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Original article

Synthesis and biological activity of some new 5′-*O*-acyl tiazofurin derivatives

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Abstract

Three new 5′-*O*-acyl tiazofurin derivatives **2**–**4** were synthesized and evaluated for their antiproliferative activity against different tumour cell lines as well as for their ability to induce apoptosis in C6 cells in vitro. Apart of the antitumour assays, the cell membrane permeation of **2**–**4** and their intracellular metabolism in C6 cells in vitro was also studied in order to evaluate their potential as possible tiazofurin bioisosteres or prodrugs.

Keywords: Tiazofurin derivatives; Prodrugs; Anticancer agents; Apoptosis; Cytotoxicity

1. Introduction

Tiazofurin (**1**, Fig. 1) is a synthetic *C*-nucleoside, which shows significant antitumour activity in a variety of tumour systems [1]. In the phase II clinical trials, it induced dramatic haematological responses in patients with acute myelogenous leukaemia, or chronic myeloid leukaemia in blast crisis [2,3]. The biological activity of tiazofurin derives from a combination of cytotoxicity and maturation-inducing activities [4–6]. Both effects are attributed to inhibition of inosine 5′-monophosphate dehydrogenase (IMPDH) by the tiazofurin adenine dinucleotide (TAD), which induces the shutdown of guanine nucleotide synthesis [7]. This results in inhibition of proliferation, induction of differentiation and apoptosis in different types of malignant cells. Despite the remarkable efficacy of tiazofurin, lack of specificity and significant toxicity remains a problem in its clinical use [1,8,9]. In order to provide an access to derivatives of reduced toxicity, a number of tiazofurin analogues have been synthesized bearing either a modified heterocyclic aglycon [10–15] or a modified sugar moiety [16–21]. Our current interest in this field is focused onto the 5′-*O*-acyl derivatives **2**, **3** and **4** (Fig. 1), designed as possible tiazofurin bioisosteres, or as the tiazofurin prodrugs cleavable by cellular esterases. A number of nucleoside prodrugs of improved selectivity and/or better uptake by target cells have already been described [22–26]. The less hydrophilic derivatives **2**, and **3** were expected to show improved transport abilities across cell membrane, while the tiazofurin/D-glucose conjugate **4**, was initially designed as a possible selective agent that would be able to pass across tumour cell membrane through D-glucose transporters. However, our preliminary studies suggested that **4** competed with free tiazofurin for the nucleoside transport sites in C6 cells, whereby its uptake was significantly lower than that observed for tiazofurin [27]. Continuing work in this field, we report herein on the synthesis of **2**–**4** for evaluation of their biological activity against some human and murine tumour cell lines (Scheme 1).

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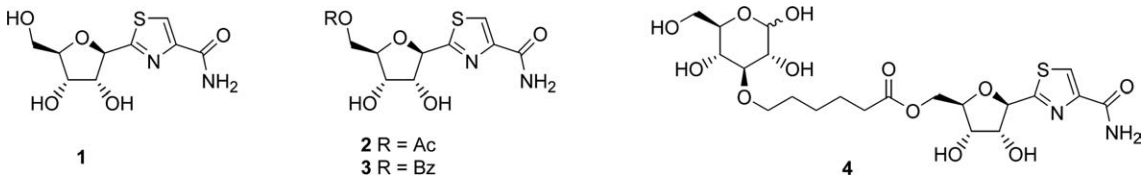


Fig. 1. Tiazofurin (**1**) and the corresponding 5-*O*-acyl derivatives **2**-**4**.

2. Results and discussion

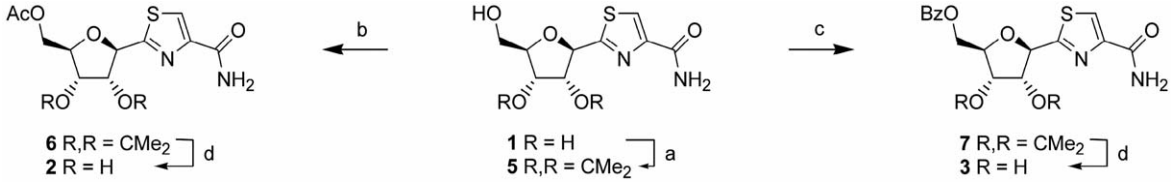
2.1. Chemistry

Our first approach to 5′-*O*-acyl tiazofurin derivatives **2** and **3** involved a direct treatment of **1** with an appropriate acyl chloride, under the conditions similar to those reported for esterification of ribavirin [28]. Although the preparation of certain 5′-*O*-acyl ribavirin derivatives was successfully achieved by an action of 1.1 molar equivalent of the appropriate acyl chloride in a mixture of pyridine and *N*,*N*-dimethylformamide, this procedure failed to afford the expected 5′-*O*-acyl tiazofurin derivatives **2** and **3**. Under these reaction conditions a mixture of the corresponding mono-, di-, and tri-*O*-acyl derivatives was obtained. To overcome this problem, a simple three-step sequence for the preparation of targets **2** and **3** was elaborated. In the first step, the secondary hydroxyl groups of **1** were first protected by acetonation with 2,2′-dimethoxypropane in boiling acetone, in the presence of *p*-toluenesulfonic acid as a catalyst. The known [29] 2,3-*O*-isopropylidene derivative **5** was thus obtained in 51% yield. Reaction of **5** with acetyl chloride in pyridine afforded the expected 5′-*O*-acetyl derivative **6** in 86% yield, while treatment of **5** with benzoyl chloride in pyridine gave the corresponding 5′-*O*-benzoyl derivative **7** in 96% yield. Both reactions must be carried out at 0 °C, in order to avoid transformations of the amide function observed at higher temperatures. Hydrolytic removal of the isopropylidene protective group in **6** and **7** gave high yields of target compounds **2** (88%) and **3** (80%) ready for biological testing. The structure and purity of **2** and **3** thus obtained was confirmed by the corresponding spectral and analytical data.

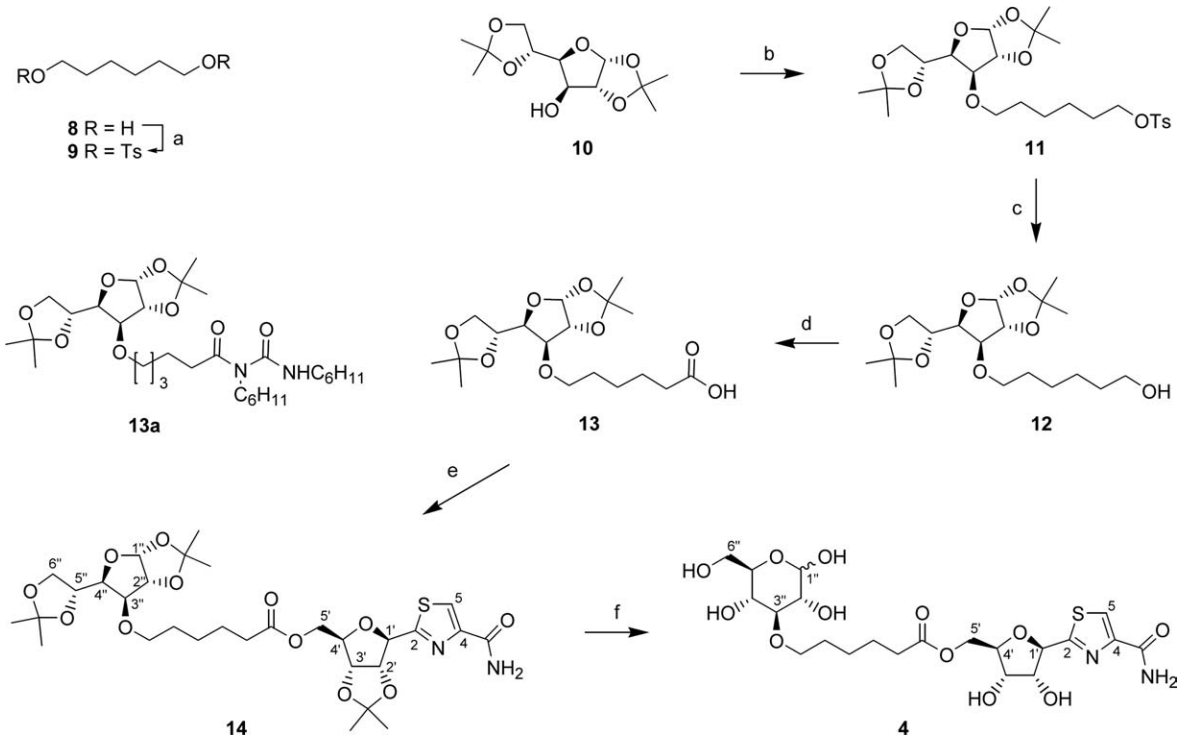
Synthesis of tiazofurin/D-glucose conjugate **4** was achieved by using an adopted procedure, recently applied for the preparation of ribavirin and thymidine analogues of **4** [30]. Commercially available 1,6-hexane diol (**8**) and 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**10**) have been used as convenient starting compounds in this part of the work (Scheme 2). Treatment of **8** with tosyl chloride in pyridine gave the expected ditosylate **9** (84%), which was further allowed to react with **10** in DMSO, in the presence of sodium hydride as a proton acceptor, whereupon the 3-*O*-(6′-tosyloxy) hexyl derivative **11** was obtained in 66% yield. The intermediate **11** was treated with an excess of potassium carbonate in aqueous DMF to give a moderate yield of the corresponding primary alcohol **12**. In this way, compound **12** was prepared in an overall yield of only 24% from two synthetic steps. However, when the last two-step sequence was carried out without purification of the intermediate **11**, the desired product **12** was obtained in a more acceptable overall yield (41% from **10**). Oxidation of the primary hydroxyl group in **12** gave the corresponding carboxylic acid **13** (67%), a key intermediate in the synthesis of target **4**. With the requisite intermediate **13** in hands, we next focused to its coupling reaction with protected tiazofurin derivative **5**. Attempted esterification of **13** with **5**, under the conditions already used for preparation of ribavirin/D-glucose conjugate (DCC, DMAP, Py, 60 °C) [30] did not give the expected product **14**. The corresponding dicyclohexylurea derivative **13a** was a major product under these reaction conditions. However, when the coupling reaction of **5** and **13** was carried out with ethyl chloroformate (Et_3N, CH_2Cl_2), the corresponding ester **14** was obtained in 36% yield. Final deprotection of **14** with aqueous trifluoroacetic acid furnished the target molecule **4** (65%), as a 1:1 mixture of the corresponding α- and β-anomers. NMR and high-resolution MS data confirmed the structure and purity of product **14**.

2.2. Cytotoxic activity

Compounds **2**–**4** were evaluated for their cytotoxic activity against murine C6 and 9L glioma, human MCF7 breast adenocarcinoma, human HTB177 lung carcinoma, human K562 and NB4 leukaemia cells, and against normal human dermal fibroblasts (NHDF). In vitro cytotoxicity was evaluated after 72-h cell treatment by using the NRU assay. The results, including the data for the reference compound **1**, are presented in Table 1. Remarkably, the 5′-*O*-acetyl derivative **2** has shown a potent cytotoxic activity against C6, MCF7, K562 and NB4 cell lines, comparable to that observed for tiazofurin. This compound also



Scheme 1. Reagents and conditions: (a) $Me_2C(OMe)_2$, TsOH, ME_2CO, reflux, 1 h, 51%; (b) AcCl, Py, 0 °C, 20 h; 86%; (c) BzCl, Py, 0 °C, 20 h; 96%; (d) 9:1 TFA-H_2O, rt, 1 h, 88% of **2**, 80% of **3**.



Scheme 2. Reagents and conditions: (a) TsCl, Py, CH_2Cl_2, −15 °C →0 °C, 24 h 84%; (b) **9**, NaH, DMSO, rt, 2 h, 66%; (c) K_2CO_3, H_2O, DMF, 125 °C, 4 h, 40%; (d) $KMnO_4$, PDC, Me_2CO, reflux, 6 h, 30%; (e) **5**, EtOCOCl, Et_3N, CH_2Cl_2, −7 °C, for 0.5 h, then rt for 19 h, 36%; (f) 9:1 TFA-H_2O, rt, 1 h, 65%.

Table 1
In vitro cytotoxicity of compounds **1**–**4**

Compds	IC_{50}, μM[a]						
	C6	9L	MCF7	HT-B177	K562	NB4	NHDF
1	3.5	18.4	5.1	28.7	6.3	ND[b]	ND[b]
2	6.4	19.1	6.8	38.1	14.7	8.0	> 100
3	27.8	74.2	37.8	73.1	> 100	13.9	> 100
4	58.4	83.9	23.3	83.8	86.0	11.9	> 100

[a] IC_{50} is a compound concentration required to inhibit the cell growth by 50% compared to untreated control.
[b] ND – not determined.

exhibited essentially the same activity as tiazofurin against murine 9L glioma cells. Compounds **3** and **4** exhibited a significant antiproliferative activity only against leukaemia NB4. The tiazofurin/D-glucose conjugate **4** was also active against MCF7 cells, but it was more than 4-fold less active with respect to the reference compound **1**.

Generally, in all murine and human tumour cells, the 5′-*O*-acetyl derivative **2** was more active than the 5′-*O*-benzoyl derivative **3**, which in turn was more active than the tiazofurin/D-glucose conjugate **4**, against both murine glioma and HTB177 cells. However, the conjugate **4** was slightly more active than 5′-*O*-benzoyl derivative **3** towards the MCF7, K562 and NB4 cell lines. Overall, these results indicate that only 5′-*O*-acetyl derivative **2** brings together structural characteristics that enable it to exert a potent antiproliferative activity towards all investigated malignant cells, but in the same time, it is y inactive against the normal human dermal fibroblast cells.

2.3. Detection of apoptosis

As the induction of apoptosis in cancer cells is an important outcome of anticancer therapy, we performed additional studies to explore the ability of tiazofurin ester derivatives **2**–**4** to promote apoptotic cell death in murine C6 glioma. The effect of tiazofurin 5′-*O*-ester derivatives **2**–**4** on the induction of apoptosis in C6 rat glioma cells was evaluated by microscopic analysis of May-Grunwald-Giemsa stained cells. Morphological changes characteristic for apoptosis – loss of cell contacts, cell rounding, nuclear condensation and fragmentation, as well as phagocytosis of apoptotic by neighbouring cells [31], were found after treatment with all tested compounds **1**–**4**. Thus, in concentration of 60 μM, 5′-*O*-acetyl derivative **2** was shown to induce 23.8% apoptotic cells (Fig. 2), almost 5 times more than 5′-*O*-benzoyl derivative **3** (4.9% apoptotic cells). The conjugate **4** induced only 2.9% of apoptotic cells, while tiazofurin itself caused 18.9% apoptotic cells after 72 hours of incubation. Morphological evidence of apoptosis was confirmed with the TUNEL assay that detected DNA internucleosomal fragments in individual cells. The number of TUNEL positive cells was proportionally higher than the number of morphologically apoptotic cells (data not shown). Such results are expected, considering the sensitivity of TUNEL method and detection of earlier stages of apoptosis [32].

2.4. Evaluation of growth inhibition

As the C6 cells were the most sensitive to tiazofurin, this cell line was used as a model for further biological testing of

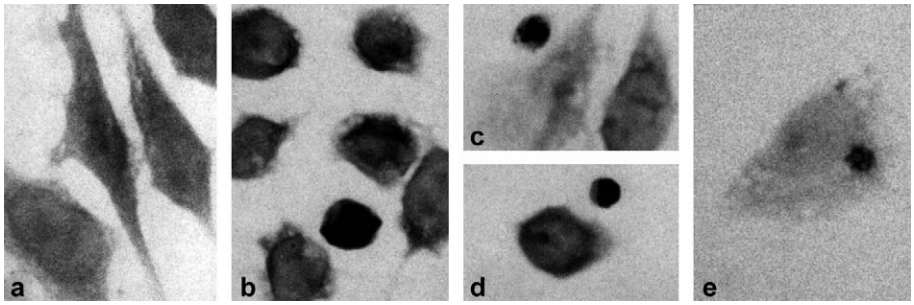


Fig. 2. Morphological evidence of apoptosis induced by 5′-*O*-acetyl-derivative **2** in C6 cells. May-Grunwald-Giemsa staining. (a) Control untreated cells. (b) Cells treated with **2** for 24 hours. Note change in cell shape – rounding and loss of cell-cell contacts. (c) and (d) Fragmentation and condensation of nuclear material seen after 48 and 72 hours of incubation. (e) Phagocytosed apoptotic cell inside one normal looking cell.

1–**4**. The viability of C6 cells was first evaluated by the trypan-blue exclusion assay. It has been found that the viability of C6 cells after their treatment with highest concentrations of **2**–**4** was higher than 90%, thus implying to their cytostatic, rather than cytotoxic effects. The similar mode of action was recently observed for tiazofurin itself [33]. The cytostatic effects were further evaluated by the NRU test at 24, 48 and 72 h after using the indicated concentrations of **1**–**4** (1–120 μM). After 24-hours treatment (Fig. 3a), both tiazofurin and the 5′-*O*-benzoyl derivative **3** inhibited the cells growth in dose-dependent manner in all tested concentrations, while the 5′-*O*-acetyl derivative **2** showed a similar dose-dependent effect in the concentrations higher than 7.5 μM. Tiazofurin/D-glucose conjugate **4** showed a weak growth inhibition only in the concentration range of 30–120 μM. Compound **4** was completely inactive in the concentration of 15 μM, but it showed a growth stimulatory activity in the concentration range of 1–7.5 μM. The most potent antiproliferative effect was induced by tiazofurin, which in concentration of 3.75 μM decreased the cells growth for 21%. The 5′-*O*-acetyl derivative **2** showed a similar activity as tiazofurin in concentrations higher than 30 μM, while compounds **3** and **4** exhibited similar antiproliferative effects at the highest concentration tested. An increased dose-dependent growth inhibition (in all concentrations) was observed when C6 cells were treated with **1** and **2** for 48 h (Fig. 3b). Conversely, compounds **3** and **4** showed a similar dose-dependent effects only in concentrations higher than 3.75 μM. Tiazofurin again showed the most potent antiproliferative activity, whereby at a concentration of 3.75 μM it induced an inhibition of 27%. In the same concentration, compound **2** was almost 3-fold less active than tiazofurin, but in the concentration of 60 μM it showed almost the same antiproliferative effect as tiazofurin itself. In the same concentration, both **3** and **4** showed significant antiproliferative effects, although almost

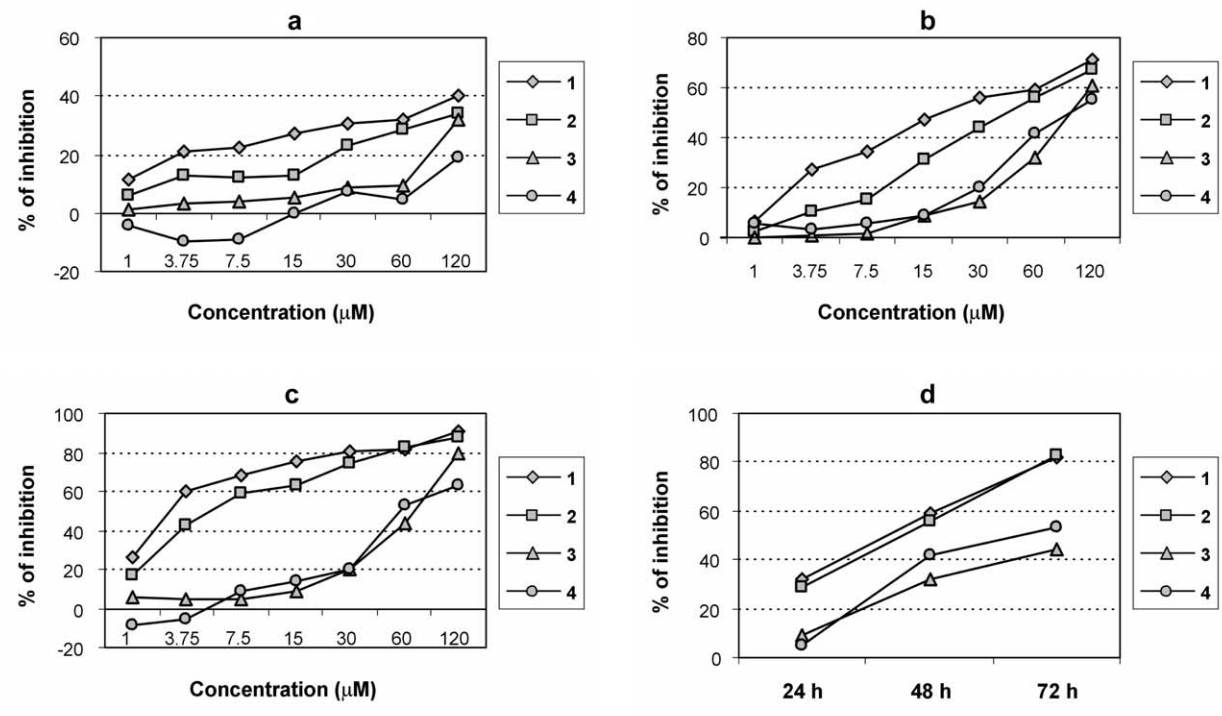


Fig. 3. Effects of tiazofurin (**1**) and ester derivatives **2**-**4** on proliferation of rat glioma C6 cells: (a) influence of concentration after 24 h, (b) influence of concentration after 48 h, (c) influence of concentration after 72 h, (d) time-dependent effects in the concentration of 60 μM.

2-fold lower with respect to tiazofurin. The growth inhibition was further increased after 72 h (Fig. 3c). At the concentration of 3.75 μM tiazofurin and 5′-*O*-acetyl derivative **2** induced growth inhibition of 60 and 42%, respectively. The 5′-*O*-benzoyl derivative **3**, at the same concentration, induced a growth inhibition of 5%, while the tiazofurin/D-glucose conjugate **4** again showed a weak growth stimulatory activity. At the concentration of 60 μM tiazofurin and 5′-*O*-acetyl derivative **2** induced the same growth inhibition of 62%, while both **3** and **4** induced growth inhibitions of 44 and 53%, respectively.

All tested compounds **1**–**4** inhibited the growth of C6 cells in time dependent manner. The growth inhibition pattern of **1**–**4** for the concentration of 60 μM is shown in Fig. 3d. The activity of all tested compounds was gradually increased in time, whereupon tiazofurin and the corresponding 5′-*O*-acetyl derivative **2** showed the most potent and the same degree of growth inhibition (cca. 30% after 24 h, 60% after 48 h, and over 80% after 72 h). After 24 h however, the 5′-*O*-benzoyl derivative **3** was almost 2-fold more active than the conjugate **4**, although the induced inhibition was very low after this time (9 and 5%, respectively). After treatment for 48 hours, compound **3** induced for 10% lower growth inhibition with respect to that induced by **4** (32 and 42%, respectively). Both **3** and **4** induced the most pronounced inhibition after 72-h treatment (44 and 53%, respectively).

2.5. In vitro uptake and intracellular conversion studies

Our previous work revealed that the actual uptake of **4** into rat C6 glioma cells was significantly lower than the uptake of tiazofurin [27]. Therefore, only the ability of **2** and **3** to enter to the murine C6 glioma cells was evaluated in the present work. Tiazofurin was used as a reference compound. Hence, the cells were incubated in the cell culture medium that contained 60 mM of tested compounds, and after 4 and 48 h of treatment the cells were collected by tripsinisation and centrifugation, homogenized and centrifuged again. The supernatant was analysed by the HPLC method recently developed in our laboratory [34]. The results are presented in Fig. 4.

The obtained results confirmed that the tiazofurin ester derivatives **2** and **3** were able to penetrate through the membrane of C6 cells, then underwent to subsequent intracellular hydrolysis to **1**, which was finally converted to TAD. The same sequence of intracellular transformations had already been established for the tiazofurin/D-glucose conjugate **4** [27]. When the C6 cells were treated with 5′-*O*-acetyl derivative **2** for 4 h, the cells contained essentially the same amounts of **1** and TAD (28.3 and 28.8 nM/mg of cells, respectively) and a somewhat lower amount (22.7 nM/mg) of the unchanged 5′-*O*-acetyl derivative **2**. The sum of intracellular concentrations of **2** and its metabolites (**1** and TAD) was for 39% higher than that observed inside the cells treated with tiazofurin for 4 h. This result implies to a faster cellular uptake of **2** with respect to tiazofurin. The cell membrane permeation of 5′-*O*-benzoyl derivative **3** is essentially the same as that observed for the parent compound **1** after 4 h. The concentration of the intact

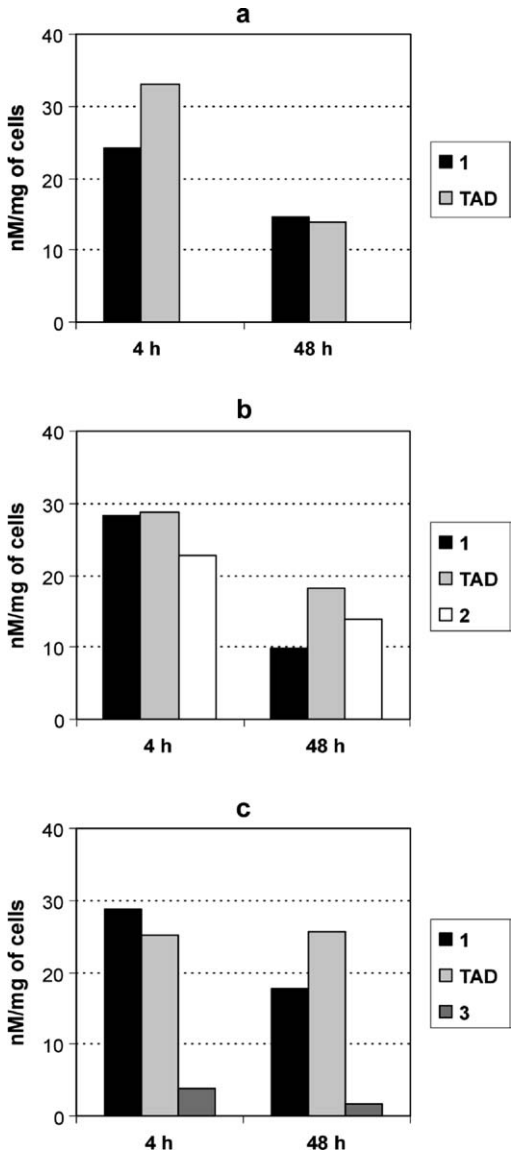


Fig. 4. Intracellular content of C6 cells after treatment with: (a) free tiazofurin **1**, (b) 2-*O*-acetyl derivative **2**, (c) 5-*O*-benzoyl derivative **3**.

5′-*O*-benzoyl derivative **3** in the cells was very low with respect to the concentrations of tiazofurin and TAD, thus implying to a rapid intracellular conversion of **3** into the corresponding metabolites (**1** and TAD). Conversely, the intracellular concentration of 5′-*O*-acetyl derivative **2** was very similar to the concentrations of tiazofurin and TAD indicating that the hydrolysis of its ester bond in the cells after 4 h might be rather slow. After 48-h of cells treatment however, the C6 cells treated with tiazofurin contained essentially the same amounts of **1** (14.5 nM/mg) and TAD (14 nM/mg). The total intracellular content was decreased for 50% compared to that established after 4-h of cells treatment, presumably due to a tight binding of TAD to the IMPDH. When the C6 cells were treated with 5′-*O*-acetyl derivative **2**, the intracellular content of TAD (18.1 nM/mg) was almost 2-fold higher than that of tiazofurin (9.8 nM/mg). In the same time, this value is for 30% higher than the concentration of TAD recorded inside the cells treated

Table 2
Correlations between the intracellular content of active metabolites (**1**+TAD) and the observed C6 cells growth inhibition

Compds	**1**+TAD [a]		Estimated amount of IMPDH-bound TAD [a]	% of inhibition
	4 h	48 h		
1	57.3	28.5	28.8	59
2	57.1	27.9	29.2	56
3	53.9	43.4	10.5	32

[a] In nM/mg of cells.

with tiazofurin for 24 hours. When the C6 cells were treated with 5′-*O*-benzoyl derivative **3** the intracellular content of TAD was essentially the same as that observed after 4 hours of cells treatment (cca. 25 nM/mg). Moreover, the total amount of active metabolites (**1** and TAD) in the cells treated for 48 h with ester derivative **3** was significantly higher with respect to that observed inside the C6 cells treated with tiazofurin or 5′-*O*-acetyl derivative **2**. These data did not apparently correlate with the observed cytotoxic patterns (Fig. 3), but enabled us to estimate the amount of IMPDH-bound TAD. Namely, it was assumed that this amount is related to the difference of intracellular concentrations of the active metabolites (**1**+TAD) detected after 4 and 48 hours of cells treatment.

As shown in Table 2, the estimated amounts of IMPDH-bound TAD after treatment with **1** and **2** are essentially the same and significantly higher with respect to that observed for **3**. These data correlate well with the growth-inhibitory activity of **1**, **2**, and **3** observed after the C6 cells treatment for 48 hours (Figs. 3b and d).

3. Conclusion

In conclusion, we have synthesized three novel tiazofurin 5′-*O*-acyl ester derivatives **2**, **3** and **4**, which have shown a potent cytotoxic activity against some human leukaemia and solid tumour cell lines, but did not exhibit any significant cytotoxicity towards the normal human dermal fibroblast cells. All newly synthesized compounds induced apoptosis in C6 rat glioma cells in vitro, whereupon the 5′-*O*-acetyl derivative **2** was shown to be the most effective (23.8% of apoptotic cells). The 5′-*O*-benzoyl derivative **3** and the conjugate **4** were significantly less effective (4.9 and 2.9% of apoptotic cells, respectively) than tiazofurin (18.9% of apoptotic cells). Compounds **2**–**4** inhibited the C6 cells growth in dose- and time-dependent manner. After 72 h and at the concentration of 60 μM, both tiazofurin and 5′-*O*-acetyl derivative **2** showed the same cytotoxicity pattern (62% growth inhibition), while compounds **3** and **4** induced growth inhibitions of 44 and 53%, respectively. Accordingly, all three esters **2**-**4** might be considered as tiazofurin bioisosteres, where by the 5′-*O*-acetyl derivative **2** has shown a similar cytotoxicity as the parent compound **1**. It was further confirmed that all tiazofurin esters **2**–**4** were intracellularly metabolised to tiazofurin and its active metabolite TAD. The intracellular concentration of these metabolites (**1** and TAD) was used to estimate the amount of IMPDH-bound TAD. The observed cytotoxicity pattern of tested compounds correlated well with these data. The sum of intracellular concentrations of **2** and its metabolites (**1** and TAD) inside the C6 cells indicated to a faster cellular uptake of **2** with respect to tiazofurin, while the cell membrane permeation of 5′-*O*-benzoyl derivative **3** is essentially the same as that observed for the parent compound **1**. The results of intracellular conversion studies of **2**–**4**, pointed to the fact that these esters could certainly serve as tiazofurin depot forms (at least in the C6 cells), but their potentiality as tiazofurin prodrugs should be further evaluated through additional in vitro and in vivo experiments. Namely, the efficacy of the reported compounds as prodrugs in vivo is strongly dependent on their stability towards plasma esterases at least. Finally, on account of the lack of the free 5′-hydroxy group in their ribose moiety, derivatives **2**–**4** should devoid of the side effects of tiazofurin on the central nervous system. Namely, it is believed that neurotoxicity of **1** originates from its ability to interact with the central nervous system adenosine receptors, whereby the presence of free 5′-hydroxyl group is essential for the ligand-receptor binding [35].

4. Experimental section

4.1. Chemistry

4.1.1. General methods

Melting points were determined using a Büchi SMP-20 apparatus and were not corrected. Optical rotations were measured on a Perkin Elmer 141 MC polarimeter. IR spectra were recorded on Perkin-Elmer 457 and Perkin-Elmer FT-IR 1725 X spectrometers and the band positions are expressed in cm^{-1}. NMR spectra were recorded on a Bruker AC 250 E instrument and chemical shifts are expressed in ppm values (δ-scale), downfield from tetramethylsilane. Low-resolution mass spectra were recorded on Finnigan-MAT 8230 (CI and EI) and VG AutoSpec (FAB) mass spectrometers. High-resolution mass spectra were taken on a Micromass LCT KA111 spectrometer. HPLC analysis was performed on a Hewlett Packard 1100 liquid chromatograph, equipped with quaternary pump and diode-array detector. The compounds of interest were separated on a Supelcosil LC-18 RP column (250×4.6 mm, 5 mm) by gradient elution. The mobile phase was composed of 0.1 M sodium-hydrogen phosphate buffer pH 5.1, and methanol. Peak identification was achieved by comparison of retention times and UV spectra of the eluting components with known standards. TLC was performed on DC Alufolien Kieselgel 60 F_{254} (E. Merck). Flash column chromatography was performed using ICN silica 32–63. Organic extracts were dried with anhydrous Na_2SO_4. Organic solutions were concentrated in a rotary evaporator under diminished pressure at a bath temperature below 30 °C. Tiazofurin [36] and TAD [37] were prepared as previously reported. All other chemicals were obtained commercially (guarantied reagent grade) and used without further purification. The trypan-blue exclusion assay was carried out as earlier reported [33].

4.1.2. 2-(2,3-O-isopropylidene-β-D-ribofuranosyl)thiazole-4-carboxamide (5)

To a solution of tiazofurin (**1**, 20 g, 74.3 mmol) in dry acetone (400 mL), was added 2,2′-dimethoxypropane (30 mL) and

TsOH×H_2O (0.8 g, 4.27 mmol). The mixture was refluxed for 1 h and then neutralized by stirring with Amberlyst IR-45 resin at room temperature for 0.5 h. The mixture was filtered and the resin washed with acetone. The combined organic solutions were evaporated to appearance of first crystals. The suspension was left at +4 °C over night to accomplish the crystallization. Pure **5** (11.7 g, 51%) was obtained as colourless crystals, mp 119–121 °C, $[\alpha]^{25}_D = -28.2$ (*c*, 0.4 in H_2O); lit. [29] mp 119–120 °C, $[\alpha]^{25}_D = -32.0$ (*c*, 0.5 in H_2O), ^{1}H NMR ($CDCl_3$): δ 1.39 and 1.63 (2×s, 3H each, Me_2C), 2.99 (dd, 1H, exchangeable with D_2O, $J_{5'a,OH} = 7.4$, $J_{5'b,OH} = 4.9$ Hz, OH), 3.73 (ddd, $J_{4',5'a} = 4.0$, $J_{5'a,5'b} = 12.2$ Hz, H-5′a), 3.89 (ddd, $J_{4',5'b} = 3.3$ Hz, H-5′b), 4.39 (m, 1H, $J_{3',4'} = 2.9$ Hz, H-4′), 4.85 (dd, 1H, $J_{2',3'} = 6.4$ Hz, H-3′), 4.96 (dd, 1H, $J_{1',2'} = 3.8$ Hz, H-2′), 5.25 (d, 1H, H-1′), 5.99 and 7.16 (2×bs, 2H, exchangeable with D_2O, $CONH_2$), 8.14 (s, 1H, H-5); ^{13}C NMR ($CDCl_3$): δ 25.38 and 27.31 (*Me*$_2$C), 63.02 (C-5′), 82.09 (C-3′), 84.27 (C-1′), 85.96 (C-2′), 86.38 (C-4′), 114.50 (Me$_2$*C*), 124.83 (C-5), 149.70 (C-4), 162.64 (C-2), 170.52 ($CONH_2$); CI MS: m/z 301 (M^++H), 300 (M^+), 285 (M^+–Me).

*4.1.3. 2-(5-O-Acetyl-2,3-O-isopropylidene-β-D-ribofuranosyl) thiazole-4-carboxamide (**6**)*

To a stirred solution of **5** (3 g, 10 mmol) in dry pyridine (70 mL) cooled at 0 °C in an ice bath, freshly distilled acetyl chloride (11 mmol) was added dropwise. The reaction mixture was stirred at 0 °C for 20 hours. After that, methanol was added and the mixture evaporated to dryness. The residue was co-evaporated twice with chloroform. Chromatographic separation on a column of silica gel (20:1 CH_2Cl_2–MeOH) gave pure **6** (2.93 g, 86%) as colourless crystals, mp 104–107 °C (from CH_2Cl_2–MeOH), $[\alpha]^{20}_D$ −22.1 (*c*, 1.0 in $CHCl_3$); IR (KBr): ν_{max} 3462, 3344, 3120, 2988, 1744, 1682, 1590, 1381, 1236, 1108, 1078; ^{1}H NMR ($CDCl_3$): δ 1.39 and 1.61 (2×s, 3H each, Me_2C), 1.93 (s, 3H, MeCO), 4.13 (dd, 1H, $J_{5'a,5'b} = 12.0$, $J_{4',5'a} = 4.6$ Hz, H-5′a), 4.30 (dd, 1H, $J_{4',5'b} = 3.9$ Hz, H-5′b), 4.47 (m, 1H, $J_{3',4'} = 3$ Hz, H-4′), 4.72 (dd, 1H, $J_{2',3'} = 6.4$ Hz, H-3′), 5.03 (dd, 1H, $J_{1',2'} = 3.5$ Hz, H-2′), 5.25 (d, 1H, H-1′), 6.22 and 7.24 (2×bs, 2H, $CONH_2$), 8.14 (s, 1H, H-5); ^{13}C NMR ($CDCl_3$): δ 20.55 (*Me*CO), 25.38 and 27.19 (*Me*$_2$C), 64.13 (C-5′), 82.24 (C-3′), 83.33 (C-4′), 84.63 (C-1′), 85.62 (C-2′), 114.54 (Me$_2$*C*), 124.83 (C-5), 149.69 (C-4), 162.36 (C-2), 170.38 and 170.78 (2×C = O); CI MS: *m/z* 685 ($2M^+$+H), 343 (M^++H).

4.1.4. 2-(5-O-Benzoyl-2,3-O-isopropylidene-β-D-ribofuranosyl) thiazole-4-carboxamide (7)

Following the same procedure as described above (preparation of **6**), by using bezoyl chloride as an acylation agent, pure **7** was obtained (3.90 g, 96%) as colourless crystals, m.p. 114–116 °C (from CH_2Cl_2–MeOH), $[\alpha]^{20}_D$ −28.1 (*c*, 1.0 in $CHCl_3$); IR (KBr): ν_{max} 3464, 3333, 2990, 1723, 1682, 1586, 1382, 1271, 1093; ^{1}H NMR ($CDCl_3$): δ 1.41 and 1.63 (2×s, 3H each, Me_2C), 4.43 (dd, 1H, $J_{5'a,5'b} = 12$, $J_{4',5'a} = 4.2$ Hz, H-5′a), 4.56 (dd, 1H, $J_{4',5'b} = 3.5$ Hz, H-5′b), 4.64 (m, 1H, $J_{3',4'} = 2.6$ Hz, H-4′), 4.86 (dd, 1H, $J_{2',3'} = 6.3$ Hz, H-3′), 5.15 (dd, 1H, $J_{1',2'} = 3.4$ Hz, H-2′), 5.30 (d, 1H, H-1′), 6.29 and 7.20 (2×bs, 2H, $CONH_2$), 7.3–7.85 (m, 5H, Ph), 8.01 (s, 1H, H-5); ^{13}C NMR ($CDCl_3$): δ 25.40 and 27.20 (*Me*$_2$C), 64.60 (C-5′), 82.30 (C-3′), 83.58 (C-4′), 84.90 (C-1′), 85.75 (C-2′), 114.43 (Me$_2$*C*), 124.77 (C-5), 149.67 (C-4), 128.35, 129.08, 129.35 and 133.30 (Ph), 162.84 (C-2), 165.93 (Ph*C*O), 170.80 ($CONH_2$); CI MS: *m/z* 405 (M^++H), 404 (M^+).

*4.1.5. 2-(5-O-Acetyl-β-D-ribofuranosyl)thiazole-4-carboxamide (**2**)*

A solution of **6** (0.684 g, 2 mmol) in 9:1 TFA/H_2O (10 mL) was stirred for 1 h at room temperature. The solvent was evaporated and the residue was co-evaporated with 1:1 MeOH–CH_2Cl_2 (2×10 mL). The remaining crude **2** was crystallised from 9:1 CH_2Cl_2–MeOH, to yield the pure **2** (0.53 g, 88%) as colourless crystals, m.p. 156–158 °C; $[\alpha]^{20}_D$ +9.8 (*c*, 2.96 in MeOH); IR (KBr): ν_{max} 3484, 3462, 3366, 1726, 1651, 1246; ^{1}H NMR (DMSO-d_6): δ 2.04 (s, 3H, MeCO), 3.91 (t, 1H, $J_{2',3'} = 5.3$, $J_{3',4'} = 5.2$ Hz, H-3′), 4.03–4.17 (m, 3H, $J_{1',2'} = 3.9$, $J_{5'a,5'b} = 13.9$ Hz, H-2′, H-4′ and H-5′a), 4.35 (dd, 1H, H-5′b), 5.00 (d, 1H, H-1′), 5.30 (bs, 2H, exchangeable with D_2O, 2×OH), 7.59 and 7.72, (2×bs, 2H, NH_2), 8.22 (s, 1H, H-5); ^{13}C NMR (DMSO-d_6): δ 20.77 (*Me*CO), 63.80 (C-5′), 71.19 (C-3′), 76.56 (C-2′), 80.91 (C-4′), 82.64 (C-1′), 124.51 (C-5), 150.40 (C-4), 162.35 (C-2), 170.27 (Me*C*O), 171.72 ($CONH_2$); CI MS: *m/z* 303 (M^++H); Anal. Calcd. for $C_{11}H_{14}N_2O_6S$: C, 43.70; H, 4.63; N, 9.27; Found: C, 43.82; H, 4.87; N, 9.12.

*4.1.6. 2-(5-O-Benzoyl-β-D-ribofuranosyl)thiazole-4-carboxamide (**3**)*

By using the same procedure as described above (preparation of **2**), pure **3** (0.58 g, 80%) was obtained, m.p. 136–138 °C (from CH_2Cl_2–MeOH); $[\alpha]^{20}_D$ +14.55 (*c*, 2.9 in MeOH); IR (KBr): ν_{max} 3478, 3356, 1715, 1682, 1277; ^{1}H NMR (DMSO-d_6): δ 4.08 (m, 1H, $J_{2',3'} = 5.2$, $J_{3',4'} = 5.1$ Hz, H-3′), 4.25 (m, 2H, $J_{1',2'} = 4.0$, $J_{4',5'a} = 4.6$, $J_{4',5'b} = 2.4$ Hz, H-2′ and H-4′), 4.41 (dd, 1H, $J_{5'a,5'b} = 12.1$ Hz, H-5′a), 4.60 (dd, 1H, H-5′b), 5.05 (d, 1H, H-1′), 5.38 (d, 1H, exchangeable with D_2O, $J_{3',OH} = 5.3$ Hz, OH-3′), 5.59 (d, 1H, exchangeable with D_2O, $J_{2',OH} = 5.6$ Hz, OH-2′), 7.45–7.95 (m, 5H, Ph), 7.62 and 7.74 (2×bs, 2H, NH_2), 8.18 (s, 1H, H-5); ^{13}C NMR (DMSO-d_6): δ 64.47 (C-5′); 71.29 (C-3′), 76.55 (C-2′), 81.19 (C-4′), 82.46 (C-1′), 124.45 (C-5), 128.82, 129.24, 129.49 and 133.52 (Ph), 150.41 (C-4), 162.38 (C-2), 165.63 (Ph*C*O), 171.48 ($CONH_2$); CI MS: *m/z* 364 (M^+); Anal. Calcd. for $C_{16}H_{16}N_2O_6S$×H_2O: C, 50.26; H, 4.71; N, 7.32; Found: C, 50.03; H, 4.93; N, 7.31.

*4.1.7. 1,6-Ditosyloxy-hexane (**9**)*

To a cooled (–15 °C) and stirred solution of dry 1,6-hexane diol (**8**; 50 g, 0.42 mol) in a mixture of dry pyridine (107 mL) and CH_2Cl_2 (180 mL) was added tosyl chloride (177.4 g, 0.93 mol) in small portions providing that temperature does not exceed 10 °C. The mixture was left for 24 h at 0 °C, then diluted with CH_2Cl_2 (550 mL) and the solution was washed succes-

sively with 10% aq HCl (5×100 mL) and with 10% aq NaCl (2×100 mL). The organic layer was dried and evaporated to a colourless solid. Recrystallization from EtOH gave an analytical sample **9** (151.3 g, 84%), mp 69–70 °C; ^{1}H NMR ($CDCl_3$): δ 1.20–1.74 (m, 8 H, 2×H-2, 2×H-3, 2×H-4 and 2×H-5), 2.46 (s, 6 H, 2×*Me*$C_6H_4SO_2$), 3.98 (m, 4H, 2×H-1 and 2×H-6), 7.32–7.80 (m, 8 H, 2×MeC_6*H*$_4SO_2$); ^{13}C NMR ($CDCl_3$): δ 21.44 (2×*Me*$C_6H_4SO_2$), 24.56 (C-3 and C-4), 23.38 and 28.44 (C-2 and C-5), 70.17 and 70.27 (C-1 and C-6), 127.64, 129.72, 132.82 and 144.67 (2×Me$C_6H_4SO_2$); CI MS: *m/z* 427 (M^++H). HR MS (ES+): *m/z* 427.1249 (M^++H). Calcd for $C_{20}H_{27}O_6S_2$: 427.1249.

*4.1.8. 1,2:5,6-di-O-isopropylidene-3-O-(6-tosyloxy-hexyl)-α-D-glucofuranose (**11**)*

To a suspension of NaH (80% in mineral oil, 1.15 g, 28.75 mmol) in dry DMSO (10 mL) was added **10** (5 g, 19.2 mmol) in small portions providing that temperature does not exceed 40 °C. After stirring the mixture at room temperature for 30 min a warm solution (35 °C) of **9** (28.4 g, 67 mmol) in DMSO (43 mL) was added. The mixture was stirred at room temperature for 2 h, and then partitioned between 10% aq NaCl (60 mL) and EtOAc (60 mL). The organic layer was separated and the aqueous phase was extracted with EtOAc (2×50 mL). The extracts were combined, washed with 10% aq NaCl (2×50 mL) dried and evaporated. Flash column chromatography (2:1 hexane–EtOAc) of the residue gave pure **11** (6.58 g, 66%) as a colourless syrup. ^{1}H NMR ($CDCl_3$): δ 1.32, 1.33, 1.42 and 1.49 (4×s, 3H each, 2×Me_2C), 1.24–1.73 (m, 8H, 4×CH_2), 2.45 (s, 3H, *Me*$C_6H_4SO_2$), 3.42–3.64 (m, 2H, CH_2O -3), 3.83 (d, 1H, $J_{3,4}$ = 3.0 Hz, H-3), 3.93–4.07 (m, 4H, CH_2OTs and 2×H-6), 4.11 (dd, 1H, $J_{4,5}$ = 7.6 Hz, H-4), 4.27 (ddd, 1H, $J_{5,6a}$ = 5.2, $J_{5,6b}$ = 4.3 Hz, H-5), 4.51 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-2), 5.86 (d, 1H, H-1), 7.35–7.78 (m, 4H, MeC_6*H*$_4SO_2$); ^{13}C NMR ($CDCl_3$): δ 21.58 (*Me*$C_6H_4SO_2$), 25.38, 26.21, 26.74 and 26.80 (2×*Me*$_2$C), 25.14, 25.43, 28.77 and 29.43 (4×CH_2), 67.22 (C-6), 70.31 (*C*H_2OTs), 70.44 (CH_2O-3), 72.44 (C-5), 81.13 (C-4), 82.09 (C-3), 82.49 (C-2), 105.22 (C-1), 108.86 and 111.70 (2×Me$_2$*C*), 127.83, 129.77, 133.20 and 144.63 (Me$C_6H_4SO_2$); CI MS: *m/z* 515 (M^++H). HR MS (ES+): *m/z* 515.2318 (M^++H). Calcd for $C_{25}H_{39}O_9S$: 515.2315.

*4.1.9. 1,2:5,6-di-O-isopropylidene-3-O-(6-hydroxy-hexyl)-α-D-glucofuranose (**12**)*

Procedure A: A mixture of **11** (17.9 g, 34 mmol), K_2CO_3 (9.6 g, 70 mmol), water (5.5 mL) and DMF (54 mL) was stirred at 125 °C for 4 h. The mixture was cooled to room temperature and divided in three portions. The first portion was poured into EtOAc (300 mL) and washed once with 10% aq NaCl (100 mL). Aqueous layer was collected and the second portion was poured into the first organic layer, which was again washed with 10% aq NaCl (100 mL). The procedure was repeated with the third reaction portion. The combined aqueous layers were extracted once with EtOAc (90 mL). Finally, the organic layers were combined, washed with 10% aq NaCl (3×60 mL), dried and evaporated. Chromatographic purification on a column of flash silica (3:2 hexane–EtOAc) gave pure **12** (5.2 g, 40%) as a colourless oil.

Procedure B: A solution of **10** (50 g, 192.1 mmol) in DMSO (150 mL), was allowed to react with a solution of **9** (300 g, 703.3 mmol) in DMSO (400 mL) in the presence of NaH (80% in mineral oil, 11.5 g, 287.5 mmol) according to procedure 4.1.7. The remaining crude **11** (118 g) was dried under high vacuum overnight and dissolved in a mixture of DMF (215 mL) and water (30 mL). To the solution was added K_2CO_3 (13 g, 95 mmol) and the obtained suspension was heated at 90 °C for 6 hours. A workup as described above, followed by flash column chromatography (procedure A) gave pure **12** (28.52 g, 41%) as a colourless oil. ^{1}H NMR ($CDCl_3$): δ 1.28, 1.31, 1.39 and 1.46 (4×s, 3H each, 2×Me_2C), 1.35–1.53 (m, 8 H, 4×CH_2), 1.96 (bs, 1H, exchangeable with D_2O, OH), 3.41–3.63 (m, 2H, CH_2O-3), 3.58 (m, 2H, C*H*$_2$OH), 3.81 (d, 1H, $J_{3,4}$ = 3.1 Hz, H-3), 3.94 (dd, 1H, $J_{6a,6b}$ = 8.5, $J_{5,6a}$ = 5.9 Hz, H-6a), 4.04 (dd, 1H, $J_{5,6b}$ = 6.1 Hz, H-6b), 4.08 (dd, 1H, $J_{4,5}$ = 7.0 Hz, H-4), 4.27 (m, 1H, H-5), 4.50 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-2), 5.84 (d, 1H, H-1); ^{13}C NMR ($CDCl_3$): δ 25.09, 25.94, 26.45 and 26.53 (2×*Me*$_2$C), 25.26, 25.57, 29.37 and 32.29 (4×CH_2), 62.10 (*C*H_2OH), 66.83 (C-6), 70.20 (CH_2O-3), 72.23 (C-5), 80.83 (C-4), 81.78 (C-3), 82.17 (C-2), 104.94 (C-1), 108.58 and 111.40 (2×Me$_2$*C*); FAB MS: *m/z* 383 (M^++Na), 359 (M^++H), 345 (M^+–Me).

*4.1.10. 1,2:5,6-di-O-isopropylidene-3-O-(6-carboxy-hexyl)-α-D-glucofuranose (**13**)*

A mixture of **12** (1 g, 2.8 mmol), pyridinium dichromate (2.12 g, 5.6 mmol) and $KMnO_4$ (0.89 g, 5.6 mmol) in dry Me_2CO (50 mL) was stirred at 50 °C for 8 h. The solution was decanted and evaporated to a yellow oil. Chromatographic purification on a column of flash silica (1:1 hexane–EtOAc) gave pure **13** (0.7 g, 67%) as a pale yellow oil. ^{1}H NMR (DMSO-d_6): δ 1.24, 1.25, 1.31 and 1.38 (4×s, 3H each, 2×Me_2C), 1.30–1.56 (m, 6 H, 3×CH_2), 2.20 (t, 2H, CH_2CO_2H), 3.72 (m, 2H, CH_2O-3), 3.81 (m, 2H, $J_{3,4}$ = 3, $J_{6a,6b}$ = 8.5, $J_{5,6a}$ = 6.2 Hz, H-3 and H-6a), 3.91 (dd, 1H, $J_{5,6b}$ = 6.5 Hz, H-6b), 4.05 (dd, 1H, $J_{4,5}$ = 6.6, $J_{3,4}$ = 3 Hz, H-4), 4.21 (m, 1H, H-5), 4.58 (d, 1H, $J_{1,2}$ = 3.7 Hz, H-2), 5.81 (d, 1H, H-1); ^{13}C NMR (DMSO-d_6): δ 24.25, 25.15, 28.90 and 33.67 (4×CH_2), 25.22, 26.04, 26.57 and 26.66 (2×*Me*$_2$C), 66.05 (C-6), 69.31 (CH_2O-3), 72.24 (C-5), 80.40 (C-4), 81.57 (C-2 and C-3), 104.69 (C-1), 108.00 and 110.81 (2×Me$_2$*C*), 174.51 (CO_2H); FAB MS: *m/z* 375 (M^++H), 359 (M^+–Me).

*4.1.11. 2-{5-O-[6-(1,2:5,6-di-O-isopropylidene-α-D-glucofuranos-3-yl)-hexanoyl]-2,3-O-isopropylidene-β-D-ribofuranosyl}thiazole-4-carboxamide (**14**)*

To a stirred and cooled (–7 °C) solution of **13**, (1.3 g, 3.5 mmol) in CH_2Cl_2 (25 mL) was added ethyl chloroformate (1.34 mL, 14 mmol) and Et_3N (2 mL, 14 mmol). The mixture was stirred at –7 °C for 30 min and then compound **5** (0.5 g, 1.67 mmol) was added to the solution. The resulting mixture was first stirred at –7 °C for 2 h, then at room temperature for

additional 19 h. The volatiles were removed by evaporation, the remaining mixture was treated with CH_2Cl_2 (3×25 mL) and evaporated. The oily residue was treated with EtOAc (8 mL), filtered and the solvent evaporated. Flash column chromatography (160:40:1 hexane–EtOAc–Et_3N) of the residue gave pure **14** (0.305 g, 36%) as a colourless oil, $[\alpha]^{20}_D = -15.0$ (*c*, 1.0 in $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 1.30, 1.32, 1.38, 1.40, 1.47 and 1.60 (6×s, 3H each, 3×Me_2C), 1.3–1.4 (m, 6 H, 3×CH_2), 2.13 (m, CH_2CO_2), 3.57 (m, 2H, CH_2O-3′) 3.82 (d, 1H, $J_{3'',4''} = 3.0$ Hz, H-3′'), 3.96 (dd, 1H, $J_{5'',6''a} = 5.8$, $J_{6''a,6''b} = 8.6$ Hz, H-6′'a), 4.04–4.20 (m, 3H, $J_{5'',6''b} = 6.1$, $J_{4'',5''} = 6.6$, $J_{5a',5b'} = 12.0$, $J_{4',5'a} = 4.6$ Hz, H-6′'b, H-4′' and H-5′a), 4.23–4.31 (m, 2H, $J_{4',5'b} = 4.1$ Hz, H-5′b and H-5′'), 4.47 (m 1H, $J_{3',4'} = 2.9$ Hz, H-4′); 4.52 (d, 1H, $J_{1'',2''} = 3.7$ Hz, H-2′'); 4.70 (dd, 1H, $J_{2',3'} = 6.3$ Hz, H-3′), 5.04 (dd, 1H, $J_{1',2'} = 3.4$ Hz, H-2′), 5.25 (d, 1H, H-1′), 5.87 (d, 1H, H-1′'), 6.19 and 7.19 (2×bs, 2H, $CONH_2$), 8.12 (s, 1H, H-5); ^{13}C NMR ($CDCl_3$): δ 24.35, 25.34 and 29.22 (3×CH_2), 33.65 (*C*H_2CO_2), 25.38, 25.48, 26.15, 26.71, 26.73 and 27.17 (3×*Me*$_2$C), 64.02 (C-5′), 67.13 (C-6′'), 70.09 (CH_2O-3′'), 72.38 (C-5′'), 81.02 (C-4′'), 82.00 (C-3′'), 82.27 (C-3′), 82.36 (C-2'), 83.36 (C-4′), 84.66 (C-1′), 85.58 (C-2′), 105.15 (C-1′'), 108.82, 111.63 and 114.45 (3×Me_2*C*), 124.71 (C-5), 149.78 (C-4), 162.68 (C-2), 170.83 (CO_2), 172.86 ($CONH_2$); EI MS: *m/z* 641 (M^+–Me).

4.1.12. 2-{5-O-[6-(α,β-D-glucopyranos-3-yl)-hexanoyl]-β-D-ribofuranosyl}thiazole-4-carboxamide (4)

A solution of **14** (1.63 g, 2.5 mmol) in 90% aq TFA (8.1 mL) was stirred for 1 h at room temperature. The mixture was evaporated by co-distillation with toluene. Flash column chromatography (4:1 $CHCl_3$–MeOH) of the residue gave pure **4** (0.87 g, 65%) as a colourless syrup, $[\alpha]^{20}_D = +23.6$ (*c*, 1.0 in MeOH); ^{1}H NMR (DMSO-d_6): δ 5.32, 5.56, 6.35 and 6.66 (4×bs, 4H, exchangeable with D_2O, 4×OH), 7.58 and 7.72 (2×bs, exchangeable with D_2O, NH_2), 4.87 (d, 1H, $J = 4.1$ Hz, H-1′α), 4.91 (d, $J = 5.2$ Hz, H-1′'β), 4.98 (d, 1H, $J = 3.4$ Hz, H-1′), 8.17 and 8.20 (2×s, 1H, H-5); ^{13}C NMR (DMSO-d_6): δ 25.13, 24.42 and 22.46 (3×CH_2), 33.59 (*C*H_2CO_2), 81.96 (C-1′), 92.38 (C-1'α), 96.95 (C-1′'β), 124.32 (C-5), 150.36 (C-4), 162.40 (C-2), 171.70 (CO_2), 172.90 ($CONH_2$); FAB MS: *m/z* 559 (M^++Na), 537 (M^++H). HR MS (ES+): *m/z* 559.1555 (M^++Na). Calcd for $C_{21}H_{32}N_2O_{12}SNa$: 559.1574.

4.2. Evaluation of growth inhibition

The target cells were maintained into the appropriate culture media as follows: murine C6 and 9L glioma cells in DMEM, human MCF7 and HTB177 cells in EMEM, leukaemia K562 cells in RPMI-1640, leukaemia NB4 cell line in SSM and NHDF cells in FGM. All media were supplemented with 10% FBS, except FGM that contained 2% of FBS. The cells were grown in a humidified atmosphere with 5% CO_2 at 37 °C. Viable cell number was determined using NRU assay (Clonetics) in 96-well plates after 72 hours of treatment with different concentrations (1–100 μM) of tested compounds **1**-**4**. The capacity of cells to concentrate Neutral Red dye (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) in their lysosomes was measured at wavelength 540 nm using Microplate cell reader (Biorad). All growth inhibition experiments were done in triplicates. Concentrations inhibiting cell growth by 50% (IC_{50}) were calculated using FORECAST programme.

4.3. Morphologic signs of apoptosis

To visualise morphological changes in C6 cells indicative of apoptosis induced by tiazofurin and the esters **2**-**4**, cells grown in chamber slides and treated with testing compounds in concentration of 60 μM for 72 hours, were fixed in 4% paraformaldehyde, post fixed in methanol, stained with May-Grunwald-Giemsa, according to the standard haematological procedure, and examined under the light microscope (Olympus). The criteria for apoptosis were the following: (1) decrease in cell size and rounding-up; (2) chromatin condensation as evidenced by the presence of dark, heavily stained nuclei; (3) chromatin fragmentation as observed by the appearance of at least three discrete masses of heavily stained nuclear material. Cells were considered apoptotic when at least two of these criteria were met. The number of morphologically apoptotic cells was counted under the microscope and the percentage of apoptotic cells was determined. At least 1000 cells were counted for each compound tested.

4.4. TUNEL in situ labelling of nuclear DNA fragments

To confirm morphological evidence of apoptosis, the terminal deoxynucleotidyl transpherase (TdT) mediated dUTP nick-end labelling (TUNEL) assay was performed, which detected DNA internucleosomal fragments in situ [32]. Cells undergoing apoptosis were detected with an in situ cell death detection kit (Boehringer-Mannheim). Procedure was followed as recommended by the manufacturer. A negative control (cells incubated with labelling solution without TdT) was included in each experiment. Cells were considered positive if they had distinct brown staining of nuclei, with or without apoptotic morphology. The number of TUNEL-positive cells was counted under the immersion objective and percentage of positive cells determined. At least 1000 cells were counted for each compound tested.

4.5. Determination of intracellular concentrations

To the C6 cells (cca. 150 mg) grown in an appropriate culture medium in triplicate was added 60 μM of tested compound. After treatment for 4 or 48 hours, the cells were collected by trypsinisation and centrifugation at 1600 rpm for 10 min. Cells were homogenised in 0.15 M KCl buffer, pH 7.4 at +4 °C and centrifuged at 40000 rpm for 0.5 h in the cold. The supernatant was heated at 100 °C for 1 min and centrifuged at 15000 rpm for 10 min. The supernatant was fil-

tered through a Sartorius filter (0.2 m) and the solution was subjected to HPLC analysis as described in general methods.

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