38 cells after 72 hours of incubation with compound **[6]** at concentrations $5x10^6$ mol/l and 10^5 mol/l and with compound **[9]** at concentrations of 10^5 mol/l and $5x10^5$ mol/l. The results confirmed those obtained by flow cytometry (percentage of cells in subG1); the compounds

Figure 5. Histograms showing the DNA content in the cell cycle phases of MCF-7 cells



(A) Untreated; **(B)** Treated with compound **[6]** at a concentration of $5x10^{-6}$ mol/l; **(C)** Treated with compound **[9]** at a concentration of 10^{-5} mol/l.

[6] and **[9]** did not induce apoptosis in the tested cell lines at concentrations of $5x10^6$ and 10^5 mol/l, respectively (Table 5). However, the Annexin V assay revealed the induction of apoptosis at higher concentrations. Compound **[6]** induced apoptosis at a concentration of 10^5 mol/l; about 10.8% of apoptotic cells were detected in MCF-7 and 10.3% in HeLa cells. Also, 10% of apoptotic cells were detected in MCF-7 and 12.9% in HeLa after the treatment with compound **[9]** at a concentration of $5x10^5$ mol/l. Interestingly, the induction of apoptosis was observed in WI 38 cells only after the treatment with compound **[9]** at a concentration of $5x10^5$ M (Table 5).

Discussion

New types of C-6 alkylated pyrimidine derivatives (**[3]**–**[16]**) were prepared via lithiation of the 2,4-dimethoxy-5,6-dimethylpyrimidine **[2]** and subsequent nucleophilic addition or substitution reactions of the organolithium intermediate with various electrophiles.

Analysis of 1D difference NOE enhancements and long range coupling constants of the ^1H and ^{13}C NMR spectra indicated that compounds **[4]**–**[6]** exist in restricted conformation which is stabilized by a N-1···H-O hydrogen bond. On the contrary, compound **[12]** containing the acetyl group in the acyclic side chain possesses unrestricted conformation. The *cis* geometry along C1′=C2′ double bond in **[6]** was deduced from analysis of the long range proton-carbon coupling constants.

Amongst all evaluated compounds, the fluorinated acyclic pyrimidine derivatives **[6]** and **[9]** showed the best cytostatic activities. Compound **[6]** exhibited pronounced effect against breast carcinoma (MCF-7, IC_{50}=8.38 μg/ml). It seems that the increase of cell population in G0/G1 and subsequent induction of apoptosis at higher concentrations are the main mechanisms of cell growth arrest for compound **[6]** in MCF-7 cells. Apoptosis was also detected in HeLa cells but, contrary to the results for MCF-7, a decrease of G0/G1 cell population was observed. Compound **[9]** exhibited moderate cytostatic effect against cervical carcinoma (HeLa, IC_{50}=19.73 μg/ml). Interestingly, no cell cycle perturbations were observed in HeLa. The cell cycle perturbations in MCF-7 were characterized by a G0/G1 increase and a G2/M decrease. Apoptosis was detected both in HeLa and MCF-7 cells, but only at higher tested concentrations.

Acknowledgements

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Table 4. Flow cytometric analysis of compound **[6] (A)** and compound **[9] (B)** on HeLa, MCF-7 and WI 38 cells

(A)

	HeLa		MCF-7		WI 38	
Cell cycle phase	Control	$5x10^{-6}$ mol/l	Control	$5x10^{-6}$ mol/l	Control	$5x10^{-6}$ mol/l
SubG1	9%	12.7%	5.2%	4.6%	6.9%	9.3%*
G0/G1	65.5%	60.8%*	55.2%	61.4%*	62.4%	62.2%
S	8.5%	6.6%*	16.3%	16%	7.25%	7.6%
G2/M	17.2%	17.9%	21.7%	17%	21.15%	19.5%*

(B)

	HeLa		MCF-7		WI 38	
Cell cycle phase	Control	10^{-5} mol/l	Control	10^{-5} mol/l	Control	10^{-5} mol/l
SubG1	9%	7.9%	5.23%	4.33%	6.9%	7.5%
G0/G1	65.5%	63.6%	55.2%	60.5%*	62.4%	63.7%
S	8.5%	8.8%	16.3%	15.9%	7.2%	8.4%*
G2/M	17.2%	17.3%	21.7%	17.3%*	21.1%	19.1%*

The results are shown as percentages of cells in a particular cell cycle phase. *Statistically significant at $P<0.05$

Table 5. Percentage of apoptotic cells after 72 h in HeLa and MCF-7 treated with compound **[6]** at a concentration of 10^{-5} mol/l and **[9]** at a concentration of $5x10^{-5}$ mol/l

		Apoptotic cells (%)		
Compound	treatment	HeLa	MCF-7	WI 38
	control	1	1	2
[6]	10^{-5} mol/l	10.3	10.7	3.8
[9]	$5x10^{-5}$ mol/l	12.9	10	8.2

cell activity assays, as well as Ann Absillis, Anita Van Lierde, Frieda De Meyer, Anita Camps and Lies Vandenheurck for excellent technical assistance in performing the antiviral activity assays.

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