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The novel pyrimidine and purine derivatives of L-ascorbic acid: synthesis, one- and two-dimensional ^{1}H and ^{13}C NMR study, cytostatic and antiviral evaluation

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Abstract—The syntheses of the novel C-5 substituted pyrimidine derivatives of L-ascorbic acid containing free hydroxy groups at C-2′ (**6**–**10**) or C-2′ and C-3′ (**11**–**15**) positions of the lactone ring are described. Debenzylation of the 6-chloro- and 6-(*N*-pyrrolyl)purine derivatives of 2,3-*O*,*O*-dibenzyl-L-ascorbic acid (**16** and **17**) gave the new compounds containing hydroxy groups at C-2′ (**18**) and C-2′ and C-3′ (**19** and **20**). *Z*- and *E*-configuration of the C4′=C5′ double bond and position of the lactone ring of the compounds **6**–**9** were deduced from their one- and two-dimensional ^{1}H and ^{13}C NMR spectra and connectivities in NOESY and HMBC spectra. Compounds **15** and **18** showed the best inhibitory activities of all evaluated compounds in the series. The compound **15** containing 5-(trifluoromethyl)uracil showed marked inhibitory activity against all human malignant cell lines (IC_{50}: 5.6–12.8 μM) except on human T-lymphocytes. Besides, this compound influenced the cell cycle by increasing the cell population in G2/M phase and induced apoptosis in SW 620 and MiaPaCa-2 cells. The compound **18** containing 6-chloropurine ring expressed the most pronounced inhibitory activities against HeLa (IC_{50}: 6.8 μM) and MiaPaCa-2 cells (IC_{50}: 6.5 μM). The compound **20** with 6-(*N*-pyrrolyl)purine moiety showed the best differential inhibitory effect against MCF-7 cells (IC_{50}: 35.9 μM).

1. Introduction

L-Ascorbic acid (vitamin C) is a biologically significant reducing agent due to its 1-oxo-2-ene-2,3-diol structure element.[1] The vitamin C is involved in a variety of key physiological processes,[2] for example, the production of collagen.[3] It is also considered to be important in the prevention of various chronic diseases such as cancer, cerebral apoplexy, diabetes, atopic dermatitis, myocardial infarction and AIDS.[4] L-Ascorbic acid functions as an electron donor and antioxidant for several enzymes and is implicated in host defence mechanisms, endocrine system function and other biological processes.[5] The hydroxy groups of the lactone ring of L-ascorbic acid at the atoms C-2′ or C-3′ were found to be particularly important to maintain its inherent biological activity in vivo.[6–10] On the contrary, the 6-hydroxy group of L-ascorbic acid proved to be unimportant in transport and function of this vitamin.[11] The 2′-hydroxy group is at the focus of the reaction site in redox processes of L-ascorbic acid and is required for the reducing properties of vitamin C. Thus, the chemical modification of the hydroxy group at C-2′ should have a marked influence on the biological properties of L-ascorbic acid.[12,13] Vitamin C has also been considered as a useful synthetic precursor to many molecules of potential biological utility on account of its inherent, varied chemical functionality.[14–16] Unsaturated analogues of nucleosides are a focus of much attention as antiviral and antitumour agents.[17–21]

Keywords: Pyrimidine and purine derivatives of L-ascorbic acid; *E* and *Z* isomers; Cytostatic activity; Antiviral activity; Apoptosis.
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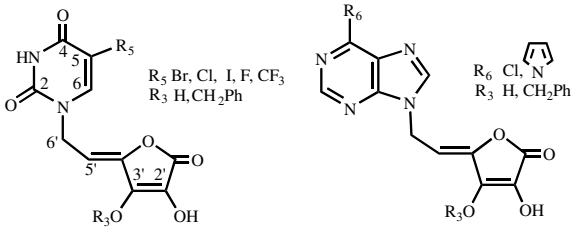


Figure 1. Pyrimidine (**6**–**15**) and purine (**18**–**20**) derivatives of 3-*O*-benzyl-2-hydroxy- and 2,3-dihydroxy-4,5-didehydro-5,6-dideoxy-L-ascorbic acid.

We have found that some pyrimidine and purine derivatives of 2,3-*O*,*O*-dibenzyl-4,5-didehydro-5,6-dideoxy-L-ascorbic acid exerted pronounced cytostatic activities against some malignant tumour cell lines.[22,23] It has also been found that the biological activity of such type of purine compounds has its origin in the reactivity of their double bond conjugated with the lactone ring toward biological nucleophiles.[24]

Taking into account the pharmacological potential of this class of compounds, we have synthesized the novel pyrimidine and purine derivatives of 3-*O*-benzyl-2-hydroxy- (**6**–**10** and **18**) and 2,3-dihydroxy-4,5-didehydro-5,6-dideoxy-L-ascorbic acid (**11**–**15**, **19** and **20**, Fig. 1) in order to evaluate their cytostatic and antiviral activities.

2. Results and discussion

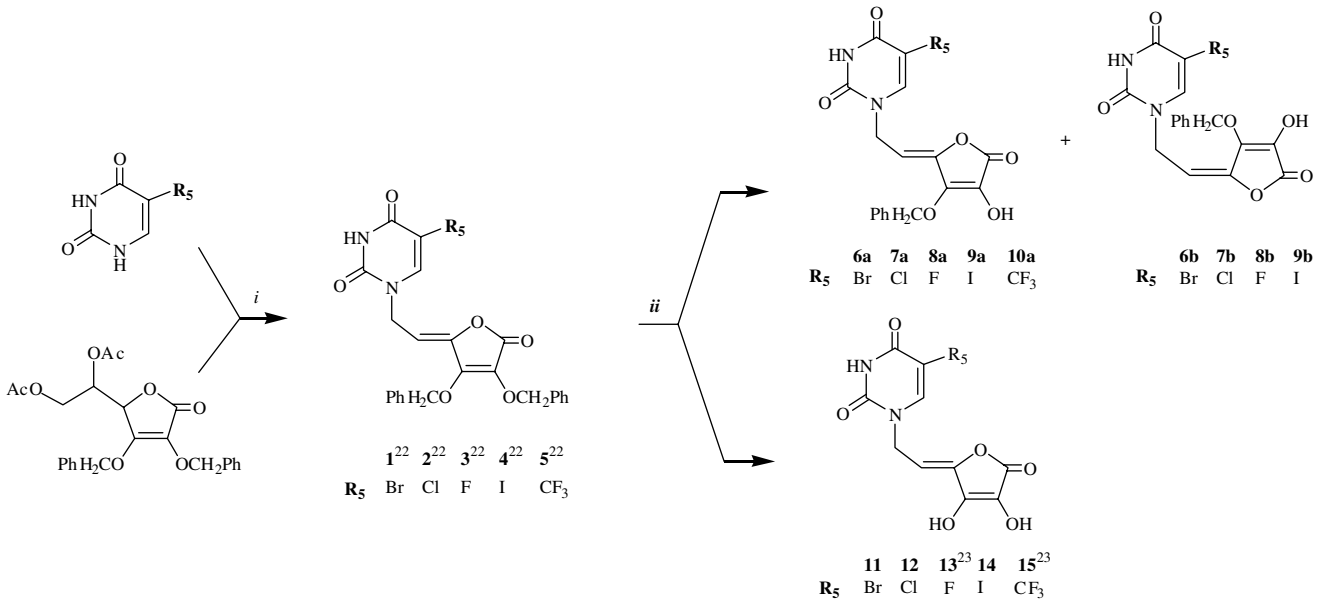
2.1. Chemistry

The 5-substituted pyrimidine (**1**–**5**) and purine (**16** and **17**) derivatives of 2,3-*O*,*O*-dibenzyl-4,5-didehydro-5,6-dideoxy-L-ascorbic acid were prepared by condensation reaction of the silylated uracil and purine derivatives with L-ascorbic acid derivatives with protected hydroxy groups as described previously.[22,23] ^{1}H NMR spectra indicated that the starting compounds **1**–**4** exist as *Z* and *E* isomers in which the *Z* isomer predominates (approximately in the range 80–90%). Debenzylation of the condensated products (**1**–**5**, **16** and **17**) by boron trichloride afforded monobenzylated 3-*O*-benzyl-2-hydroxy pyrimidine **6**–**10** (Scheme 1) and purine derivative **18** (Scheme 2), as well as 2,3-dihydroxy pyrimidine **11**, **12** and **14** (Scheme 1) and purine derivatives **19** and **20** (Scheme 2) of L-ascorbic acid. The monobenzylated pyrimidine derivatives **6**–**9** were found to exist as a mixture of *Z* (**a** series) and *E* (**b** series) isomers in which prevails the isomer **a**.

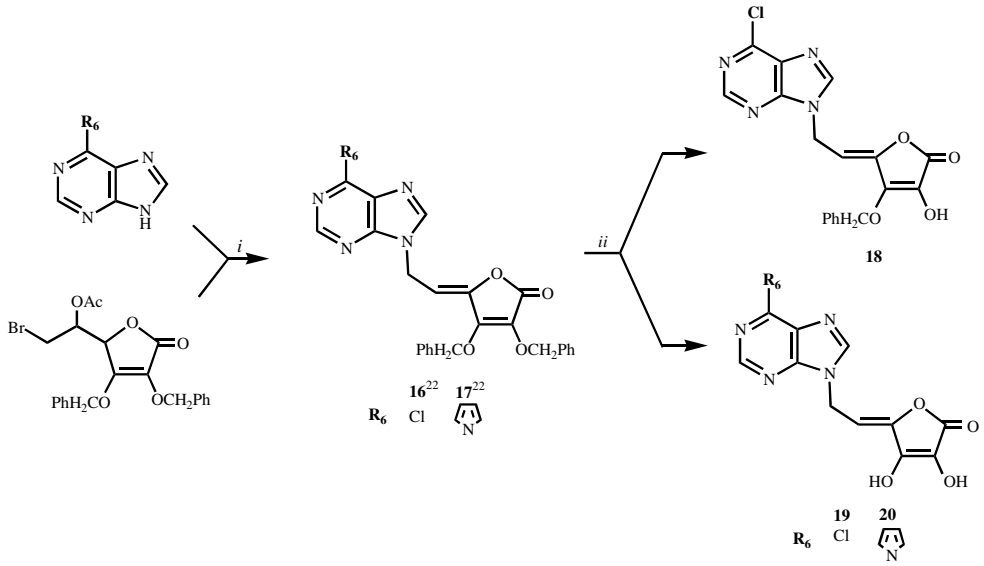
2.2. ^{1}H and ^{13}C NMR studies

Assignment of ^{1}H and ^{13}C resonances was performed with the use of decoupling experiments and 2D NOESY and HMBC correlation experiments (Tables 1 and 2).

The perusal of ^{1}H NMR spectrum of sample of **7** clearly showed the presence of two stereoisomers *Z* and *E*, designated as **7a** and **7b**. The prevailing isomer **7a** was present in 76%. Several resonances showed distinct chemical shifts for both isomers (Table 1). Analysis of the long-range carbon–proton correlations showed that benzyl group is attached to C3′ in both products. HMBC spectrum for **7a** showed the following cross-peaks: C*H*$_2$–Ph to C3′, H5′ to C3′ and H6′ to C3′. Similarly, cross-peaks of C*H*$_2$–Ph to C3′ and H5′ to C3′ were observed for **7b**. No NOE interactions between H6 and protons of benzyl group in both products were observed. On the other hand, H6 showed strong NOE interactions with H6′ and H5′ in both isomers. H6′ pro-



Scheme 1. Procedure for preparation of pyrimidine derivatives of 3-*O*-benzyl-2-hydroxy- (**6**–**10**) and 2,3-dihydroxy-4,5-didehydro-5,6-dideoxy-L-ascorbic acid (**11**–**15**). Reaction conditions: (i) HMDS, $(NH_4)_2SO_4$/Ar atmosphere/reflux/3 h, then trimethylsilyl triflate/anhydrous CH_3CN/55–70 °C/12 h; (ii) BCl_3, CH_2Cl_2/−40 °C/3 h. The predominant *Z* isomer of **1**–**4** was depicted only. Compound **5** was found to exist solely as *Z* isomer.



Scheme 2. Procedure for preparation of purine derivatives of 3-*O*-benzyl-2-hydroxy- (**18**) and 2,3-dihydroxy-4,5-didehydro-5,6-dideoxy-L-ascorbic acid (**19** and **20**). Reaction conditions: (i) Et_3N/anhydrous DMF/70 °C/11 h; (ii) BCl_3, CH_2Cl_2/−78 °C/3 h.

Table 1. Selected chemical shifts (δ/ppm)[a] and $^3J_{HH}$ coupling constants (J/Hz) in ^{1}H NMR spectra of *Z*- (**a**) and *E*- (**b**) isomers of **6**–**9** (cf. Scheme 1)

Compound	H6	NH	H5′	H6′	CH_2Ph	$^3J_{H5'-H6'}$
6a	8.17	11.78	5.36	4.50	5.46	6.8
6b	8.00	11.78	5.61	4.69	5.52	7.3
7a	8.11	11.81	5.36	4.50	5.46	6.8
7b	7.93	11.65	5.61	4.68	5.52	7.3
8a	8.04	11.78	5.33	4.44	5.45	6.8
8b	7.86	11.78	5.58	4.62	5.52	7.4
9a	8.15	11.64	5.34	4.49	5.45	6.7
9b	8.00	11.64	5.59	4.68	5.51	7.6

[a] (DMSO-d_6), chemical shifts referred to TMS.

tons in isomer **7b** in turn showed NOE contacts with phenyl protons. This NOE interaction suggested that H6′ and benzyl group are on the same side of the double bond, which led us to conclude that **7b** exhibits *E* configurations across the C4′–C5′ double bond. Furthermore, almost all ^{13}C chemical shifts were comparable for both products ($\Delta\delta < 0.5$ ppm), except for C5′ where they differed by approximately 5 ppm (Table 2). The major product **7a** exhibited the *Z* configuration across C4′–C5′ bond. Probable NOE interactions between H5′ and methylene protons of benzyl group in isomers **7a** and **7b** could not be unequivocally established due to their small chemical shift difference ($\Delta\delta \approx 0.1$ ppm). This observation was also true for analogues **6**, **8** and **9**. Protons H6′ of isomers **7a** and **7b** were isochronous, which suggested fast rotation across C5′–C6′–N1 bonds. This observation was supported with $^3J_{H5'-H6'}$ coupling constants of 6.8 Hz for isomer **7a** and 7.3 Hz for isomer **7b**, which are typical for proton–proton coupling constants along freely rotatable bonds.

The NMR analysis of **6**, **8** and **9** was done in an analogous way as described above for **7**. The ratio of two isomers **a** and **b** was found by ^{1}H NMR to be 10:1 for **6**, **8** and **9**. Analysis of the long-range carbon–proton correlations (HMBC) and comparison of ^{1}H and ^{13}C chemical shifts to other analogues showed that the benzyl group is attached to the atom C3′ in both **a** and **b** isomers of **6**, **8** and **9** (Tables 1 and 2). Based on NMR data, major isomers **6a** and **9a** were assigned to have *Z* configuration across the C4′–C5′ bond, whereas minor isomers **6b** and **9b** exhibited the *E* configuration across C4′–C5′ bond (Fig. 2).

NMR data for **20** showed the presence of single species: aromatic H8 showed NOE contacts with H6′ and H5′. Although no benzyl group is present in **20**, chemical

Table 2. Selected chemical shifts (δ/ppm)[a] in ^{13}C NMR spectra *Z*- (**a**) and *E*- (**b**) isomers of **6**–**9** (cf. Scheme 1)

Compound	C1′	C4	C2	C4′	C6	C2′	C3′	C_1Ph	C5	C5′	CH_2Ph	C6′
6a	164.66	159.69	150.18	143.60	145.06	123.39	141.18	136.12	94.98	100.88	72.21	43.16
6b	164.66	159.69	150.18	143.40	144.63	123.39	141.26	136.12	94.98	106.13	72.21	43.40
7a	164.65	159.52	149.96	143.61	142.70	123.37	141.16	136.12	106.34	100.83	72.51	43.16
7b	164.65	159.52	149.62	143.41	142.27	123.37	141.67	136.12	106.34	106.07	72.20	43.16
8a	164.64	157.29	149.44	143.65	129.82	123.36	141.15	136.11	138.15	100.74	72.18	43.07
8b	164.64	157.29	149.44	143.65	129.82	123.36	141.15	136.11	138.15	106.00	72.18	43.07
9a	164.64	161.06	150.50	143.51	149.61	123.37	141.17	136.11	68.32	101.03	72.17	43.07
9b	164.64	161.06	150.50	143.51	149.18	123.37	141.17	136.11	68.53	106.28	72.48	43.32

[a] (DMSO-d_6), chemical shifts referred to TMS.

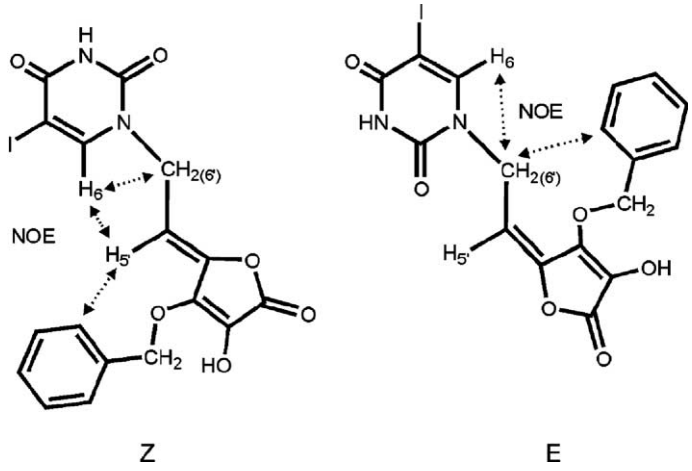


Figure 2. Selected key NOE contacts in *Z* and *E* isomers of **9**.

shifts for C2′ and C3′ are almost identical. In fact, perusal of chemical shift data (Section 4) shows that chemical shifts of C2′ and C3′ in the studied compounds change only marginally upon removal of the benzyl groups.

Protons H6′ of **20** and **a** and **b** isomers of **6**, **8** and **9** were isochronous, which suggests fast rotation across the C5′–C6′–N1 bonds. In full agreement, $^{3}J_{H5'-H6'}$ 7.1 Hz (**20**), 6.8 Hz (**6a**), 7.3 Hz (**6b**), 6.8 Hz (**8a**), 7.4 Hz (**8b**), 6.7 Hz (**9a**) and 7.6 Hz (**9b**) were measured (Table 1). In addition, the following scalar coupling constants were measured for **8**: $^{4}J_{NH-F}$ of 5.2 Hz (**8a**), $^{3}J_{H6-F}$ of 6.7 Hz (**8a**) and 6.7 Hz (**8b**), $^{1}J_{C5-F}$ of 220 Hz (**8a**) and $^{2}J_{C6-F}$ of 34 Hz (**8a**).

2.3. Cytostatic and antiviral activities

Compounds **6**–**15** and **18**–**20** were evaluated for their cytostatic activity against malignant tumour cell lines: murine leukaemia (L1210), human T-lymphocytes (Molt4/C8 and CEM), cervical carcinoma (HeLa), breast carcinoma (MCF-7), pancreatic carcinoma (MiaPaCa-2), laryngeal carcinoma (Hep-2), colon carcinoma (SW620) and human normal fibroblasts (WI38) (Table 3). Cytostatic activities of conjugated compounds **6**–**15** and **18**–**20** were compared with those of 5-fluoro-uracil (**5-FU**), 5-(trifluoromethyl)uracil (**5-TFMU**), 6-chloropurine (**6-ClPu**), L-ascorbic acid (**AA**) and 2,3-dibenzyl-L-ascorbic acid (**DiBnAA**).

Among the pyrimidine derivatives of L-ascorbic acid, 5-fluoro-uracil (**13**) and 5-(trifluoromethyl)uracil (**15**) derivatives of 2,3-dihydroxy-L-ascorbic acid showed rather marked cytostatic activities. Compound **13** had the highest inhibitory activity against murine leukaemia (L1210) cells (IC_{50}: 5.2 μM), while **15** showed pronounced inhibitory activity against all human malignant cell lines (IC_{50}: 5.6–12.8 μM) except for human T-lymphocytes. However, these compounds also exhibited

Table 3. Inhibitory effects of **6**–**15** and **18**–**20** on the growth of malignant tumour cell lines and normal human fibroblasts (WI 38)

Compound	IC_{50}[a] (μM)								
	L1210	Molt4/C8	CEM	HeLa	MCF-7	MiaPaCa-2	Hep-2	SW 620	WI 38
6	>200	>200	>200	>200	>200	>200	>200	>200	>200
7	>200	170 ± 24	175 ± 24	>200	>200	>200	>200	>200	>200
8	33.3 ± 5.5	>200	>200	⩾100	86 ± 29.4	>200	⩾100	>200	26.4 ± 13.6
9	180 ± 45	177.4 ± 25.6	181.6 ± 10.7	>200	>200	>200	>200	>200	>200
10	>122	>122	>122	>200	>200	>200	⩾100	>200	>200
11	>76	>76	>76	>200	>200	>200	>200	>200	>200
12	196 ± 7	>200	>200	>200	>200	>200	>200	>200	>200
13[b]	5.2 ± 2.2	118 ± 87	77.4 ± 4.8	50 ± 5.2	59.3 ± 34.1	58.1 ± 7.4	>100	>100	42.6 ± 23.3
14	>200	>200	>200	>200	>200	>200	>200	>200	>200
15[b]	>200	>200	>200	5.6 ± 1.6	8.8 ± 1.3	12.8 ± 12.5	5.6 ± 2.5	8.8 ± 5.0	11.6 ± 10.9
18	21.8 ± 1.0	19.8 ± 0.3	22.9 ± 0	6.8 ± 4.2	14.3 ± 14.6	6.5 ± 1.0	14.8 ± 3.4	20.0 ± 9.9	16.1 ± 1.6
19	>200	>200	>200	66.9 ± 14.9	>100	>100	>100	>100	>100
20	>200	>200	>200	⩾100	35.9 ± 32.9	>100	>100	>100	>100
5-FU	0.69 ± 0.01	20 ± 0.76	9.23 ± 3.08	16 ± 15	4.5 ± 2	6.5 ± 0.5	51 ± 34	8.7 ± 12	10 ± 9
5-TFMU	>200	>200	>200	>200	>200	>200	>200	>200	>200
6-ClPu	>200	>200	>200	>100	>200	>200	>100	>200	>200
AA	>200	>200	>200	>200	>200	>200	>200	>100	>200
DiBnAA	199.4 ± 143.2	143.2 ± 25.2	151.7	>100	>200	>100	>200	>100	>100

[a] The concentration that causes 50% growth inhibition.
[b] The cytostatic effects of **13** and **15** against L1210, Molt4/C8 and CEM cells were published.

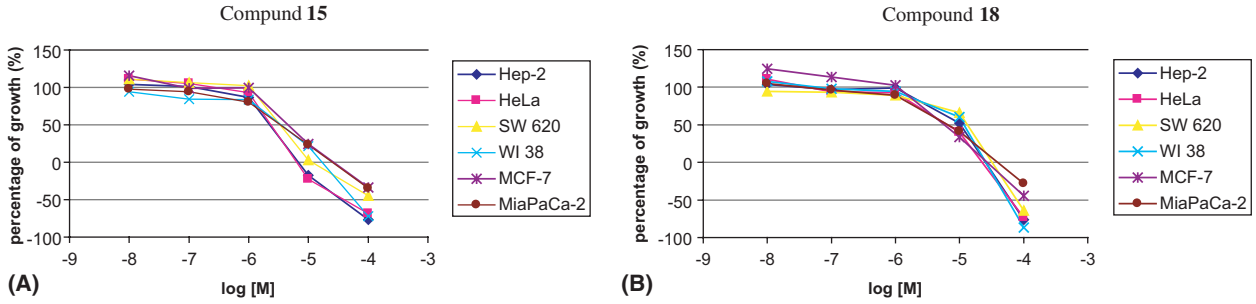


Figure 3. Dose–response profiles for compounds **15** (A) and **18** (B).

cytostatic activities against normal human fibroblasts (WI38) (Fig. 3A). 5-Fluoro-uracil derivative of L-ascorbic acid with 2-hydroxy group free (**8**) was approximately 6-fold less active against L1210 cells than 2,3-dihydroxy derivative (**13**). The compound **18** containing 6-chloropurine moiety exhibited the best cytostatic effect against MiaPaCa-2 (IC_{50}: 6.5 μM) and HeLa cells (IC_{50}: 6.8 μM) (Fig. 3B). Its dihydroxy derivative **19** showed only slight inhibition of the growth of HeLa cells (IC_{50}: 66.9 μM). The compound **20** with the 6-(*N*-pyrrolyl)purine moiety showed only moderate antitumoral activity against MCF-7 (IC_{50}: 35.9 μM).

Comparison of the cytostatic activities of the pyrimidine and purine derivatives of 2,3-*O*,*O*-dibenzyl-L-ascorbic acid (**1**–**5**, **16** and **17**)[22] revealed that these compounds exhibited better inhibitory effects on the growth of malignant tumour cell lines than the corresponding 2,3-dihydroxy compounds (**11**–**15**, **19** and **20**), except for **13** and **15**. 5-Fluoro-uracil (**13**) and 5-(trifluoromethyl)uracil (**15**) containing 2,3-dihydroxy-L-ascorbic acid moiety showed better cytostatic effects against most of the cell lines than their 2,3-*O*,*O*-dibenzylated derivatives (**8** and **10**).

5-Fluoro-uracil (**5-FU**) showed better cytostatic activities against all cell lines, particularly against leukaemia cells (IC_{50}: 0.69 μM), than its conjugated derivatives of L-ascorbic acid (**8** and **13**). However, **5-FU** showed also cytotoxic effect against normal fibroblasts (WI38). On the contrary to this, other pyrimidine (**5-TFMU**) and purine (**6-ClPu**) bases, as well as L-ascorbic acid (**AA**) and L-ascorbic acid with protected hydroxy groups of the lactone ring (**DiBnAA**) did not exhibit any or exhibit slight antitumoral activities. Substitution of bromine, chlorine and iodine at C-5 position of pyrimidine ring had no influence on cytostatic effect.

We can conclude that 5-(trifluoromethyl)uracil (**15**) and 6-chloropurine (**18**) derivatives of L-ascorbic acid had marked cytostatic activity, while its entities **5-TFMU**, **6-ClPu** and **AA** did not show any effects.

Flow cytometric analysis was performed for compounds **15** and **18** at concentrations 5×10^{-6} and 10^{-5} mol/L after 24, 48 and 72 h to identify whether the cell growth was caused by specific perturbation of cell cycle-related events. DNA contents of MiaPaCa-2 and SW620 were measured. DNA histograms of both cell lines showed that compound **15** increased the population of subG1 (apoptotic cells) and G2/M cells and decreased the population of G0/G1 cells at concentration 10^{-5} mol/L after 72 h (Table 4). No significant changes were observed in the cell cycle population after treatment with compound **18** (data not shown).

Annexin V assay was performed on SW620 and MiaPaCa-2 cells after 24 and 72 h of incubation with compound **15** at concentrations 5×10^{-6} and 10^{-5} mol/L. The results confirmed those obtained by flow cytometry; the compound **15** induced apoptosis in both cell lines at concentration 10^{-5} mol/L (Table 5). However, unlike the flow cytometric analysis, the Annexin V assay revealed about 9% apoptotic cells even after treatment with the lower concentration. Interestingly, the percentage of apoptotic cells did not vary substantially between tested concentrations, except in SW620 after 72 h (9.8% vs 14.5%).

Compounds **7**–**9**, **14**, **19** and **20** were also evaluated against herpes simplex virus type 1 and 2, vaccinia virus, cytomegalovirus, varicella-zoster virus, vesicular stomatitis virus, Coxsackie virus B4, respiratory syncytial virus, parainfluenza-3 virus, reovirus-1, Sindbis virus and Punta Toro virus. None of the evaluated

Table 4. Flow cytometric analysis of the cell cycle after the treatment of MiaPaCa-2 and SW620 cell lines with the compound **15**

Cell cycle phase (%)	MiaPaCa-2			SW620		
	Control	5×10^{-6} mol/L	10^{-5} mol/L	Control	5×10^{-6} mol/L	10^{-5} mol/L
Sub G1	1.75 ± 0.28	2.06 ± 0.80	4.51 ± 0.11[a]	0.45 ± 0.15	0.67 ± 0.21	2.81 ± 0.30[a]
G0/G1	83.38 ± 1.16	83.38 ± 2.81	78.32 ± 1.50[a]	67.46 ± 3.19	68.64 ± 3.35	58.28 ± 2.23[a]
S	4.30 ± 0.14	2.93 ± 0.50[a]	4.03 ± 0.96	13.02 ± 0.98	8.60 ± 1.54[a]	12.16 ± 0.88
G2/M	9.61 ± 1.14	9.18 ± 1.46	12.37 ± 0.89[a]	18.19 ± 2.02	21.55 ± 2.80	25.48 ± 1.96[a]

Results represent means of three experiments ± standard deviation.
[a] Statistically significant at $p < 0.05$.

Table 5. Percentage of apoptosis induced by compound **15** in MiaPaCa-2 and SW620 cells

Compound **15**	MiaPaCa-2		SW620	
	24 h	72 h	24 h	72 h
Control	2%	1%	1%	3%
5×10^{-6} mol/L	8.5%	9%	9.7%	9.5%
10^{-5} mol/L	8.7%	10.5%	9.8%	14.4%

compounds showed appreciable antiviral activity at subtoxic concentrations, except for the slight activity of **9** (IC_{50}: 102.6 μM) against vesicular stomatitis virus in HeLa cell cultures.

3. Conclusions

The 5-substituted pyrimidine and purine derivatives of 3-*O*-benzyl-2-hydroxy-L-ascorbic acid (**6**–**10** and **18**) and 2,3-dihydroxy-L-ascorbic acid (**11**–**15**, **19** and **20**) were prepared by debenzylation of the condensated products (**1**–**6**, **16** and **17**) with boron trichloride. The compounds **6**–**9** were established from NOE interactions in NOESY spectra and heteronuclear correlations in HMBC spectra to be a mixture of *Z* and *E* isomers at a 10:1 ratio, except for **7** in which the prevailing isomer *Z* was present in 76%.

The 6-chloropurine derivative of L-ascorbic acid (**18**) showed the most pronounced cytostatic activity against pancreatic carcinoma (MiaPaCa-2) (IC_{50}: 6.5 μM) and cervical carcinoma (HeLa) cells (IC_{50}: 6.8 μM). However, this compound was also cytostatic towards human normal fibroblasts (WI38). Compound **15** showed a marked cytostatic activity against all human malignant cell lines (IC_{50}: 5.6–12.8 μM) except for human T-lymphocytes. Besides, this compound influenced the cell cycle by increasing the cell population in G2/M phase and induced apoptosis in SW620 and MiaPaCa-2 cells. The compound **20** containing 6-(*N*-pyrrolyl)purine moiety exhibited moderate activity against breast carcinoma (MCF-7) cells. Effects of conjugation of nucleoside bases with L-ascorbic acid was observed for 5-(trifluoromethyl)uracil (**15**) and 6-chloropurine (**18**) derivatives of L-ascorbic acid. The evaluated compounds did not exhibit appreciable antiviral activity at subtoxic concentrations, except for the monobenzylated 5-iodo-uracil derivative **9**, which showed slight activity against vesicular stomatitis virus (IC_{50}: 102.6 μM).

4. Experimental

4.1. General methods

New compounds were characterized by ^1H and ^{13}C NMR, electron impact mass and UV spectra. Melting points were determined on a Kofler micro hot-stage instrument (Reichter, Wien) and are uncorrected. 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 was performed using silica gel (0.05–0.2 mm) Merck; glass column was slurry packed under gravity. Solvent systems used for column chromatography were CH_2Cl_2–MeOH, 30:1 (S_1) and 40:1 (S_2). Additional purification of compounds by recrystallization from ethanol afforded the analytical samples.

4.2. NMR measurements

All ^1D and ^2D NMR spectra were acquired with high-resolution NMR Spectrometer Varian Unity Inova 300 (^1H at 298.64 MHz, ^{13}C at 75.10 MHz). DMSO-d_6 (99.8% deuterium) was used as solvent with tetramethylsilane as internal standard. Samples were dissolved in DMSO-d_6 with concentrations of 6–7 mg in 0.5 mL. The sample temperature was set at 298 K and controlled to approximately ±0.5 K. ^1H NMR measurements were performed under the following spectral and processing conditions: 4.0 kHz sweep width, 90° pulse (11 μs), 2 s relaxation delay, 32 K time domain, zero filling to 64 K and line broadening of 0.5 Hz. ^2D NMR spectra: HMBC: [^1H–^{13}C] with gradients for coherence selection; 8192 ($\omega 2$) × 256 ($\omega 1$) data points, 32 scans per FID, 16 dummy scans, a pulse delay of 2 s, appropriate delays were calculated from $^1J_{CH}$ = 140 Hz and $^nJ_{CH}$ = 8 Hz, 4.0 kHz ($\omega 2$) × 16.5 kHz ($\omega 1$) spectral width transformed after multiplication with a sine square filter in both $\omega 2$ and $\omega 1$ to give $4K \times 1K$ matrix. NOESY: 4096 ($\omega 2$) × 256 ($\omega 1$) data points, 16 scans per FID, 32 dummy scans, a pulse delay of 2 s, with mixing time of 150 ms, 4.0 kHz ($\omega 2$) × 4.0 kHz ($\omega 1$) spectral width, transformed after multiplication with a sine-bell squared filter shifted by π/2 in both $\omega 2$ and $\omega 1$ to give $4K \times 1K$ matrix.

4.2.1. 1-[2,4-Dioxo-5-bromo-(1*H*,3*H*)-pyrimidin-1-yl]-2-(3-*O*-benzyl-2-hydroxy-2-butene-4-olidylidene)ethane (6) and 1-[2,4-dioxo-5-bromo(1*H*,3*H*)-pyrimidin-1-yl]-2-(2,3-dihydroxy-2-butene-4-olidylidene)ethane (11). To a stirred solution of **1** (200 mg, 0.42 mmol) in anhydrous CH_2Cl_2 1 M solution of BCl_3 in CH_2Cl_2 (0.5 mL) was added under argon at −78 °C. The reaction mixture was stirred at −40 °C for 4 h, and then 1 M solution of BCl_3 in CH_2Cl_2 (0.5 mL) was added. The reaction mixture was additionally stirred at 0 °C for 2 h, then the temperature was raised to room temperature and stirred overnight. A solvent mixture of CH_2Cl_2/MeOH (1:1) was added to deactivate unreacted BCl_3 and the solvent was then removed under reduced pressure. The oily residue was purified by column chromatography (S_1) to yield **6a** and **6b** as a mixture at 10:1 ratio (42 mg, 23.7%) and **11** (8 mg, 5.8%). Compound **6**: UV (MeOH) λ_{max} (log ε) 206, 256 (2.88, 2.61); **6a**: ^{13}C NMR (DMSO-d_6): δ 164.66 (C-1′), 159.69 (C-4), 150.18 (C-2), 123.39 (C-2′), 143.60 (C-4′), 136.12 (C_1Ph), 141.18 (C-3′), 94.98 (C-5), 145.06 (C-6), 100.88 (C-5′), 72.21 (CH_2Ph), 43.16 (C-6′). Compound **6b**: 164.66 (C-1′), 123.39 (C-2′), 141.26 (C-3′), 136.12 (C_1Ph), 106.13 (C-5′), 72.21 (CH_2Ph), 43.40 (C-6′); MS *m*/*z* 420.0 ($M^{+\cdot}$). Compound **11**: UV (MeOH) λ_{max} (log ε) 208, 256 (2.64, 2.56); ^1H NMR (DMSO-d_6): δ 7.55 (H-6, 1H, s), 11.33 (N*H*, 1H, s), 5.45 (H-5′, 1H, t, *J* = 6.75 Hz), 4.57 (H-6′, 2H, d, *J* = 6.81); ^{13}C NMR (DMSO-d_6): δ 164.67 (C-1′), 159.71 (C-4), 150.20 (C-2), 123.12 (C-2′), 143.17 (C-4′),

141.16 (C-3′), 94.87 (C-5), 144.58 (C-6), 102.35 (C-5′), 43.17 (C-6′); MS *m*/*z* 329.9 ($M^{+\cdot}$).

4.2.2. 1-[2,4-Dioxo-5-chloro-(1*H*,3*H*)-pyrimidin-1-yl]-2-(3-*O*-benzyl-2-hydroxy-2-butene-4-olidylidene)ethane (7) and 1-[2,4-dioxo-5-chloro(1*H*,3*H*)-pyrimidin-1-yl]-2-(2,3-dihydroxy-2-butene-4-olidylidene)ethane (12). To a solution of **2** (50 mg, 0.11 mmol) in anhydrous CH_2Cl_2 1 M solution of BCl_3 in CH_2Cl_2 (0.2 mL) was added under argon at −78 °C. The mixture was stirred at −78 °C for 3 h, then the temperature was raised to 10 °C and the reaction was continued for next 1 h. A mixture of CH_2Cl_2/MeOH (1:1) was added and the solvent was then removed under reduced pressure. The crude product purified by column chromatography (S_2), yielded isomers **7a** and **7b** at a 3:1 ratio (15 mg, 52.2%, mp 191–193 °C) and **12** (18 mg, 44.7%, mp 211–212 °C). Compound **7**: UV (MeOH) λ_{max} (log ε) 208, 268 (4.13, 3.88). Compound **7a**: ^{13}C NMR (DMSO-d_6): δ 164.65 (C-1′), 159.52 (C-4), 149.96 (C-2), 123.37 (C-2′), 143.61 (C-4′), 136.12 (C_1Ph), 141.16 (C-3′), 106.34 (C-5), 142.70 (C-6), 100.83 (C-5′), 72.51 (CH$_2$Ph), 43.16 (C-6′). Compound **7b**: ^{13}C NMR (DMSO-d_6): δ 164.65 (C-1′), 159.52 (C-4), 149.62 (C-2), 123.37 (C-2′), 143.41 (C-4′), 136.12 (C_1Ph), 141.67 (C-3′), 106.34 (C-5), 142.27 (C-6), 106.07 (C-5′), 72.20 (CH$_2$Ph), 43.16 (C-6′); MS *m*/*z* 376.1 ($M^{+\cdot}$). Compound **12**: UV (MeOH) λ_{max} (log ε) 208, 270 (4.12, 4.02); ^{1}H NMR (DMSO-d_6): δ 8.09 (H-6, 1H, s), 11.80 (N*H*, 1H, s), 5.33 (H-5′, 1H, t, J = 6.65), 4.46 (H-6′, 2H, d, J = 6.71); ^{13}C NMR (DMSO-d_6): δ 164.66 (C-1′), 159.52 (C-4), 149.96 (C-2), 123.61 (C-2′), 143.17 (C-4′), 141.38 (C-3′), 106.34 (C-5), 142.46 (C-6), 106.08 (C-5′), 43.16 (C-6′); MS *m*/*z* 286.0 ($M^{+\cdot}$).

4.2.3. 1-[2,4-Dioxo-5-fluoro-(1*H*,3*H*)-pyrimidin-1-yl]-2-(3-*O*-benzyl-2-hydroxy-2-butene-4-olidylidene)ethane (8) and 1-[2,4-dioxo-5-fluoro-(1*H*,3*H*)-pyrimidin-1-yl]-2-(2,3-dihydroxy-2-butene-4-olidylidene)ethane (13). To a solution of 5-fluoro-uracil-2,3-*O*,*O*-dibenzyl-L-ascorbic acid (**3**) (250 mg, 0.56 mmol) in dry CH_2Cl_2 1 M solution of BCl_3 in CH_2Cl_2 (0.65 mL) was added at −78 °C under argon. The mixture was stirred at −78 °C for 2 h, then the temperature was raised to −40 °C and the reaction was continued for 6 h. A mixture of CH_2Cl_2/MeOH (1:1) was added and the solvent was removed under reduced pressure. The crude product, purified by column chromatography (S_2), yielded **8a** and **8b** at a 10:1 ratio (96 mg, 47.6%, mp 178–179 °C) and **13**23. Compound **8**: UV (MeOH) λ_{max} (log ε) 208, 269 (4.05, 4.12). Compound **8a**: ^{13}C NMR (DMSO-d_6) 164.64 (C-1′), 157.29 (C-4), 149.44 (C-2), 123.36 (C-2′), 143.65 (C-4′), 136.11 (C_1Ph), 141.15 (C-3′), 138.15 (C-5), 129.82 (C-6), 100.74 (C-5′), 72.18 (CH$_2$Ph), 43.07 (C-6′). Compound **8b**: ^{13}C NMR (DMSO-d_6): δ 164.64 (C-1′), 157.29 (C-4), 149.44 (C-2), 123.36 (C-2′), 143.65 (C-4′), 136.11 (C_1Ph), 141.15 (C-3′), 138.15 (C-5), 129.82 (C-6), 106.00 (C-5′), 72.18 (CH$_2$Ph), 43.07 (C-6′); MS *m*/*z* 360.1 ($M^{+\cdot}$). Compound **13**: UV (MeOH) λ_{max} (log ε) 208, 276 (4.28, 4.12); ^{13}C NMR (DMSO-d_6) 164.64 (C-1′), 157.62 (C-4, J = 25.6), 149.60 (C-2), 123.38 (C-2′), 141.17 (C-3′), 143.61 (C-4′), 106.34 (C-5′), 106.08 (C-6), 100.83 (C-5′), 43.16 (C-6′); MS *m*/*z* 270.0 ($M^{+\cdot}$).

4.2.4. 1-[2,4-Dioxo-5-iodo-(1*H*,3*H*)-pyrimidin-1-yl]-2-(3-*O*-benzyl-2-hydroxy-2-butene-4-olidylidene)ethane (9) and 1-[2,4-dioxo-5-iodo-(1*H*,3*H*)-pyrimidin-1-yl]-2-(2,3-dihydroxy-2-butene-4-olidylidene)ethane (14). To a solution of 5-iodo-uracil-2,3-*O*,*O*-dibenzyl-L-ascorbic acid (**4**) (100 mg, 0.19 mol) in dry CH_2Cl_2 1 M solution of BCl_3 in CH_2Cl_2 (0.6 mL) was added at −78 °C under argon. The mixture was stirred at −78 °C for 2 h, then BCl_3 in CH_2Cl_2 (0.6 mL) was added and the temperature was raised to −40 °C and the reaction was continued for 1 h. A mixture of CH_2Cl_2/MeOH (1:1) was added and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (S_1) to yield **9a** and **9b** at a 10:1 ratio (36 mg, 40.4%, mp 216–217 °C) and **14** (18 mg, 25.1%, mp 183–185 °C). Compound **9**: UV (MeOH) λ_{max} (log ε) 208, 287 (4.38, 4.18). Compound **9a**: ^{13}C NMR (DMSO-d_6): δ 164.64 (C-1′), 161.06 (C-4), 150.50 (C-2), 123.37 (C-2′), 143.51 (C-4′), 136.11 (C_1Ph), 141.17 (C-3′), 68.32 (C-5), 149.61 (C-6), 101.03 (C-5′), 72.17 (CH$_2$Ph), 43.07 (C-6′). Compound **9b**: 164.64 (C-1′), 161.06 (C-4), 150.50 (C-2), 123.37 (C-2′), 143.51 (C-4′), 136.11 (C_1Ph), 141.17 (C-3′), 68.32 (C-5), 149.18 (C-6), 106.28 (C-5′), 72.48 (CH$_2$Ph), 43.32 (C-6′); MS *m*/*z* 468.0 ($M^{+\cdot}$). Compound **14**: UV (MeOH) λ_{max} (log ε) 206, 276 (4.11, 4.02); ^{1}H NMR (DMSO-d_6): δ 8.12 (H-6, 1H, s), 11.63 (N*H*, 1H, s), 5.31 (H-5′, 1H, t, J = 6.72), 4.45 (H-6′, 2H, d, J = 6.68); ^{13}C NMR (DMSO-d_6): δ 164.64 (C-1′), 161.00 (C-4), 150.50 (C-2), 123.37 (C-2′), 142.44 (C-4′), 140.96 (C-3′), 68.30 (C-5), 149.40 (C-6), 100.03 (C-5′), 43.07 (C-6′); MS *m*/*z* 378.0 ($M^{+\cdot}$).

4.2.5. 1-[2,4-Dioxo-5-trifluoromethyl-(1*H*,3*H*)-pyrimidin-1-yl]-2-(3-*O*-benzyl-2-hydroxy-2-butene-4-olidylidene)-ethane (10) and 1-[2,4-dioxo-5-iodo(1*H*,3*H*)-pyrimidin-1-yl]-2-(2,3-dihydroxy-2-butene-4-olidylidene)ethane (15). Compound **10** (150 mg, 0.3 mmol) was treated according to a procedure that was analogous to that for the preparation of compounds **9** and **14** to give **10** (32 mg, 26%, mp 160–162 °C) and **15** (50 mg, 52.1%, mp 292–294 °C).[23] Compound **10**: UV (MeOH) λ_{max} (log ε) 206, 269 (4.40, 4.22); ^{1}H NMR (DMSO-d_6): δ 7.24 (H-6, 1H, d, J = 7.36 Hz), 11.92 (*N*H, 1H, s), 5.01 (H-5′, 1H, s), 4.24 (H-6′, 2H, d, J = 5.98 Hz), 8.58 (OH-2′, 1H, s), 7.37–7.29 (C$_6H_5$, 5H, m), 4.96 (CH_2Ph, 2H, s); ^{13}C NMR (DMSO-d_6): δ 164.35 (C-1), 148.06 (C-2), 158.22 (C-4), 103.45 (C-5), 147.28 (C-6), 123.20 (CF_3), 121.40 (C-2′), 141.64 (C-3′), 73.20 (CH$_2$Ph), 136.05 (C_1Ph), 143.44 (C-4′), 102.15 (C-5′), 43.80 (C-6′); MS *m*/*z* 410.1 ($M^{+\cdot}$).

4.2.6. 1-(6-Chloropurine-9-yl)-2-(3-*O*-benzyl-2-hydroxy-2-buten-4-olidylidene)ethane (18) and 1-(6-chloropurine-9-yl)-2-(2,3-dihydroxy-2-buten-4-olidylidene)ethane (19). To a cooled solution of 6-chloropurine derivative of 2,3-*O*,*O*-dibenzyl-L-ascorbic acid (**16**) (60 mg, 0.13 mmol) in dry CH_2Cl_2, a 1 M solution of BCl_3 in CH_2Cl_2 (0.3 mL) was added at −78 °C under argon. The mixture was stirred at −78 °C for 2 h, then was added BCl_3 in CH_2Cl_2 (0.3 mL), the temperature was raised to −40 °C and the reaction was continued for 1 h. A mixture of CH_2Cl_2/MeOH (1:1) was added and the solvent was then removed under reduced pressure. The crude product was

purified by column chromatography (S_2) yielding **18** (21 mg, 43.3%, mp 141–143 °C) and **19** (8 mg, 21.5%, mp 109–111C). **18**: UV (MeOH) λ_{max} (log ε) 207, 261 (4.49, 4.13); ^{1}H NMR (DMSO-d_6): δ 4.02 (H-5′, 1H, dd, J = 6.0 Hz), 4.83 (H-6′, 2H, d, J = 6.3), 8.44 (H-2, 1H, s), 8.92 (H-8, 1H, s), 5.49 and 5.39 (CH_2Ph, 2H, 2d, J = 11.80 Hz), 12.45 (OH-2′, 1H, s), 7.33–7.45 (C_6H_5, 5H, m); ^{13}C NMR (DMSO-d_6): δ 152.10 (C-4), 149.35 (C-2), 121.12 (C-5), 147.31 (C-6), 145.22 (C-8), 163.25 (C-1′), 129.20 (C-2′), 143.64 (C-3′), 145.84 (C-4′), 99.91 (C-5′), 38.12 (C-6′), 73.92 (CH$_2$Ph), 135.41 (C_1Ph); MS m/z 384.4 ($M^{+\cdot}$). Compound **19**: UV (MeOH) λ_{max} (log ε) 208, 258 (4.45, 4.11); ^{1}H NMR (DMSO-d_6): δ 5.47 (H-5′, 1H, s), 5.08 (H-6′, 2H, d, J = 6.1), 8.51 (H-2, 1H, s), 8.17 (H-8, 1H, s), 12.66 (OH-2′, 1H, s), 11.28 (OH-3′, 1H, s); ^{13}C NMR (DMSO-d_6): δ 154.20 (C-4), 149.15 (C-2), 123.44 (C-5), 149.10 (C-6), 147.17 (C-8), 164.25 (C-1′), 125.83 (C-2′), 142.33 (C-3′), 144.30 (C-4′), 99.51 (C-5′), 42.42 (C-6′); MS m/z 294.6 ($M^{+\cdot}$).

4.2.7. 1-[6-(*N*-Pyrrolyl)purine-9-yl]-2-(2,3-dihydroxy-2-buten-4-olidylidene)ethane (20). Compound **17** (100 mg, 0.2 mmol) was treated according to a procedure that was analogous to that for the preparation of compounds **18** and **19** to give **20** (22 mg, 33.8%, mp 142–144 °C); UV (MeOH) λ_{max} (log ε) 204, 289 (4.37, 4.31); ^{1}H NMR (DMSO-d_6): δ 5.61 (H-5′, 1H, t, J = 7.1 Hz), 5.16 (H-6′, 2H, d), 8.76 (H-2, 1H, s), 8.68 (H-8, 1H, s), 8.30 (H-2″, H-5″, 2H, t), 6.45 (H-3″, H-4″, 2H, t); ^{13}C NMR (DMSO-d_6): δ 152.04 (C-2), 153.02 (C-4), 121.73 (C-5), 146.41 (C-6), 145.82 (C-8), 164.75 (C-1′), 121.18 (C-2′), 143.23 (C-3′), 144.89 (C-4′), 99.56 (C-5′); MS m/z 325.3 ($M^{+\cdot}$).

4.3. Antitumour activity assays

Antitumour activity against L1210 (murine leukaemia), Molt4/C8 and CEM (human T-lymphocytes) cell lines were measured essentially as originally described for the mouse leukaemia (L1210) cell lines.[25]

The HeLa (cervical carcinoma), MCF-7 (breast carcinoma), SW620 (colon carcinoma), MiaPaCa-2 (pancreatic carcinoma), Hep-2 (laryngeal carcinoma) and WI38 (diploid fibroblasts) cells were cultured as monolayers and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal 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 cells were inoculated onto standard 96-well microtitre plates on day 0. Test agents were then added in 5-, 10-fold dilutions (10^{-8}–10^{-4} mol/L) and incubated for 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 performing the MTT assay,[26] which detects dehydrogenase activity in viable cells. The absorbency (OD, optical density) was measured on 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

$$PG = 100 \times (\text{mean } OD_{test} - \text{mean } OD_{tzero})/(\text{mean } OD_{ctrl} - \text{mean } OD_{tzero}).$$

If (mean OD_{test} − mean OD_{tzero} < 0 then:

$$PG = 100 \times (\text{mean } OD_{test} - \text{mean } OD_{tzero})/OD_{tzero},$$

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}, which is the concentration necessary for 50% of inhibition. The IC_{50} values for each compound are calculated from dose–response curves using linear regression analysis by fitting the test concentrations that give PG values above and below the reference value (i.e., 50%). Each result is a mean value from three separate experiments.

4.4. Antiviral activity assays

Antiviral activity against herpes simplex virus type 1 and 2, vaccinia virus, cytomegalovirus, varicella-zoster 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.[27,28]

4.5. Cell cycle analysis

Cells (1×10^6) were seeded per 100 mm plate. After 24 h the tested compounds were added at concentrations 5×10^{-6} mol/L and 10^{-5} mol/L. After the desired length of time the attached cells were trypsinized, combined with floating cells, washed with phosphate buffer saline (PBS) and fixed with 70% ethanol. 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 analyzed with Becton Dickinson FACScalibur flow cytometer (20,000 counts were measured). The percentage of the cells in each cell cycle phase was determined using the WINMDI software based on the DNA histogram. Statistical analysis was performed in Microsoft Excel by using the ANOVA single factor test.

4.6. Annexin V test

Detection and quantification of apoptotic cells at single cell level, was performed using Annexin V-FLUOS

staining kit (Roche), according to the manufacturer's recommendations. After the desired length of time, both floating and attached cells were collected. The cells were then 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 analyzed 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 nonfluorescent cells), which was expressed as 100%.

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