Synthesis and Anti-Virus Activity of Some Nucleosides Analogues

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New 3'-, 5'-, 5-bromo-2'-deoxyuridine (**3a–g**) and 3'-, 5'- thymidine (**4a–i**) analogues with amino acid and peptide residues were synthesized and evaluated for antiviral activity. The influence of long peptide chains, essential amino acids and the effect of this structural modification on the antiviral activity has been also reported.

Three 5-bromo-2'-deoxyuridine derivatives containing glycyl-, glycyl-glycyl- and glycyl-glycyl-glycyl- residues (**3a, 3b, 3c**) showed a strong activity against the herpes virus PsRV and a moderate one vs. HSV-1.

The corresponding thymidine analogues were considerably less effective, and only compounds **4d** and **4h** showed a borderline effect against PsRV.

Introduction

Analogues of 2'-deoxynucleosides modified at the 3'- and/or 5'- position are of a great importance for medicinal chemistry and biochemistry as potential anticancer and/or antiviral agents (Uhlmann *et al.*, 1990; Holy *et al.*, 1994). Some of these compounds are rather efficient therapeutic agents for the treatment of patients with AIDS, but some of them showed marked toxic side effects. In attempts to overcome the problem of rapid elimination and decreased permeability of 3'-azido-2', 3'-dideoxythymidine, AZT through the blood-brain barrier and to increase its therapeutics efficiency, Aggarwal *et al.* (1990) studied a variety of 5'-esters of AZT. They prepared N-substituted piperazine and morpholine esters, and 1,4- dihydro-1-methyl-3-[(pyridylcarbonyl) oxy] ester for transport into neuron tissues (Haines *et al.*, 1987), ester of retinoic acid which itself inhibits HIV replication and selected amino acids to use their specific transport properties. Since bone marrow cells lack amino acid transport system (Vistica, 1980) it has been presumed that such an AZT derivatives may be less toxic for bone marrow cells. These derivatives are generally less toxic and they are taken up by cells more easily.

The modification of various biological active compounds with amino acids allows them to be hydrolyzed more quickly under the influence of plasma enzymes, thus leading to their transformation as prodrugs. On the other hand these compounds exhibited poor stability in aqueous solution as exemplified with esters of metronidazole (Cho *et al.*, 1985), acyclovir (Colla *et al.*, 1983; Bundgaard *et al.*, 1991), ganciclovir (Bundgaard *et al.*, 1991), corticosteroids (Kawamura *et al.*, 1971; Jonson *et al.*, 1985), and paracetamol (Jensen *et al.*, 1991; Kovach *et al.*, 1981). One of the solution to overcome such problem is the use of adequately selected spacer groups.

Considering all these facts, we have been interested to estimate the role of modification in the

Abbreviations: Z, benzyloxycarbonyl; Boc, *N-tert*-butoxycarbonyl; TBTU, N-[(1-H-benzotriazol-1-yloxy) (dimethylamino) methylene]-N-methyl-, tetrafluoroborate; DMF, N, N-dimethylformamide; DMAP, 4-dime- thylaminopyridine; DIEA, diizopropylethyl amine; BUdR, 5-bromo-2'-deoxyuridine.
HIV, human immunodeficiency virus; HSV-1, herpes simplex virus 1; PsRV, pseudo Rabies virus; MIC_{50}, minimal 50% inhibitory concentration; $CCID_{50}$, cell culture 50% infections dose; MTC, maximum tolerated concentration; SI, selectivity index.

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3'- and 5'- position with selected amino acid and peptide residues (Stankova *et al.*, 1996; Alargov *et al.*, 1997). We report here the synthesis and biological (antiviral) evaluation of several amino acid and peptide derivatives of 5-bromo-2'-deoxyuridine and thymidine shown in Fig. 1.

We have also considered the role of chirality, aromatic, heterocyclic amino acid residues, the addition of the long peptide chains formed of glycine to 5-bromo-2'-deoxyuridine and thymidine, and the effect of these structural modifications on antiviral activity.

Results and Discussion

Nucleosides modified with amino acids are not so many, but nucleosides containing peptide residues are not known at all. In order to obtain antimetabolites with antiviral activity that have more desirable characteristics than those analogues now known, we synthesized a series of new 3'-, 5'-, 5-bromo-2'-deoxyuridine as well as thymidine analogues containing amino acid and peptide residues. The α-amino group of glycine, phenylalanine, alanine and valine (**1a, 1d, 1h, 1i**) was protected by the appropriate groups of urethane type – such as benzyloxycarbonyl (Z) (Bergmann *et al.*, 1932), and in the case of thiazole containing glycine (**1 g**) with butoxycarbonyl (Boc) (Pozdnev, 1977).

To obtain di- and tri- peptides (**1b, 1c**) the models was used the two well known methods for peptide bond formation: the active ester method (Anderson *et al.*, 1964), and those of the mixed anhydrides (Zaoral, 1962). Optically pure N-α-Z-(4-F)-phenylalanine (*S, R*) (**1e, 1f**) we obtained from a suitable protected ester by enzymatic hydrolysis using the alkaline proteinase, subtilisin DY (Tong *et al.*, 1971; Aleksiev *et al.*, 1981). N-*tert*- butoxycarbonyl-2-aminomethyl-thiazole-4-carboxylic acid (**1 g**) was synthesized from a corresponding protected amino acid by the modified Hantsch method according to (Videnov *et al.*, 1996). The target acylated **3a–g, 4a–i** compounds were prepared by previous activation *in situ* of the amino acids or peptides using the reagent TBTU in the presence of 4-dimethylamino-pyridine (DMAP), (Knorr, 1989). The esterification was carried out in dimethylformamide (DMF) solution

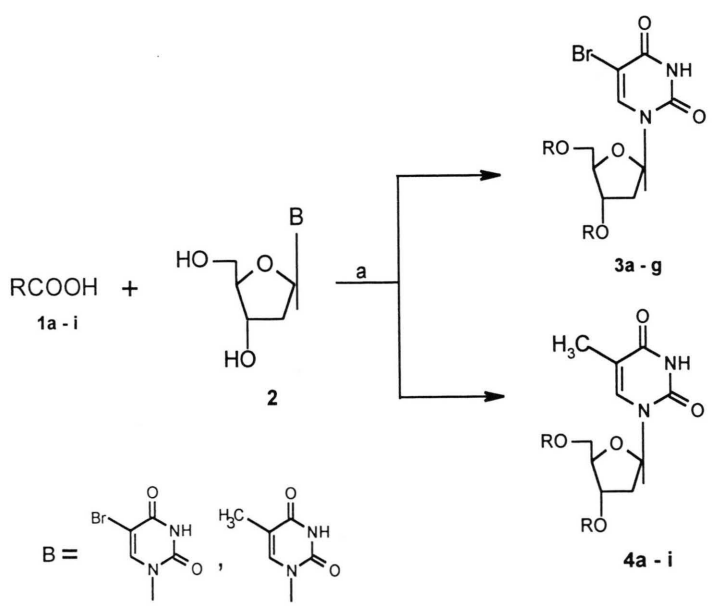

Fig. 1. Synthesis of amino acids and peptides derivatives of BUdR and thymidine.
a: R = *N*-α-Z-glycyl; **b:** R = *N*-α-Z-glycyl-glycyl; **c:** R = *N*-α-Z-glycyl-glycyl-glycyl; **d:** R = *N*-α-Z-phenylalanyl; **e:** R = *N*-α-Z-(4-F)-phenylalanyl (S); **f:** R = *N*-α-Z-(4-F)-phenylalanyl (R); **g:** R = *N*-*t*-Boc-2-aminomethyl-thiazolyl-4-carboxyl; **h:** R = *N*-α-Z-alanyl; **i:** R = *N*-α-Z-valyl.
a: Reagents: TBTU, DIEA, DMAP, room temperature.

at room temperature. Purification of the crude product was achieved by column chromatography which gave a yield of 3',5'-O-bis-5-bromo-2'-deoxyuridine derivatives of 43–60%, and 3',5'-O-bis-thymidine analogues of 70–79%.

It should be noted that our chosen approach yielded the desired products with a higher degree of optical purity and a faster condensation than those currently known.

Another advantage of the condensation method we have favored is lack of undesirable side processes including racemization.

Biological activity

Initially the new analogues **3a–g, 4a–i** were evaluated for their antiviral activity towards influenza virus A / chicken / Germany / 27 / Weybridge (H7N7) (FPV) and pseudorabies virus (Aujeszky, A2 strain) (PsRV) growth in primary chick embryo fibroblasts cultures (CEC). Results of the antiviral screening of 5-bromo-2'-deoxyuridine and thymidine derivatives by the agar diffusion plaque – inhibition test are shown in Table I and Table II.

These data clearly demonstrate the marked anti – herpes virus potential of these compounds, comparable of that of 5-bromo-2'-deoxyuridine in the case of **3b**.

Table I. Screening of BUdR derivatives **3a–g** for antiviral activity by the agar-diffusion plaque inhibition test.

Compounds* No.	FPV Φ_i	FPV Φ_t	FPV E	PsRV Φ_i	PsRV Φ_t	PsRV E
3a	0	9.2	–	35.7	9.5	++
3b	0	0	–	51.0	10.5	+++
3c	0	11.2	–	29.0	8.5	++
3d	0	0	–	13.2	7.7	–
3e	0	8.2	–	0	0	–
3f	0	9.0	–	12.2	10.5	–
3 g	8.7	7.7	–	0	8.0	–
Ribavirin	27.5	9.2	+		N. T.	
BUdR		N. T.		63.2	10	+++

* All tested compounds were applied at the concentration of 5 x 10^{-3} M.
Φ_i = diameter of inhibition zone (mm);
Φ_t = diameter of toxicity zone (mm);
E = antiviral effect;
BUdR = 5-bromo-2'-deoxyuridine;
FPV, influenza virus A (H7N7);
PsRV, pseudorabies virus;
N. T., not tested.

Table II. Screening of thymidine derivatives **4a–i** for antiherpes virus activity.

Compounds* No.	PsRV Φ_i	PsRV Φ_t	PsRV E
4a	0	7.9	–
4b	13.9	9.7	–
4c	0	7.9	–
4d	17.4	8.2	±
4e	9.5	8.1	–
4f	10.6	8.4	–
4 g	17.2	11.1	±
4h	12.5	10.2	–
4i	0	8.7	–
BUdR	63.2	10	+++

* All tested compounds were applied at the concentration of 5 x 10^{-3} M.
Φ_i = diameter of inhibition zone (mm);
Φ_t = diameter of toxicity zone (mm);
E = antiviral effect;
BUdR = 5-bromo-2'-deoxyuridine;
PsRV, pseudorabies virus.

It is important to stress that there is no difference in activity between the *R* and *S* isomers **3e, f** and **4e, f**.

Analogue of phenylalanine with 5-bromo-2'-deoxyuridine (**3d**) do not show activity, and analogue of thymidine (**4d**) exhibited a borderline activity.

As seen in Table I compound **3b** has a strong activity against herpes virus PsRV. This activity was comparable with that of BUdR used as a reference antiherpes virus compound. Compounds **3a, 3c**, showed a marked but less pronounced effect as compared to **3b**. The rest of the compounds was inactive, and neither analogue showed activity against the influenza virus model.

The corresponding derivatives of the normal metabolite – thymidine – do not show any antivirus activity as seen in Table II.

The three compounds **3a, 3b, 3c** showed antiherpes activities to HSV-1 in the cytopathic effect (CPE) inhibition test. However, they showed a decrease of the effect selectivity, especially strongly expressed in **3c** (Table III).

In conclusion, these results demonstrated that among the compounds tested, peptide analogues possessed a marked antiviral activity in comparison with these of the amino acids. In addition, elongation of the peptide chain enhanced antiviral activity.

Table III. Activity of compounds **3a, 3b** and **3c** towards HSV-1, in cytopathic effect (CPE) inhibition multicycle test.

Derivatives No.	MIC_{50} (M)	MTC (M)	SI (MTC/MIC_{50}) (M)
3a	9.5 x 10-6	3.2 x 10-5	3.4
3b	3.7 x 10-6	1 x 10-5	2.7
3c	2.5 x 10-6	3.2 x 10-6	1.28
BUdR	1.15 x 10-6	3.2 x 10-5	27.8

HSV-1 = herpes simplex virus 1;
MIC_{50} = minimal 50% inhibitory concentration;
MTC = maximum tolerated concentration;
SI = selectivity index;
BUdR = 5-bromo-2'-deoxyuridine.

The rational synthesis of our compounds was based on the fact that some amino acid esters of nucleosides are known as prodrugs (Perry *et al.*, 1996). It is possible that the compounds obtained are prodrugs of BUdR (the active antiherpes virus moiety). An implicit proof of this assumption lies in the fact that the activity of these three compounds (**3a, 3b, 3c**) does not exceed that of BUdR. Further systematic research *in vivo* is needed for additional proof of the above assumption.

Materials and Methods

All reagents were from Fluka AG, Buchs (CH). TLC analysis was performed on aluminum sheets Silica gel 60 F_{254} (Merck, Germany) using the eluent systems: A-ethyl acetate/n-hexane (6:1 v:v) and B-CHCI3/MeOH (9:1 v:v). Silica gel 60 (230–400 mesh), also a Merck product, was used for flash chromatography. The derivatives were visualized with UV light (254 nm), charring reagent and/or ninhydrin.

Melting points were determined using a Kofler apparatus. Optical rotations were determined with a Perkin – Elmer 241 polarimeter at 20 °C.

NMR spectra were recorded on a Bruker AC – 250 – Spektrometer.

^{1}H NMR chemical shifts are reported in δ ppm relative to DMSO (2.49 ppm) as an internal standard, and ^{13}C chemical shifts are reported in ppm relative to MeOH (49.15 ppm) unless specified otherwise. Multiplicities in ^{1}H NMR are reported as (s) singlet, (d) doublet, (dd) doublet of doublets, (t) triplet, (q) quartet, and (m) multiplet. Mass spectrometry: API III triple quadrupole mass spectrometer equipped with an electrospray ion source at atmospheric pressure (Sciex, Thornhill, Canada); electrospray ionization mass spectra (ESI-MS) were recorded in the positive mode.

Synthesis

General procedure for the synthesis of the protected 3', 5'-O-bis-5-bromo-2'-deoxyuridine (**3a–g**) and 3', 5'-O-bis-thymidine (**4a–i**) derivatives.

The compounds **1a–i** were synthesized according to a known procedure (Bergmann *et al.*, 1932; Pozdnev, 1974; Anderson, 1964; Zaoral, 1962; Tong, 1971; Aleksiev *et al.*, 1981; Videnov *et al.*, 1996).

1a–i (3 mmol), DIEA (3.1 mmol) was added to a solution of TBTU (3 mmol) in DMF (15 ml) (Knorr, 1989). After stirred the mixture was treated with 5-bromo-2'-deoxyuridine or thymidine (1.5 mmol) along with DMAP (3 mmol). This mixture was stirred at room temperature for 3 h, and then evaporated to dryness. The residue dissolved in ethyl acetate, washed with 10% $NaHCO_3$, water, brine and dried over Na_2SO_4. After evaporation the residue was purified by flash chromatography with ethyl acetate/n-hexane (6/1 v/v) mixtures as eluent.

3a: 3', 5'-bis-O-(*N*-benzyloxycarbonyl-glycyl)-5-bromo-2'-deoxyuridine

m.p. = 74–76 °C. ^{1}H-NMR, δ = 2.27 (m, 1H, H2''), 2.5 (m, 1H, H2'), 3.86 (m, 2H, αHGly), 4.16 (dd, 1H, ^3J = 7.9, 4 Hz; H4'), 4.33 (br.s, 2H,H5'), 5.04-5.03 (s, 2H ,C_6H_5-CH_2), 5.22 (m, 1H,H3'), 6.11 (dd, 1H, ^3J = 7.0, 7.0 Hz; H1'), 7.33 (m, 10H, C_6H_5), 7.74 (m, 1H, NH), 8.06 (s, 1H, H6), 11.89 (br.s, 1H, H3). ^{13}C-NMR: δ = 35.87 (C-2'), 42.36, 42.19 (Cα-Gly), 64.13 (C-5',5''), 65.65 (CH_2-O), 74.38 (C-3'), 81.26 (C-4'), 84.94 (C-1'), 96.42 (C-5), 127.89, 127.77, 128.39 (C_6H_5, C-3, 4), 136.89 (C_6H_5, C-1), 140.09 (C-6), 149.70 (C-2), 156.57 (C-4), 159.07 (OCO), 170.01, 176.91 (COO). ESI-MS: *m/z*: 690, 691 $[M+H]^+$.

3b: 3', 5'-bis-O-(*N*-benzyloxycarbonyl-glycyl-glycyl)-5-bromo-2'-deoxyuridine

m. p. = 89–91 °C. ^{1}H-NMR, δ = 2.3 (m, 1H, H2''), 2.53 (dd, 1H,^3J = 14.8, 7.5 Hz; H2'), 3.93 (m, 2H, αH-Gly), 4.17 (dd, 1H, ^3J = 7.6, 4.3 Hz; H4'),

4.32 (br.s., 2H, H5'), 5.02 (s, 2H, C_6H_5-CH_2), 5.21 (m, 1H ,H3'), 6.13 (t, 1H, ^3J = 7.4 Hz; H1'), 7.31-7.34 (m, 10H, C_6H_5), 7.52 (dd, 1H, ^3J = 11.6, 6.0 Hz; NH), 8.09 (s, 1H, H_6), 8.35 (dd, 1H, ^3J = 11.1, 6.1 Hz; NH'), 11.88 (br.s., 1H, H3). ^{13}C-NMR: δ = 35.79 (C-2'), 40.85, 40.65, 40.44 (Cα-Gly), 43.34, 43.23 (Cα-Gly), 64.08 (C-5',5''), 65.54 (CH2-O), 76.36 (C-3'), 81.3 (C-4'), 85.01 (C-1'), 96.4 (C-5), 127.75, 127.83, 127.83 (C_6H_5, C-3,4), 136.99 (C_6H_5, C-1), 140.22 (C-6), 149.70 (C-2), 159.04 (C-4), 156.44, 159.06 (OCO), 169, 169.8 (COO). ESI-MS: *m/z:* 804, 805 [M+H]+.

3c: 3', 5'-bis-O-(*N*-benzyloxycarbonyl-glycyl-glycyl-glycyl)-5-bromo-2'-deoxyuridine

m.p. = 139–141 °C. ^{1}H-NMR, δ = 2.3 (m, 1H, H2''), 2.53 (dd, 1H, ^3J = 14.8, 7.5 Hz; H2'), 3.86 (m, 2H, αHGly), 4.17 (t, 1H, ^3J = 6.5 Hz; H4'), 4.33 (br.s, 2H, H5'), 5.04 (s, 2H, C_6H_5-CH_2), 5.21 (m, 1H, H3'), 6.19 (dd, 1H, ^3J = 8.1, 6.4 Hz; H1'), 7.31-7.39 (m, 10H, C_6H_5), 8.09 (s, 1H, H6), 8.19 (dd,1H, ^3J = 10.7, 5.4, 5.5 Hz; NH'), 8.35 (dd, 1H, ^3J = 11.1, 5.6 Hz; NH''), 11.88 (br.s, 1H, H3). ^{13}C-NMR: δ = 35.7 (C-2'), 41.75, 41.45, 41.24 (Cα-Gly), 42.34, 42.23 (Cα-Gly), 64.12 (C-5', 5''), 65.74 (CH_2-O), 74.39 (C-3'),81.51 (C-4'), 85.0 (C-1'), 96.2 4 (C-5), 127.55, 127.73, 127.8 (C_6H_5, C-3,4), 136.99 (C_6H_5, C-1), 140 (C-6), 149.70 (C-2), 156.5 (C-4), 156.54, 159 (OCO), 170, 171.8 (COO). ESI-MS: *m/z:* 917, 918 $[M+H]^+$.

3d: 3', 5'-bis-O-(*N*-benzyloxycarbonyl-phenyl-alanyl)-5-bromo-2'-deoxyuridine

m.p. = 79–81 °C. ^{1}H-NMR, δ = 2.5 (m, 1H, H2') ,3.3 (dd, 1H, ^3J = 13.8 Hz; β-CH_2), 4.17 (m, 1H, H4'), 4.13 (br.s., 1H, H5'', H5'), 4.69 (m, 1H, α-CH), 5.04 (s, 2H, C_6H_5-CH_2), 5.09 (m, 1H, H3'), 6.01 (t, ^3J = 7.0 Hz; H1'), 7.31-7.34 (m, 20H, C_6H_5), 7.7 (m, 1H, NH), 8.04 (s, 1H, H6), 11.88 (br. s, 1H, H3). ^{13}C-NMR: δ = 35.8 (C-2'), 38.10, 38.68 (Cβ, -Phe), 57.02, 57.36 (Cα, α'-Phe), 67.67 (CH_2-O), 64.01 (C-5'), 76.13 (C-3'), 83.81 (C-4'), 87.0 (C-1'), 96.43 (C-5), 128.05 (Cζ-Phe), 128.74, 128.88, 129.41 (C_6H_5, C-3,4), 129.58 (Cε-Phe), 130.28 (Cδ-Phe), 136.76 (C_6H_5, C-1), 137.98 (Cγ-Phe), 140.2 (C-6), 150.5 (C-2), 159.06 (OCO), 172.76 (COO). ESI-MS: *m/z:* 870, 871 $[M+H]^+$.

3e: 3', 5'-bis-O-(*N*-benzyloxycarbonyl-(4-F)-phenylalanyl)-5-bromo-2'-deoxyuridine (S)

m.p. = 75–77 °C. $[\alpha]^{20}_D$ = –1.1° (c = 1, CH_3OH). ^{1}H-NMR, δ = 2.5 (m, 1H, H2'), 3.6 (d, 1H, ^3J = 14.3 Hz, βH-Phe), 4.17 (m, 1H, H4'), 4.32 (br.s., 1H, H5'', H5'), 4.4 (dd, 1H, ^3J = 7.9, 5.3 Hz, α-CH), 5.03 (s, 2H, C_6H_5-C H_2), 5.09 (m,1H, H3'), 6.01 (t, 1H, ^3J = 7.0 Hz; H1'), 7.33-7.34 (m, 18H, C_6H_5), 7.7 (m, 1H, NH), 8.04 (s, 1H, H_6), 11.88 (br.s, 1H, H3). ^{13}C-NMR: δ = 35.52 (C-2'), 38.2, 38.72 (Cβ,β'-Phe), 56.64, 56.89 (Cα,α'-Phe), 64.12 (C-5'), 65.57 (CH_2-O), 76.28 (C-3'), 83.82 (C-4'), 83.82 (C-1'), 96.52 (C-5), 114.96, 116.06 ($^3J_{CF}$ = 21.4 Hz, Cε,ε'-Phe), 128.78, 129.40, 128.89 (C_6H_5, C-3,4), 131.96 (Cδ-Phe), 133.85, 134.0 ($^2J_{CF}$ = 9.4 Hz; Cγ,γ'-Phe), 136.07 (C_6H_5, C-1), 140.17 (C-6), 149.32 (C-2), 158.07 (OCO), 159.08 (C-4), 161.14 ($^1J_{CF}$ = 243 Hz; Cζ - Phe), 171.8 (COO). ESI-MS: *m/z:* 906 $[M+H]^+$.

3f: 3', 5'-bis-O-(*N*-benzyloxycarbonyl-(4-F)-phenylalanyl)-5-bromo-2-deoxyuridine (R)

m.p. = 63–65 °C. $[\alpha]^{20}_D$ = +1.5° (c = 1,CH_3OH). ^{1}H-NMR, δ = 2.51 (m, 1H, H2'), 3.7 (d, 1H, ^3J = 13.9 Hz; βH-Phe), 4.17 (m, 1H, H4'), 4.32 (br.s., 1H, H5'', H5'), 4.4 (dd, 1H, ^3J = 8.0, 5.3 Hz; αCH), 5.03 (s, 2H, C_6H_5-CH_2), 5.22 (m, 1H, H3'), 6.11 (dd, 1H, ^3J = 7.0, 7.0 Hz, H1'), 7.31-7.34 (m, 18H, C_6H_5), 8.0 (m, 1H, NH), 8.09 (s, 1H, H6), 11.89 (br.s, 1H, H3). ^{13}C-NMR: δ = 35.23 (C2'), 37.93, 38.43 (Cβ,β'-Phe), 56.89 (Cα,α'-Phe), 65.51 (CH_2-O), 64.13 (C-5'), 76.19 (C-3), 81.26 (C-4), 83.31 (C-1), 96.7 (C-5), 115.89, 116.23 ($^3J_{CF}$ = 21.4 Hz; Cε,ε'-Phe), 128.05, 128.78, 128.98 (C_6H_5, C-3,4), 131.98 (Cδ-Phe), 133.85 ($^2J_{CF}$ = 9.4Hz; Cγ- Phe), 140.23 (C-6) ,149.3 (C-2), 159.4 (OCO), 161.14 ($^1J_{CF}$ = 243 Hz; Cζ-Phe), 163 (C-4), 172.9 (COO). ESI-MS: *m/z:* 906 $[M+H]^+$.

3 g: 3',5'-bis-O-(*N-tert*-butoxycarbonyl-2-amino-methylthiazol-4-carboxyl)-5-bromo-2'-deoxy-uridine

m.p. = 106–108 °C. ^{1}H-NMR, δ = 1.41 (s, 9H, $3CH_3$), 2.40 (m, 1H, H-2'), 4.13 (br.s., 1H, H4'), 4.26 (dd, 2H, ^3J = 12.0, 5.8 Hz; H5'), 4.39 (d, 2H, ^3J = 5.83 Hz, CH_2), 5.22 (m, 1H, H3'), 6.17 (dd, 1H, ^3J = 6.2, 79 Hz; H1'), 8.05 (s, 1H, H6), 7.82 (t, 1H, ^3J = 5.62 Hz; NH), 8.34 (s, 1H, CH-Thz), 11.89

(s, 1H, H3). ^{13}C-NMR, δ = 28.14 (Boc-3CH_3), 36.34 (C-2'), 42.0 (CH_2), 64.61 (C-5'), 75.44 (C-3'), 78.74 (Boc-Cq), 81.53 (C-4'), 85.01 (C-1'), 96.47 (C-5), 129.82, 130.16 (C-5_{Thz}), 139.88 (C-6), 149.8 (C-4_{Thz}), 149.82 (C-2), 155.76 (Boc-CO), 158.98 (C-4), 160.07 (C-2_{Thz}), 171.7 (COO). ESI-MS: *m/z:* 787, 788 $[M+H]^+$.

4a: 3', 5'-bis-O-(*N*-benzyloxycarbonyl-glycyl)-thymidine

m.p. = 144–146 °C. ^{1}H-NMR, δ = 1.78 (s, 3H, CH_3), 2.2 (dd, 1H, ^3J = 13.9, 5.8 Hz; H2'), 2.4 (m, 1H, H2''), 3.8 (m, 2H, αH-Gly), 4.13 (s, 1H, H4'), 4.29 (br.s, 2H, H5', 5''), 5.04-5.03 (s, 2H, C_6H_5-CH_2), 5.22 (m, 1H, H3'), 6.17 (dd, 1H, ^3J = 6.2, 7.9 Hz; H1'), 7.33-7.34 (m, 10H ,C_6H_5), 7.46 (s, 1H, H6), 7.75 (m, 1H, NH), 11.39 (br.s, 1H, H3). ^{13}C-NMR: δ = 12.15 (CH_3-Thym), 35.44 (C-2'), 42.38, 42.25 (Cα-Gly), 64.19 (C-5'), 65.65 (CH_2-O), 74.5 (C-3'), 80.83 (C-4'), 83.78 (C-1'), 110.12 (C-5), 127.88, 127.77, 128.34 (C_6H_5, C-3, 4), 135.68 (C-6), 136.85 (C_6H_5, C-1), 150.34 (C-2), 156.56 (OCO), 163.54 (C-4), 170.04, 169.90 (COO). ESI-MS: *m/z:* 625 $[M+H]^+$.

4b: 3', 5'-bis-O-(*N*-Benzyloxycarbonyl-glycyl-glycyl)-thymidine

m.p. = 114–116 °C. ^{1}H-NMR, δ = 1.79 (s, 3H, CH_3), 2.24 (m, 1H, H2'), 2.42 (m, 1H, H2''), 3.86 (dd, 2H, ^3J = 17.6, 6.4 Hz; αH-Gly), 3.95 (dd, 1H, ^3J = 17.6, 6.4 Hz; αH-Gly), 4.14 (dd, 1H, ^3J = 4.5, 7.7, 12.7 Hz, H4'), 4.26 (dd, 1H, ^3J = 12.0, 5.8 Hz; H5''),4.32 (dd, 1H, ^3J = 11.8, 3.9 Hz; H5'), 5.02-5.01 (s, 2H, C_6H_5-CH_2), 5.22 (m, 1H, H3'), 6.17 (dd, 1H, ^3J = 6.5, 7.8 Hz; H1'), 7.31-7.34 (m, 10H, C_6H_5), 7.49 (s, 1H, H6), 7.52 (dd, 1H, ^3J = 9.6, 6.5 Hz; NH), 8.35 (dd, 1H, ^3J = 6.7, 4 Hz; NH'), 11.32 (br.s, 1H, H3). ^{13}C NMR: δ = 12.72 (CH_3-Thym), 35.32 (C-2'), 40.53, 40.64, 40.85 (Cα-Gly), 43.31 (Cα'-Gly), 64.09 (C-5'), 65.51 (CH_2-O), 74.49 (C-3'), 81.63 (C-4'), 84.64 (C-1'), 110.15 (C-5), 129.15, 128.61, 127.57 (C_6H_5, C-3,4), 136.73, (C_6H_5, C-1), 137.0 (C-6), 156.07 (OCO), 163.63 (C-4) 169.93, 169.57 (COO). ESI-MS: *m/z:* 739 $[M+H]^+$.

4c: 3', 5'-bis-O-(*N*-benzyloxycarbonyl-glycyl-glycyl-glycyl)-thymidine

m.p. = 127–129 °C. ^{1}H-NMR, δ = 1.8 (s, 3H, CH_3), 2.25 (m, 1H, H2') ,2.43 (m, 1H, H2''), 3.75 (s, 2H, αH-Gly), 3.99 (s, 2H, α'H-Gly), 4.15 (dd, 1H, ^3J = 4.1, 7.2 Hz; H4'), 4.28 (dd, 1H, ^3J = 11.9, 5.5 Hz; H5''), 4.32 (dd, 1H, ^3J = 12.5, 4.1 Hz; H5'), 5.03 (s, 2H, C_6H_5-CH_2), 5.23 (m, 1H, H3'), 6.19 (dd, 1H, ^3J = 8.1, 6.4 Hz; H1'), 7.3-7.39 (m, 10H, C_6H_5), 7.49 (dd, 1H, ^3J = 11.8, 6.0 Hz; NH), 7.51 (s, 1H, H6), 8.2 (dd, 1H,^3J = 10.7, 5.4 Hz; NH'), 8.34 (t, 1H, ^3J = 5.0 Hz; NH''), 11.4 (br.s, 1H, H3). ^{13}C-NMR: δ = 12.13 (CH_3 -Thym), 35.30 (C-2'), 40.78, 40.67, 40.55 (Cα-Gly), 41.65, 41.55 (Cα''-Gly), 43.48, 43.35 (Cα'''-Gly), 64.13 (C-5'), 64.50 (CH_2-O), 74.49 (C-3'), 80.78 (C-4'), 83.83 (C-1'), 110.08(C-5), 127.79, 127.10, 127.81 (C_6H_5, C-3,4), 135.83 (C-6), 136.99 (C_6H_5, C-1), 150.41 (C-2), 164.0 (C-4), 156.46, 156.51 (OCO), 169.56, 169.47 (COO). ESI-MS: *m/z:* 853 $[M+H]^+$.

4d: 3', 5'-bis-O-(*N*-benzyloxycarbonyl-phenyl-alanyl)-thymidine

m.p. = 83–85 °C. ^{1}H-NMR, δ = 1.82 (s, 3H, CH_3), 2.21 (m, 1H, H2''), 2.87 (m, 1H, H2'), 3.23 (dd, 2H, ^3J = 6, 14 Hz; β-CH_2), 4.17 (m, 1H, H4'), 4.33 (m, 1H, H5'', H5'), 4.88 (dd, 1H, ^3J = 14.4, 6.3 Hz; α-CH), 5.03 (s, 2H, C_6H_5-CH_2), 5.09 (m, 1H, H3'), 6.01 (t, 1H, ^3J = 7.0 Hz; H1'), 7.33-7.34 (m, 20H, C_6H_5), 7.34 (s, 1H, H6), 8.35 (dd, 1H, ^3J = 6.7, 4.0, Hz; NH), 11.4 (br.s, 1H, H3). ^{13}C-NMR: δ = 12.18 (CH_3-Thym), 35,62 (C-2'), 38.5, 38.96 (Cβ,β'-Phe), 57.02, 57.36 (Cα,α'-Phe), 64.2 (C-5'), 65.53 (CH_2-O), 74.52 (C-3'), 80.78 (C-4'), 83.69 (C-1'), 110.11 (C-5), 127.76, 127.8, 128.23 (C_6H_5, C-3,4), 128.34 (Cζ-Phe), 130.15 (Cε-Phe), 130.86 (Cδ-Phe), 137.96 (Cγ-Phe), 135.51 (C-6), 136.79 (C_6H_5, C-1), 150.42 (C-2), 156.43 (OCO), 163.52 (C-4), 171.26, 171.56 (COO). ESI-MS: *m/z:* 805 $[M+H]^+$.

4e: 3', 5'-bis-O-(*N*-benzyloxycarbonyl-(4-F)-phenylalanyl)-thymidine (S)

m.p. = 73–75 °C, $[\alpha]^{20}_D$ = –0.8° (c = 1, CH_3OH). ^{1}H-NMR, δ = 1.78 (s, 3H, CH_3), 2.2–2.5 (m, 2H, H2'), 2.94–3.04 (m, 4H, βH-Phe), 3.97 (s, 1H, H4'), 4.2–4.4 (m, 4H, αH-Phe, H5'), 4.97, 5.01 (s, 2H, C_6H_5-CH_2), 5.09 (br.s., 1H, H3'), 6.12 (t, 1H, ^3J = 7.2 Hz; H1'), 7.08-7.40 (m, 18H, H-C_6H_5), 7.44 (s ,1H, H6), 7.94 (t, 2H, ^3J = 8.3 Hz; NH), 11.41 (s, 1H, H3). ^{13}C-NMR: δ = 12.15 (CH_3-Thym), 35,54 (C2'), 38.56, 38.90 (Cβ,β'-Phe), 55.49, 55.73 (Cα,α'-Phe), 64.40 (C-5'), 65.56 (CH_2-O), 74.56

(C-3'), 80.88 (C-4'), 83.90 (C-1'), 110.08 (C-5), 114.80, 115.14 ($^3J_{CF}$ = 21.0 Hz; Cε,ε'-Phe), 127.65, 127.81, 128.23 (C_6H_5, C-3,4), 131.0 (Cδ-Phe), 133.49, 133.34 ($^2J_{CF}$ = 9.4 Hz; Cγ,γ'-Phe), 136.07 (C_6H_5, C-1), 135.49 (C-6), 136.77 (C_6H_5, C-1), 150.39 (C-2), 155.99 (OCO), 161.14 ($^1J_{CF}$ = 243 Hz; Cζ-Phe), 163 (C-4), 172.9 (COO). ESI-MS: *m/z:* 841 $[M+H]^+$.

4f: 3', 5'- bis-O-(*N*-benzyloxycarbonyl-(4-F)-phenylalanyl)-thymidine (R)

m.p. = 69–71 °C, $[\alpha]^{20}{}_D$ = +1.2° (c = 1, CH_3OH). ^{1}H-NMR, δ = 1.75 (s, 1H, CH_3), 2.3–2.4 (m, 1H, H2'), 2.91–3.08 (m, 4H, βH-Phe), 4.09 (br.s., 1H, H4'), 4.29–4.36 (m, 4H, αH-Phe, H5'), 4.97, 5.0 (s, $2HC_6H_5$-C H_2), 5.16 (m, 1H, H3'), 6.14 (t, 1H, ^3J = 7.16 Hz; H1'), 7.04-7.4 (m, 18H, C_6H_5), 7.51 (s, 1H, H6), 7.92 (t, 1H, 1H, ^3J = 8.3 Hz; NH), 11.41 (s, 1H, H3). ^{13}C-NMR: δ = 12.07 (CH_3-Thym), 35.48 (C-2'), 38.4, 38.52 (Cβ,β'-Phe), 55.35, 55.50 (Cα,α'-Phe), 64.36 (C-5'), 65.58 (CH_2-O), 74.63 (C-3'), 80.88 (C-4'), 83.74 (C-1'), 110.14 (C-5), 114.80, 114.13 ($^3J_{CF}$ = 20.7 Hz; Cε,ε'-Phe), 127.54, 127.66, 127.82 (C_6H_5, C-3,4), 131.07 (Cδ-Phe), 133.49, 133.34 ($^2J_{CF}$ = 9.4 Hz; Cγ,γ'-Phe), 135.54 (C-6), 136.82 (C_6H_5, C-1), 150.38 (C-2), 156.04 (OCO), 161.14 ($^1J_{CF}$ = 243 Hz, Cζ-Phe), 163.62 (C-4), 171.29, 171.44 (COO). ESI-MS: *m/z:* 841 $[M+H]^+$.

4 g: 3',5'-bis-O-(*N-tert* -butoxycarbonyl-2-amino-methylthiazol-4-carboxyl)-thymidine

m.p. = 120–123 °C. ^{1}H-NMR, δ = 1.78 (s, 3H, CH_3), 1.41 (s, 9H, $3CH_3$), 2.28–2.52 (m, 1H, H-2'), 4.20 (br.s., 1H, H4'), 4.32 (m, 2H, H5'), 4.39 (d, 2H, ^3J = 5.83 Hz; CH2), 5.23 (m, 1H, H3'), 6.17 (br.s, 1H, H1'),7.68 (s, 1H, H6), 7.81 (t, 1H, ^3J = 5.82 Hz; NH), 8.3 (s,1 H, CH-Thz), 11.39 (s, 1H, H3). ^{13}C-NMR: δ = 11.84 (CH_3- Thym), 28.12 (Boc-CH_3), 35.88 (C-2'), 41.96 (CH_2), 64.64 (C-5'), 75.44 (C-3'), 78.73 (Boc-Cq), 81.11 (C-4'), 84.10 (C-1'), 110.03(C-5), 129.86, 130.11 ($C\text{-}5_{Thz}$), 135.63(C-6), 145.0 ($C\text{-}4_{Thz}$), 150.5 (C-2), 155.76 (Boc-CO), 160.09, 160.38 ($C\text{-}2_{Thz}$), 163 (C-4), 172.16 (COO). ESI-MS: *m/z:* 723 $[M+H]^+$.

4h: 3', 5'-bis-O-(*N*-benzyloxycarbonyl-alanyl)-thymidine

m.p. = 79–81 °C. ^{1}H-NMR, δ =1 .58 (d, 3H, ^3J = 5 Hz; βH-Ala), 1.78 (s, 3H, CH_3), 2.2–2.5 (m, 1H, H2'), 4.17 (s, 1H, H4'), 4.3 (s, 2H, H5'), 4.63 (m, 1H, α-CH), 5.03–5.09 (s, 2H, C_6H_5-CH_2), 5.21 (m, 1H, H3'), 6.13 (t, 1H, ^3J = 7.4 Hz; H1'), 7.31–7.35 (m, 10H, C_6H_5), 7.49 (s, 1H, H6), 8.77 (d, 1H, ^3J = 6.3 Hz; NH), 11.88 (s, 1H, H3). ^{13}C-NMR: δ = 12.47 (CH_3-Thym), 17.25, 17.49 (Cβ,β'-Ala), 37.65 (C-2'), 49.65, 49.99 (Cα,α'-Ala), 65.56 (C-5'), 67.68 (CH_2-O), 76.23 (C-3'), 83.23 (C-4'), 86.41 (C-1'), 112.01 (C-5), 128.88, 129.44 (C_6H_5, C-3,4), 135.18 (C-6), 137.17 (C_6H_5, C-1), 152.08 (C-2), 156.51 (OCO), 164.21 (C-4), 174.04, 174.35 (COO). ESI-MS: *m/z:* 653 $[M+H]^+$.

4i: 3', 5'-bis-O-(*N*-benzyloxycarbonyl-valyl)-thymidine

m.p. = 59–61 °C. ^{1}H-NMR, δ = 0.83-0.93 (m, 12H, γCH3-Val), 1.79 (s, 3H, CH_3), 1.99–2.13 (m, 2H, βH- Val), 2.22–2.47 (m, 1H, H2'), 3.96 (m, 2H, αH-Val), 4.14 (br.s, 1H, H4'), 4.3 (s, 2H, H5'), 5.03–5.05 (s, 2H, C_6H_5-CH_2), 5.22 (br.s, 1H, H3'), 6.20 (t, 1H, ^3J =7 .0 Hz; H1'), 7.35 (br.s, 10H, C_6H_5), 7.45 (s, 1H, H6), 7.8 (t, 1H, ^3J = 8.4 Hz; NH), 11.38 (s, 1H, H3). ^{13}C-NMR: δ = 12.06 (CH_3-Thym), 18.29, 18.88 ($C\gamma_1$, γ_2-Val), 29.62, 29.90 (Cβ,β'-Val), 35.66 (C-2'), 59.78, 60.01 (Cα,α'-Val), 64.12 (C-5'), 65.66 (CH_2-O), 74.34 (C-3'), 80.86 (C-4'), 84.0 (C-1'), 109.98 (C-5), 127.85, 128.34 (C_6H_5, C-3, 4), 135.57 (C-6), 136.81 (C_6H_5, C-1), 150.41 (C-2), 156.42 (OCO), 163.62 (C-4), 171.30, 171.64 (COO). ESI-MS: *m/z:* 709 $[M+H]^+$.

Viruses

Influenza virus A / chicken / Germany / 27 / Weybridge (H7N7) (FPV) (Institute of Virology, Bratislava, Slovak Republic), pseudorabies virus (Aujeszky, A2 strain) (PsRV) (Central Veterinary Research Institute, Sofia) and herpes simplex virus 1 DA strain (HSV-1) (National Center for Infections and Parasitic Diseases, Sofia) were used.

Cell cultures

Primary chick embryo fibroblasts cultures (CEC) were prepared according to Porterfield (1960) and cell suspension (1–1.5x10^6 cells/ml) was seeded in Eagle's MEM (Difco) growth medium supplemented with 10% calf serum and antibiotics. Human diploid foreskin fibroblast cultures

(HDFFC) were grown in DMEM (Gibco) supplement with 10% calf serum and antibiotics.

Antiviral test

Primary screening for antiviral activity was carried out using the agar-diffusion plaque-inhibition method with cylinders (Rada *et al.*, 1962; Galabov *et al.*, 1980). It included testing of compounds against representatives of two taxonomic viral groups, namely orthomyxo and herpes viruses, which represent a few of the most important families of human pathogens. The viruses used were FPV and PsRV, respectively. Monolayer cell cultures of CEC in 90-mm petri dishes were inoculated (60 min adsorption at 20 °C) with a virus dose giving semiconfluent plaques after incubation at 37 °C (48h with FPV, 72 h with PsRV). Test compounds (0.005 mol/l solutions in DMSO) were added dropwise within 6-mm glass cylinders fixed in the agar overlay [1% Bactoagar (Difco) in Eagle's MEM (Gibco) medium with heated calf serum, 1.65 mg/ml sodium bicarbonate and antibiotics, penicillin 100 IU/ml and streptomycin 100 μg/ml]. A second overlay containing 1.5% (w/v) agar and 0.002% (w/v) neutral red in physiological saline was added after the incubation. The antiviral effect (E) of a given compound was recorded on the basis of the difference ($\Delta\Phi$) between the size of the zone of plaque inhibition (diameter $\Delta\Phi_i$ in mm) and zone of cytotoxycity ($\Delta\Phi_t$) (four cylinders per compound, placed in a separate petri dish each) and designated as follows: -, $\Delta\Phi \leq 5$ mm; ± $\Delta\Phi = 5-10$ mm; +, $\Delta\Phi = 11-20$ mm; ++, $\Delta\Phi = 21-40$ mm; +++, $\Delta\Phi > 40$ mm.

Cytopathic effect (CPE) inhibition multicycle test

Monolayer cell cultures of HDFFC grown in 96 – wells plastic microplates (Flow, UK) were used. Compounds (at subsequent 0.5 $\log_{10}$ dilution) were applied in the maintenance medium [DMEM (Gibco, USA) with 2% calf serum and antibiotics] immediately after virus inoculation at three different viral doses (10, 100 and 1000 $CCID_{50}$, well), three wells per test sample were used. CPE was scored an a 0–4 basis with 4 representing the total cell destruction. These data were used to obtain dose-response curves for each compound at a given viral dose. From these graphs the minimum concentration causing a 50% reduction of CPE as compared to the untreated controls (MIC_{50} value) was determined.

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