Searching for anti-glioma activity. Ribonucleoside analogues with modifications in nucleobase and sugar moieties

Grzegorz Framski, Dariusz Wawrzyniak, Zofia Jahnz-Wechmann, Agnieszka Szymanska-Michalak, Adam Kraszewski✉, Jan Barciszewski, Jerzy Boryski and Jacek Stawinski✉

Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznań, Poland

Several ribonucleoside analogues with modifications in the nucleobase and sugar moiety have been screened for anti-glioma activity in the T98G glioma cell line using cervical (HeLa) cell line as reference human malignant cells, and lung fibroblast (MCR-5) cell line as non-cancerous reference cells. Among the investigated compounds, ribonucleosides containing 6-chloropurine (**3**), 7-guanine (**5**) and a pyrrolopyrimidine (**18**) as nucleobases, show promising anti-glioma activity with good selectivity indices, and can be considered as lead structures for further anti-cancer studies.

Key words: ribonucleoside analogues, anti-glioblastoma activity, anticancer ribonucleotides

INTRODUCTION

Glioblastoma or grade IV astrocytoma, known also as glioblastoma multiforme (GBM), belongs to the most devastating brain cancers that is characterized by high malignancy and poor clinical outcome. This cancer invades the glial cells of the brain and often is malignant by the time of diagnosis. A conventional treatment of this type of cancer involves surgical resection, followed by chemotherapy and radiation therapy (Gallego, 2015). Unfortunately, this does little to improve life expectancy since the cancer usually recurs, and the length of survival after diagnosis is typically about 12 months.

Treatment of primary brain tumors is a very difficult task for several reasons. The most important are: (i) the tumor cells are resistant to conventional anticancer therapies, (ii) the brain is particularly prone to damages imparted by such interventions, (iii) the brain has a poor capacity to repair itself from the damage inflicted by chemical or radiation therapy, and finally, (iv) many drugs cannot surmount the blood-brain barrier to reach the targeted cancerous cells (Lawson *et al.*, 2007).

To overcome these inherent problems of the GBM anticancer therapy, alternative approaches were explored. The most prominent among them are those making use of the body's immune system to eradicate malignant cells (anticancer vaccine immunotherapies) (Xu *et al.*, 2014; Yang *et al.*, 2006), and those based on nucleic acid technologies (Catuogno *et al.*, 2012). Immunotherapy is appealing due to its potential of high tumor specificity, which is particularly important for brain cancers (Prins *et al.*, 2011). Some of the anti-GBM vaccines are at present in phase III clinical trials (NTC00045968) (Xu *et al.*, 2014). As to nucleic acids as potential therapeutics, the most promising in anti-glioma therapy is a sequence-specific inhibition of RNA expressed in tumor cells by interference RNA intervention (iRNAi) (Catuogno *et al.*, 2012; Piwecka *et al.*, 2011; Rolle *et al.*, 2010). Preliminary results using this technique had shown a significant improvement in the overall survival without compromising the quality of life of the patients (Rolle *et al.*, 2010).

Apart from these, there are numerous experimental therapies investigated that involve targeting various molecules expressed in GBM in the form of inhibitors, alkylating agents, or antibodies (Agnihotri *et al.*, 2013; Chen *et al.*, 2014).

As part of our interest in the development of new anticancer agents, we recently investigated various 3'-O-acyl-5-fluoro-2'-deoxyuridine derivatives as potential drugs (Szymanska-Michalak *et al.*, 2016). Although some of them displayed higher cytotoxicity against GBM of the T98G cell line than the parent 5-fluoro-2'-deoxyuridine, their SI selectivity indices were rather poor (< 5). Since until now the only preferred therapy against GBM is surgery, followed by a chemistry supported radiation (notably, with temozolomide as a sensitizing agent) (Neidle & Thurston, 2005; Stupp *et al.*, 2005), we have been searching for compounds with increased selective toxicity that could act *per se* as anticancer agents against this type of brain tumor. In this paper we report our results on screening of several ribonucleoside analogues with diverse structural features as potential low molecular weight selective cytotoxic agent against GBM.

MATERIALS AND METHODS

Reagents. Adenosine (**1**), 6-chloro-9-(β-D-ribofuranosyl)purine (**3**), inosine (**6**), and cytidine (**10**) were of commercial grades obtained from Sigma. Other ribonucleosides used in this study were prepared according to the published procedures. Specifically, 7-(β-D-ribofuranosyl)adenine (**2**) and 1-(β-D-ribofuranosyl)adenine (**7**) were obtained as kinetic products in the ribosylation of adenine (Framski *et al.*, 2006); 6-methyl-9-(β-D-ribofuranosyl)purine (**4**) and 7-(β-D-ribofuranosyl)guanine (**5**), *via* transglycosylation of inosine (Boryski, 1998) and guanosine (Boryski, 2008), respectively; 1-(β-ribofuranosyl)indazole (**8**) and 2-(β-D-ribofuranosyl)indazole (**9**) *via* direct ribosylation of indazole (Boryski, 1995); 5-azacytidine (**11**), *via* coupling of the silylated 5-azacytosine with peracetylated ribofuranose accord-

ing to the Vorbrüggen method (Vorbrüggen & Bennua, 1978); 1-(β-D-ribofuranosyl)cyanuric acid (**12**), from silylated cyanuric acid and 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-ribofuranose (Khaled *et al.*, 2004); 6-methyluridine (**13**), by Lewis acid catalyzed condensation of a silylated 6-methyl-4-methylthiouracyl with suitably protected ribofuranose (Felczak *et al.*, 1996); 2'-*C*-β-methyl-D-cytidine (**14**), 5-aza-2'-*C*-β-methyl-D-cytidine (**15**), 5-fluoro-2'-*C*-β-methyl-D-cytidine (**16**), and 2'-*C*-β-methyl-D-guanosine (**17**), were obtained by ribosylation of cytosine, its analogues, or guanine with 1,2,3,5-tetra-*O*-benzoyl-2'-*C*-methyl-β-D-ribofuranose (Fogt *et al.*, 2008). The series of furano[2,3-*d*]pyrimidine ribonucleoside derivatives (**18**, **19**, and **20**) were synthesized by the Pd-catalyzed cross-coupling of 5-iodouridine with appropriate alkyne derivatives (Jahnz-Wechmann *et al.*, 2015; McGuigan *et al.*, 2001; Tolstikov *et al.*, 1993), and the pyrollo[2,3-*d*]pyrimidine ribonucleoside derivatives (**21-26**), by the ammonia treatment of the corresponding furanopyrimidine nucleoside precursors (Diez-Torrubia *et al.*, 2011; Januszczyk *et al.*, 2009)

Biological assays. Cell line and culture conditions. Glioblastoma cell line (T98G), cervical cancer cell line (HeLa), and non-cancerous lung fibroblast cell line (MRC-5), were purchased from ATCC (Manassas, USA). All cell lines were of human origin. HeLa were cultured in RPMI 1640 medium, and T98G as well as MRC-5, were cultured in EMEM medium. Each medium was supplemented with 10% (v/v) foetal bovine serum (FBS) and 10 mg/mL antibiotics (penicillin and streptomycin). Cells were cultured at 37°C with 5% CO_2 in humidified air. Cell media (RPMI 1640 and EMEM) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and ATCC. Cell concentrations in the culture were adjusted to allow for an exponential growth.

Cell viability/proliferation assays. The assays were performed essentially as described in our recent paper (Szymanska-Michalak *et al.*, 2016). Cell viability/proliferation was evaluated by a dye staining method using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT). The protocol was adapted from methods found in the literature (Xavier *et al.*, 2011). The monolayer cell culture was trypsinized and counted. To each well of the 96-well plate, 100 μL of the diluted cell suspension (1×10^4 cells) was added. After 24 hours, when a partial monolayer was formed, 100 μL of the fresh medium with different compound concentrations (7.81, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 μg/mL) were added to the cells. After 48 hours, the supernatant was washed out and 100 μL of MTT solution in the medium (final concentration 0.5 mg/mL) were added to each well for 2 h. After the incubation time was complete, unreacted dye was removed by aspiration. The formazan crystals were dissolved in 100 μL/well DMSO and measured spectrophotometrically in a multi-well Synergy2 plate reader (BioTek Instruments, USA) at a test wavelength of 492 nm and a reference wavelength of 690 nm. The half-maximal inhibitory concentrations (IC_{50}) were calculated by fitting experimental values to sigmoidal bell-shaped equation using GraphPad Prism v5.01 (GraphPad Software, Inc., USA). Results are presented as mean of at least three independent experiments.

RESULTS AND DISCUSSION

Since most studies until now show no benefit from the addition of chemotherapy in treatment of GBM (Stupp *et al.*, 2005), we searched for low molecular weight compounds with increased selective cytotoxicity against GBM that could constitute a lead structure for further developments. To this end we selected four groups of ribonucleoside analogues with diverse structural and conformational features, and estimated their effect on the T98G glioblastoma cell line and HeLa as standard human malignant cells. The compounds investigated were either ribonucleoside analogues with known antiviral/anticancer properties or those being subjects of recent investigations in this laboratory. As a reference of non-cancerous cells, lung fibroblast (MCR-5) cells were used.

Purine nucleoside analogues

Adenosine receptors (ARs) emerged in the last three decades as important targets for drug development (Baraldi *et al.*, 2008). Apart from adenosine, the natural ligand for these receptors, other nucleosides, *e.g.* inosine, were found to be potent triggers of these receptors (Baraldi *et al.*, 2008; Gomez and Sitkovsky, 2003). Of particular interest in the context of the present study was the finding that activation of ARs is implicated in inhibition of tumor growth both *in vitro* and *in vivo* (Merighi *et al.*, 2003). As potential modulators of the ARs' activity, or other unknown biological targets, nine ribonucleosides with diverse purine base modifications were selected from our nucleoside library.

Anticancer drugs should not only have potent activity at low concentrations but also should exhibit a high degree of selectivity. Therefore, all studied ribonucleoside analogues were also tested for their anti-proliferative activity against non-cancerous lung fibroblasts (MRC-5). In the selection of the least toxic compounds among the most active ones, a special parameter, known as Selectivity Index (SI) was applied (Badisa *et al.*, 2009). The *in vitro* selectivity index of a drug is defined as the ratio of the toxic dose to the therapeutic dose (SI = IC_{50} non-tumor cell line/IC_{50} tumor cell line) and is a selectivity indicator of the tested compounds towards tumor cells.
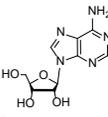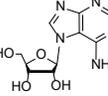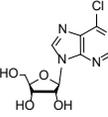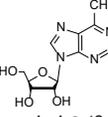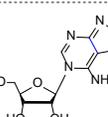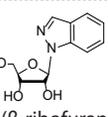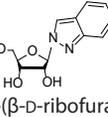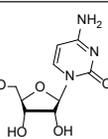
The screening experiments showed that that most of the investigated ribonucleoside **1–9**, with a notable exception of 6-methyl-9-(β-D-ribofuranosyl)purine (**4**), were not cytotoxic against the non-cancerous MRC-5 cells (Table 1). 6-Methylpurine nucleoside **4**, that is known for its potent anti-herpes simplex virus 1 activity and non-selective cytotoxicity for cancerous cells (Montgomery and Hewson, 1968; Wu *et al.*, 2010), in our screening also displayed high toxicity against both, HeLa and T98G cell lines, with rather low selectivity indices relative to the non-cancerous cells (Table 1).
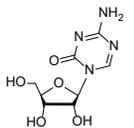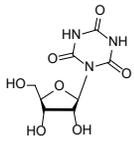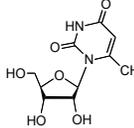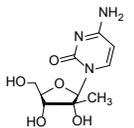
In this subgroup of the ribonucleosides investigated, a regioisomer of natural guanosine, 7-(β-D-ribofuranosyl)guanine (**5**), clearly stands out from the rest, with its high, selective cytotoxicity against glioblastoma (IC_{50} = 1.6) *vs* HeLa (IC_{50} > 177), and *vs* MCR-5 cell lines (IC_{50} > 100). These values translated into SI of 111 for HeLa/T98G, and SI = 64 for MRC-5/glioblastoma T98G cell lines. Also, 6-chloro-9-(β-D-ribofuranosyl)purine (**3**) displayed some selective cytotoxicity for glioblastoma *vs* MRC-5 cells, but the selectivity index was rather modest (SI = 23).

Pyrimidine nucleoside analogues

The pyrimidine ring, apart from being a part of natural nucleosides, constitutes an important pharmacophore endowed with drug like properties (Radi *et al.*, 2009), and when appended with a sugar moiety, may show a wide

Table 1. Cytotoxic activity of selected classes of ribonucleoside analogues*

Cmpd	Structure	IC$_{50}$ [µM] (MTT)		
		HeLa	T98G	MRC-5
Purine nucleoside analogues				
1	adenosine	>187 SI =1.0	110 SI =1.7	>187
2	7-(β-D-ribofuranosyl)adenine	>187 SI =1.0	41 SI =4.6	>187
3	6-chloro-9-(β-D-ribofuranosyl)purine	29.3 SI =5.8	7.32 SI =23	>170
4	6-methyl-9-(β-D-ribofuranosyl)purine	0.65 SI =5.1	0.83 SI =4.0	3.3
5	7-(β-D-ribofuranosyl)guanine	>177 SI =0.6	1.6 SI =63	>100
6	inosine	>180 SI =1.0	95 SI =1.9	>180
7	1-(β-D-ribofuranosyl)adenine	>180 SI =0.6	>180 SI =0.6	<100
8	1-(β-ribofuranosyl)indazole	101 SI =2.0	58.1 SI =3.4	>199
9	2-(β-D-ribofuranosyl)indazole	27.6 SI =1.5	52.8 SI =0.8	42.3
Pyrimidine nucleoside analogues				
10	cytidine	130 SI =1.1	138 SI =1.0	138
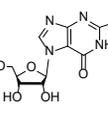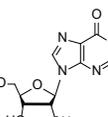
11	5-azacytidine	15.89 SI =1.4	12.48 SI =1.8	22.65
12	1-(β-D-ribofuranosyl)cyanuric acid	> 191 SI =1.0	48 SI =4.0	> 191
13	6-methyluridine	>190 SI =1.0	138 SI =1.4	>190

Pyrimidine 2′-C-methyl nucleoside analogues

14	2′-C-β-methyl-D-cytidine	60.91 SI =0.8	76.31 SI =0.7	49.62
15	5-aza-2′-C-β-methyl-D-cytidine	>190 SI =1.0	>190 SI =1.0	>190
16	5-fluoro-2′-C-β-methyl-D-cytidine	>180 SI =1.0	>180 SI =1.0	>180
17	2′-C-β-methyl-D-guanosine	>170 SI =1.0	>170 SI =1.0	>170
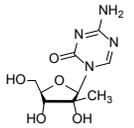
Pyrimidine bicyclo nucleoside analogues

18	6-octylpyrrolo[2,3-d]pyrimidine ribonucleoside	17.8 SI =7.3	0.75 SI =173	>130
19	6-(2-methylbutyl)pyrrolo[2,3-d]pyrimidine ribonucleoside	9.79 SI =4.1	14.31 SI =2.8	40.18
20	6-decylpyrrolo[2,3-d]pyrimidine ribonucleoside	18.58 SI =1.3	33.07 SI =0.7	23.64

21	furano[2,3-*d*]pyrimidine ribonucleoside	48.6 SI =2.9	187 SI =0.8	143.3
22	6-hydroxymethylfurano[2,3-*d*]pyrimidine ribonucleoside	>100 SI =1.0	>100 SI =1.0	>100
23	6-(1-hydroxybutylfurano[2,3-*d*]pyrimidine ribonucleoside	96.68 SI =1.0	>100 SI =1.0	>100
24	6-octyl[2,3-*d*]pyrimidine ribonucleoside	80.9 SI =0.9	20.01 SI =3.7	74.83
25	6-decyl[2,3-*d*]pyrimidine ribonucleoside	17.15 SI =1.1	29.71 SI =0.7	19.46
26	6-(2-methylbutylpyrrolo[2,3-*d*]pyrimidine ribonucleoside	>100 SI =1.0	>100 SI =1.0	>100
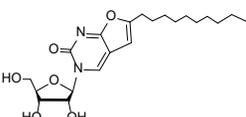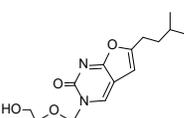
*HeLa, cervical cancer cell line; T98G, glioblastoma cancer cell line; MCR-5, non-cancerous lung fibroblast cell line; IC_{50}, concentration of the compound that inhibits cells proliferation by 50%; SI, selectivity index; IC_{50} non-tumor cell line/IC_{50} tumor cell line.

range of pharmacological activities (Walker *et al.*, 1979). For this study, we chose 5-azacytidine (**11**) and natural cytidine (**10**) as a reference, and two ribonucleosides with a non-typical syn conformation around the glycosidic bond, namely, 1-(β-D-ribofuranosyl)cyanuric acid (**12**) (Khaled *et al.*, 2004) and 6-methyluridine (**13**) (Felczak *et al.*, 1996).

5-Azacytidine, which is an US FDA approved drug (Vidaza, Pharmion; inhibitor of DNA methylation) for treatment of the myelodysplastic syndrome (MDS) (Issa *et al.*, 2005), is an analogue of cytidine in which carbon-5 in the heterocyclic ring is replaced by nitrogen. This changes the number and pattern of the hydrogen-acceptor centres when compared to cytidine **10**, and may be responsible for its known general cytotoxicity. A possible role of DNA methylation in cancer therapy (Christman, 2001) prompted us to include 5-azacytidine **11** in our screening experiments.

Among the four investigated pyrimidine ribonucleoside analogues (Table 1), only 5-azacytidine **11** was cytotoxic, but with practically no selectivity against cancerous and non-cancerous cell lines. Slightly better selective toxicity against T98G was displayed by cyanuric acid nucleoside **12**, but the SI index was still low (SI = 4).

Pyrimidine 2'-C-methylnucleoside analogues

This class of analogues retains the skeleton of natural ribonucleosides, but possesses a β-methyl substituent at the 2'-position of the D-ribose moiety. This 2'-C-methyl branching structural element (Walton *et al.*, 1969) transforms natural ribonucleosides into potent, broad-spectrum antiviral species (Benzaria *et al.*, 2007). For our studies, we selected 2'-C-β-methyl-D-cytidine (**14**), 5-aza-2'-C-β-methyl-D-cytidine (**15**), 5-fluoro-2'-C-β-methyl-D-cytidine (**16**), and 2'-C-β-methyl-D-guanosine (**17**).

As for toxicity of the investigated compounds **14**-**17**, only cytidine derivative **14** displayed a moderate, non-selective cytotoxicity ($IC_{50} > 50$, Table 1), while the rest was essentially non-toxic. From comparison with the group of pyrimidine ribonucleoside analogues (*vide supra*), it seems apparent that the presence of 2'-C-methyl group in the sugar moiety completely suppressed toxicity of 5-azacytidine (**15** *vs* **11**), but noticeably increased cytotoxicity of cytidine (**14** *vs* **10**).

Pyrimidine bicyclo nucleoside analogues

Addition of a furano or pyrrolo fused ring structure to pyrimidine nucleosides transformed these compounds into fluorescent, potent antiviral agents (Carangio *et al.*,

2001; McGuigan *et al.*, 2001). Properties of the bicyclic, furano-pyrimidine and pyrrolo-pyrimidine, pharmacophores have been investigated mainly for deoxyribonucleoside (Carangio *et al.*, 2001; McGuigan *et al.*, 2001), and to lesser extent for ribonucleoside (Jahnz-Wechmann *et al.*, 2015; Koh *et al.*, 2007), or dideoxy derivatives (McGuigan *et al.*, 2013).

Selected examples of this type of nucleoside analogues in our investigated series included: three lipophilic pyrrolo derivatives [6-octyl- (**18**), 6-(2-methylbutyl)- (**19**), and 6-decylpyrrolo [2,3-*d*]pyrimidine ribonucleosides (**20**)], unsubstituted furano[2,3-*d*]pyrimidine ribonucleoside **21**, 6-hydroxymethylfurano[2,3-*d*]pyrimidine ribonucleoside **22**, 6-(1-hydroxybutylfurano[2,3-*d*]pyrimidine ribonucleoside **23**, and three lipophilic furano derivatives [6-octyl- (**24**), 6-decyl- (**25**), and 6-(2-methylbutylpyrrolo[2,3-*d*]pyrimidine ribonucleosides (**26**)].

Although most of the investigated compounds in this subgroup were not cytotoxic or displayed a moderate to low, not selective cytotoxicity, the 6-octylpyrol derivative **18** clearly stood out with its high selective cytotoxicity against the T98G glioblastoma cells (IC$_{50}$ = 0.74), and high selectivity index, IC$_{50}$ MRC-5/IC$_{50}$ T98G, SI = 173.

It seems that the pyrrolo bicyclo nucleosides in this series are on average more cytotoxic than the furano derivatives, however, a pairwise comparison, *e.g.* **18** *vs* **24**, **19** *vs* **26**, **20** *vs* **26**, does not support this notion.

CONCLUDING REMARKS

We have screened selected compounds from our ribonucleoside library to find nucleosides with an anti-glioma activity. Three of them, namely 6-chloro-9-(β-D-ribofuranosyl)purine (**3**), 7-(β-D-ribofuranosyl)guanine (**5**), and 6-octyl-furano[2,3-*d*]pyrimidine ribonucleoside (**18**) were found to have appreciable selective cytotoxicity against T98G glioblastoma when compared to non-cancerous MRC-5 cells, and also HeLa human cancer cell line, and deserve some further studies as possible lead structures for development of an anti-glioma drug.

With the growing understanding of molecular alterations and complexity found in gliomas (Bleeker *et al.*, 2012; Zhang *et al.*, 2012) it seems, however, that a single-agent therapy in treatment of this kind of cancer will be insufficient; rather, a multi drug combination treatment will be necessary. This calls for further searching for anti-glioma activity in structurally diverse classes of compounds that could selectively interact with therapeutically important molecular targets revealed by the GBM cancer.

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