Bioactive Sulfated Saponins from Sea Cucumber *Holothuria moebii*

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Abstract

The bioactive ingredients of sea cucumber *Holothuria moebii* were investigated, and four sulfated saponins (**1**–**4**) and one desulfated saponin (**3B**) with an unusual 3,4-epoxy xylose were obtained from this study. Compound **2** is a new triterpenoid saponin and **3B** is a new artificial compound. On the basis of the extensive NMR and HRESIMS data, their structures were assigned as 3-*O*-[*β*-D-quinovopyranosyl-(1 → 2)-4-sodium sulfato-*β*-D-xylopyranosyl]-25-acetoxy-22-oxo-9(11)-holostene-3*β*,12*α*,17*α*-triol (**2**) and 3-*O*-[*β*-D-quinovopyranosyl-(1 → 2)-3,4-epoxy-*β*-xylopyranosyl]-22,25-epoxy-9(11)-holostene-3*β*,12*α*,17*α*-triol (**3B**). Compounds **1**–**4** showed activity suppressing the proliferation of four different glioma cells with IC_{50} values ranging from 0.99 to 8.64 μM. New saponin **2** significantly induced apoptosis in human glioblastoma U87-MG cells and reduced the expression levels of several glioma metabolic enzymes of glycolysis and glutaminolysis. This study reveals for the first time that selectively targeting multiple glioma metabolic regulators of glycolysis and glutaminolysis might be one of the anti-glioma mechanisms of saponin **2**.

Supporting information available online at http://www.thieme-connect.de/products

Introduction

Sea cucumbers are soft-bodied worm-like echinoderms and belong to the class Holothuroidea, which has 25 families, including about 200 genera with more than 1400 species [1]. Sea cucumbers have economic importance in Asian countries, especially in China where some sea cucumbers are used in traditional medicine [2] and are also perceived as a delicacy. In recent decades, sea cucumbers have gained great attention among researchers around the world, not only due to their nutritive value, but also for their potential health benefits and therapeutic uses [3]. A number of biological activities including anticancer, antiangiogenic, anticoagulant, antihypertension, and anti-inflammatory effects are ascribed to various species of sea cucumbers. Different classes of compounds such as triterpenoid glycosides (saponins), sulfated polysaccharides, glycosaminoglycan, sterols, phenolics, lectins, peptides, glycosphingolipids, and fatty acids were found to be present in sea cucumbers [3]. To date, more than 100 triterpenoid glycosides with widely studied anticancer and antiviral activities have been isolated and identified from sea cucumbers [1].

Holothuria moebii Ludwig is a species of sea cucumbers of the Holothuriidae family. Members of this species are always found under rocks near the ebb tide line and distributed mainly in the seas of Mauritius, Seychelles, Sri Lanka, South Japan, South China, Philippines, Indonesia, South Pacific, and North Austria [2]. Until now, there have been no reports on the constituents or medicinal uses of this species. As a part of our ongoing project for the discovery of novel antitumor agents from natural resources [4–7], we have conducted chemical studies on a methanol extract prepared from the whole bodies of *H. moebii* and investigated the bioactivity of the isolated compounds. Four sulfated saponins, **1**–**4** (**Fig. 1**), were isolated from sea cucumber *H. moebii*. Alkaline hydrolysis of saponin **3**, the major compound in the crude saponins, produced desulfated saponin **3B** with an unusual modified xylose. Compound **2** is a new triterpenoid glycoside and **3B** is a new artificial compound. We report herein the isolation

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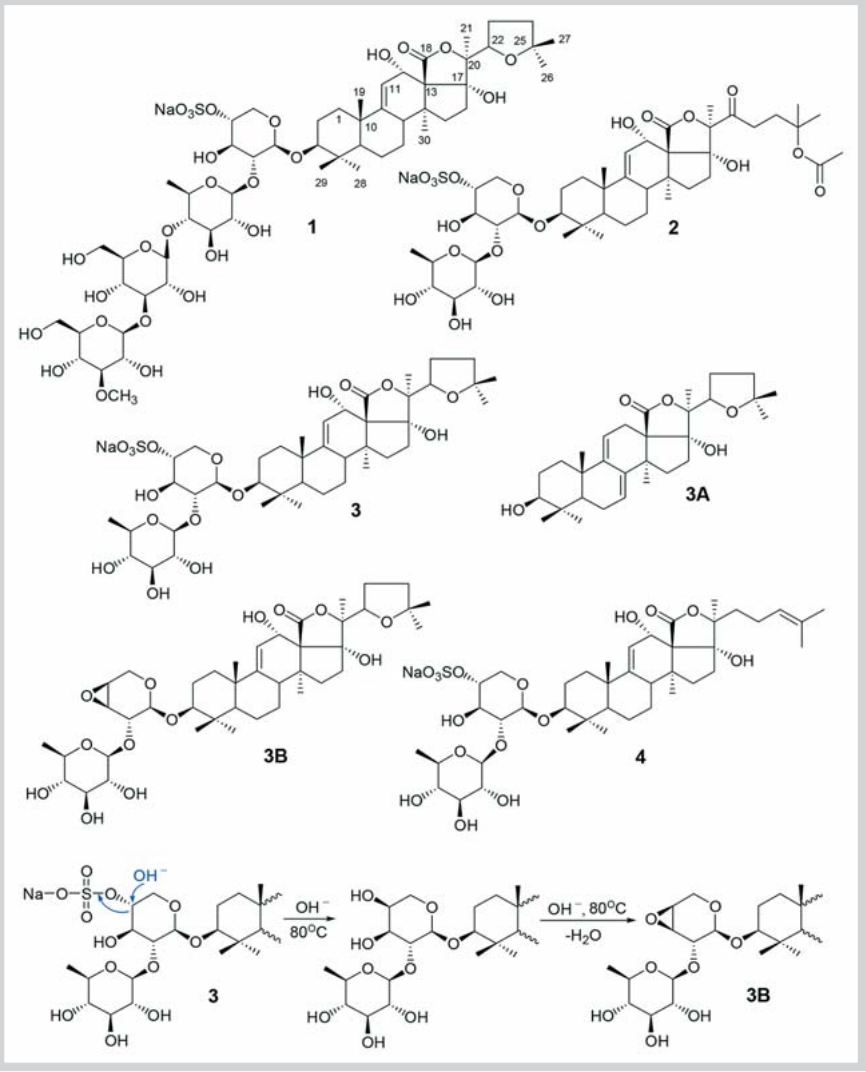

Fig. 1 Structures of compounds **1–4, 3A**, and **3B**. (Color figure available online only.)

and structural elucidation of the new compounds, the activity of the isolated compounds inhibiting the proliferation of different glioma cells, and the effects of the new sulfated saponin **2** on several important glioma metabolic enzymes, hexokinase 2 (HK2), 6-phosphofructo-2-kinase/2,6-bisphosphatase 3 (PFKFB3), pyruvate kinase (PKM2), and glutaminase (GLS). It has been revealed that these metabolic regulators are related to the tumorigenesis of gliomas [8, 9].

Results and Discussion

The bioactive components of the sea cucumber *H. moebii* were investigated. A BuOH partition of the MeOH extract prepared from *H. moebii* was repeatedly separated by ODS columns to give four sulfated triterpenoid saponins, **1**–**4**. On the basis of their NMR analysis and comparison with published data, saponins **1, 3**, and **4** were proven to be identical to holothurin A (**1**) [10], holothurin B (**3**) [11], and 24-dehydroechinoside B (**4**) [12]. The ^{13}C and ^{1}H NMR data for these known compounds are summarized in **Table 1S** and **2S** (Supporting Information). Holothurin B (**3**) is the major component in the mixed saponins.

Compound **2** was obtained as an amorphous solid and has the molecular formula $C_{43}H_{65}NaO_{19}S$, deduced from its negative HRESIMS $[M - Na]^-$ ion at *m/z* 917.3842 (calcd. for $C_{43}H_{65}O_{19}S$, 917.3841) and ^{13}C NMR data. The IR spectrum showed the presence of OH (3423 cm^{-1}), C=O (1741 cm^{-1}), and an olefinic bond (1633 cm^{-1}). Two anomeric protons at δ 4.73 (1H, d, 7.2 Hz) and 5.16 (1H, d, 7.6 Hz) were correlated to two anomeric carbons at δ 105.7 and δ 106.3 in the HMQC spectrum, suggesting that **2** had two monosaccharides. The ^{13}C NMR spectrum of **2** displayed 43 carbon signals, of which 30 were assigned to the triterpenoid backbone, two to the acetyl group, and the remaining 11 to the disaccharide. An extensive NMR interpretation concluded that the aglycone of **2** was 25-acetoxy-22-oxo-9(11)-holostene-3β,12α,17α-triol, the aglycone of marmoroside C [13]. The two sugars were assigned as xylose and quinovose based on their NMR data and comparison with those of xylose and quinovose in **3**. Acidic hydrolysis of **2** produced D-xylose and D-quinovose as detected by GC analysis using authentic sugars as references. Both sugars were determined to be in the pyranose form from analysis of their ^{13}C NMR data, and the β-anomeric configuration of the monosaccharides was defined from the $^{3}J_{H1,H2}$ coupling constants and NOE results (**Fig. 39S**, Supporting Information). The sequence and linkage of the disaccharide chain at C-3 of the aglycone were established from the following HMBC correlations: Xyl H-1 (δ 4.73) with aglycone C-3 (δ 89.0), Qui H-1 (δ 5.16) with Xyl C-2 (δ 83.7), aglycone H-3 (δ 3.16) with Xyl C-1 (δ

Table 1 ^{13}C and ^{1}H NMR data of compounds **2** and **3B** (in pyridine-d_5).

No.	2			3B		
	δ_C	δ_H (*J* in Hz)	HMBC	δ_C	δ_H (*J* in Hz)	HMBC
1	36.7, CH_2	1.42, m; 1.83, m	3	36.7, CH_2	1.44, m; 1.85, m	3
2	27.4, CH_2	1.94, m; 2.11, m	10	27.3, CH_2	1.93, m; 2.08, m	10
3	89.0, CH	3.16, dd (4.1, 11.8)	4, 29, Xyl-1	89.0, CH	3.13, dd (4.2, 11.9)	4, 28, Mxyl-1
4	40.4, C	–	–	40.4, C	–	–
5	53.0, CH	0.99, d (11.7)	4, 10	53.0, CH	1.01, d (11.3)	4, 28
6	21.4, CH_2	1.55, m; 1.76, m	–	21.6, CH_2	1.54, m; 1.73, m	–
7	28.7, CH_2	1.49, m; 1.75, m	14	28.7, CH_2	1.53, m; 1.78, m	–
8	41.2, CH	3.32, m	14	41.3, CH	3.35, m	13
9	154.6, C	–	–	154.1, C	–	–
10	40.1, C	–	–	40.1, C	–	–
11	115.6, CH	5.61, d (5.1)	10, 13	115.9, CH	5.64, d (5.6)	10, 12, 13
12	71.3, CH	4.99, d (5.1)	9, 11, 14, 18	71.9, CH	4.99[a]	9, 14
13	58.9, C	–	–	59.2, C	–	–
14	46.6, C	–	–	46.3, C	–	–
15	37.1, CH_2	1.42, m; 1.83, m	13	37.2, CH_2	1.44, m; 1.87, m	13, 14, 17
16	39.3, CH_2	2.18, m; 2.47, m	13, 17	35.9, CH_2	2.43, m; 2.98, dd (8.7, 15.0)	13, 14, 17, 20
17	92.6, C	–	–	90.1, C	–	–
18	173.9, C	–	–	174.8, C	–	–
19	22.9, CH_3	1.37, s	1, 5, 9, 10	22.8, CH_3	1.36, s	1, 5, 9, 10
20	87.7, C	–	–	87.0, C	–	–
21	21.7, CH_3	1.94, s	17, 20, 22	19.2, CH_3	1.77, s	17, 20, 22
22	209.3, C	–	–	81.0, CH	4.34, t (7.0)	–
23	34.3, CH_2	2.99, m; 3.12, m	22	28.5, CH_2	2.05, m	20, 22, 24
24	34.9, CH_2	2.35, m	24, 26, 27	38.8, CH_2	1.64, m	22
25	81.6, C	–	–	81.8, C	–	–
26	26.3, CH_3	1.53, s	24, 25, 27	29.0, CH_3	1.21, s	24, 25, 27
27	26.2, CH_3	1.51, s	24, 25, 26	27.8, CH_3	1.20, s	24, 25, 26
28	28.3, CH_3	1.30, s	3, 4, 5, 29	28.5, CH_3	1.26, s	3, 4, 5, 29
29	17.1, CH_3	1.15, s	3, 4, 5, 28	17.1, CH_3	1.11, s	3, 4, 5, 28
30	20.2, CH_3	1.64, s	8, 13, 14, 15	20.7, CH_3	1.70, s	8, 13, 14, 15
Ac-1	170.6, C	–	–	–	–	–
2	22.5, CH_3	2.0, s	Ac-1	–	–	–
Xyl-1	105.7, CH	4.73, d (7.2)	Agl-3	104.2, CH	4.56, d (7.1)	5, Agl-3
2	83.7, CH	4.10, t (7.2, 8.7)	1, 3, Qui-1	73.1, CH	4.40, d (7.1)	1, 3, Qui-1
3	76.0, CH	4.35, t (8.7)	2, 4	53.4, CH	3.73, d (3.8)	1, 2, 4
4	76.4, CH	5.14, m	2	50.0, CH	3.10, d (3.8)	5
5	64.7, CH_2	3.77, m; 4.81, dd (5.2, 11.7)	4	62.9, CH_2	3.92, d (13.2); 4.27, d (13.2)	1, 3, 4
Qui-1	106.3, CH	5.16, d (7.6)	Xyl-2	103.5, CH	5.02, d (7.6)	Mxyl-2
2	77.3, CH	4.07, t (7.6, 8.7)	1, 3	75.9, CH	4.06, t (7.6, 8.7)	3
3	78.1, CH	4.16, t (8.7)	4	78.6, CH	4.16, t (8.7)	4
4	77.0, CH	3.73, t (8.7)	3, 5, 6	77.1, CH	3.76, t (8.7)	5
5	73.8, CH	3.79, m	3	73.5, CH	3.75, m	1, 4
6	19.0, CH_3	1.67, d (6.0)	4, 5	19.0, CH_3	1.65, d (5.6)	4, 5

[a] *J* value was not given because of overlap

105.7), and Xyl H-2 (δ 4.10) with Qui C-1 (δ 106.3). The NOE information, as described in **Fig. 39S**, also supported the above conclusions. The full assignment (**Table 1**) of carbons and protons of **2** was achieved by a combination of extensive NMR experiments. The structure of **2** was elucidated as 3-*O*-[β-D-quinovopyranosyl-(1 → 2)-4-sodium sulfato-β-D-xylopyranosyl]-25-acetoxy-22-oxo-9(11)-holostene-3β,12α,17α-triol, which is a new triterpenoid saponin.

To understand the structure-activity relationship (SAR) of bioactive saponins **1**–**4**, the major saponin holothurin B (**3**) was hydrolyzed by HCl and NaOH to obtain its aglycone and desulfated prosapogenin for the bioactive assay. Acidic hydrolysis of **3** produced 22,25-oxidoholothurinogenin (**3A**), a modified sapogenin of holothurigenol (the native aglycone of holothurin saponins) [11]. Alkaline hydrolysate of **3** furnished desulfated saponin **3B** with an unusual modified xylose.

Compound **3B** has a molecular formula of $C_{41}H_{62}O_{13}$, as deduced from its negative HRESIMS ion at *m/z* 761.4115 $[M - H]^-$ (calcd. for $C_{41}H_{61}O_{13}$, 761.4112) and ^{13}C NMR data. The ^{13}C NMR spectrum of **3B** displayed 41 carbon signals including 30 for the aglycone and 11 for the sugar part. Careful comparison of the NMR data of compounds **3B** and **3** showed that both compounds shared the same quinovose moiety and the same aglycone of 22,25-oxidoholothurinogenin (**3A**). This was further confirmed by the fact that the acidic hydrolysis of **3B** produced **3A** and D-quinovose as detected by TLC and GC analysis, respectively. The structural difference between **3B** and **3** was the xylose-related monosaccharide, as indicated by their large different ^{13}C shifts

Compounds	Glioma cell lines			
	C6	U87-MG	U251	SHG-44
1	0.99 ± 0.17	4.03 ± 0.55	3.76 ± 0.08	3.68 ± 0.16
2	1.22 ± 0.10	3.81 ± 0.07	4.39 ± 0.52	2.80 ± 0.69
3	2.86 ± 0.23	3.00 ± 0.13	8.64 ± 2.30	1.39 ± 0.83
4	2.09 ± 0.72	2.72 ± 0.04	6.10 ± 0.71	1.99 ± 0.18
3A	19.27 ± 0.88	52.46 ± 3.41	23.26 ± 1.25	45.12 ± 10.47
3B	27.97 ± 6.63	53.01 ± 1.64	51.43 ± 3.66	16.43 ± 1.33
TMZ	69.58 ± 6.10	> 100.00	> 100.00	85.80 ± 4.10

Table 2 Isolated compounds inhibiting the proliferation of glioma cells (IC_{50}: μM).

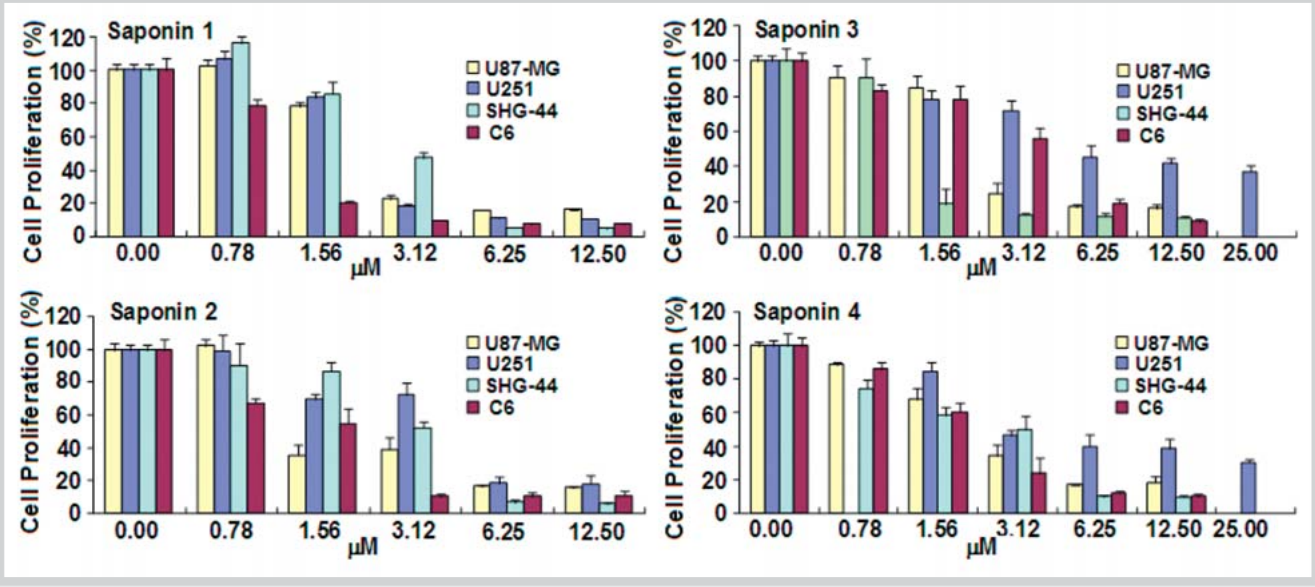

Fig. 2 Saponins **1**–**4** inhibited the proliferation of four different glioma cells, U87-MG, U251, SHG-44, and C6. (Color figure available online only.)

(**Table 1**; **Table 1S**, Supporting Information) between xylose in **3** and modified xylose in **3B**. In compound **3**, the xylose resonated at δ 105.5 (C-1), δ 83.4 (C-2), δ 75.9 (C-3), δ 76.2 (C-4), and δ 64.5 (C-5), while the modified xylose in **3B** resonated at δ 104.2 (C-1), δ 73.1 (C-2), δ 53.4 (C-3), δ 50.0 (C-4), and δ 62.9 (C-5). COSY and HMBC correlations (**Table 1**), in combination with HRESIMS data, indicated that the modified xylose in **3B** had an epoxy group at positions C-3 and C-4. The anomeric configuration of the modified xylose was defined to have the same β form as xylose based on their $^3J_{H1,H2}$ coupling constant. A doublet signal (δ 4.40, 7.1 Hz) for H-2 due to the vicinal coupling to the H-1 signal (δ 4.56, 7.1 Hz) indicated a β-face of H-2. No vicinal coupling between H-2 and H-3 was observed, suggesting that the dihedral angle between H-2 and H-3 was around 90° [14]. By carefully examining the energy-minimized model, it was found that the dihedral angle mentioned above was about 90° when the epoxy group at C-3 and C-4 was at the β-orientation. A small $^3J_{H3,H4}$ coupling constant (3.8 Hz) also indicated that H-3 and H-4 had the same orientation. A possible mechanism for the formation of this epoxy xylose has been proposed as described in **Fig. 1**. Based on the above evidence, compound **3B** was assigned as 3-*O*-[β-D-quinovopyranosyl-(1 → 2)-3,4-epoxy-β-xylopyranosyl]-22,25-epoxy-9(11)-holostene-3β,12α, 17α-triol, a new artificial compound.

All compounds obtained from this study were assayed for their activity inhibiting the proliferation of rat glioma C6 cells and human glioma U87-MG, U251, and SHG-44 cells. Temozolomide (TMZ), the most used chemotherapy for the treatment of gliomas [15], was used as a positive control. The results showed that the sulfated saponins **1**–**4** had potent dose-dependent activity with IC_{50} values of 0.99–8.64 μM, while the desulfated prosapogenin **3B** and the modified sapogenin **3A** had moderate or weak activity with IC_{50} values in the range of 16.43–53.01 μM (**Table 2** and **Fig. 2**). These results suggested that the sulfate group at C-4 of xylose might be important for the activity of this type of triterpenoid saponin. One of the reasons for the reduction of the bioactivity of compounds **3A** and **3B** might be the reduction of the polarity and water solubility of these two artificial compounds. The control, TMZ, showed activity against C6 and SHG-44 but was inactive for U87-MG and U251 cells at 100 μM. This is probably because these cell lines were resistant to TMZ [16, 17].

The new sulfated saponin **2** was tested by DAPI (4,6-diamidino-2-phenylindole) and PI (propidium iodide) double staining to determine its ability to induce apoptosis and necrosis in U87-MG cells. Curcumin, a well-studied antitumor polyphenolic compound [18], was used as a positive control. Saponin **2** (2.0 μM or 4.0 μM) induced apoptosis and necrosis in U87-MG cells after 24 h treatment (**Fig. 3A**; **Fig. 40SA**, Supporting Information). Annexin V-FITC/PI double staining using flow cytometry was applied to quantify the apoptotic and necrotic cells. The results indicated that **2** caused a 60.25% (2.0 μM) or 55.02% (4.0 μM) increase in total apoptotic cells, and a 60.48% (2.0 μM) or 65.49% (4.0 μM) increase in total apoptotic and necrotic cells, compared to the control (CON) (**Table 3** and **Fig. 3B**; **Fig. 40SB**, Supporting Information). The positive control curcumin (25.0 μM) also

Table 3 Quantification of apoptosis and necrosis induced by saponin 2 in U87-MG cells.

	CON	2 (2.0 µM)	2 (4.0 µM)	2 (2.0 µM)–CON	2 (4.0 µM)–CON
Early apoptotic cells	4.48	59.94	49.50	55.46	45.02
Late apoptotic cells	3.94	8.73	13.94	4.79	10.0
Necrotic cells	0.77	1.00	11.24	0.23	10.47
Total apoptotic cells	8.42	68.67	63.44	60.25	55.02
Total apoptotic and necrotic cells	9.19	69.67	74.68	60.48	65.49

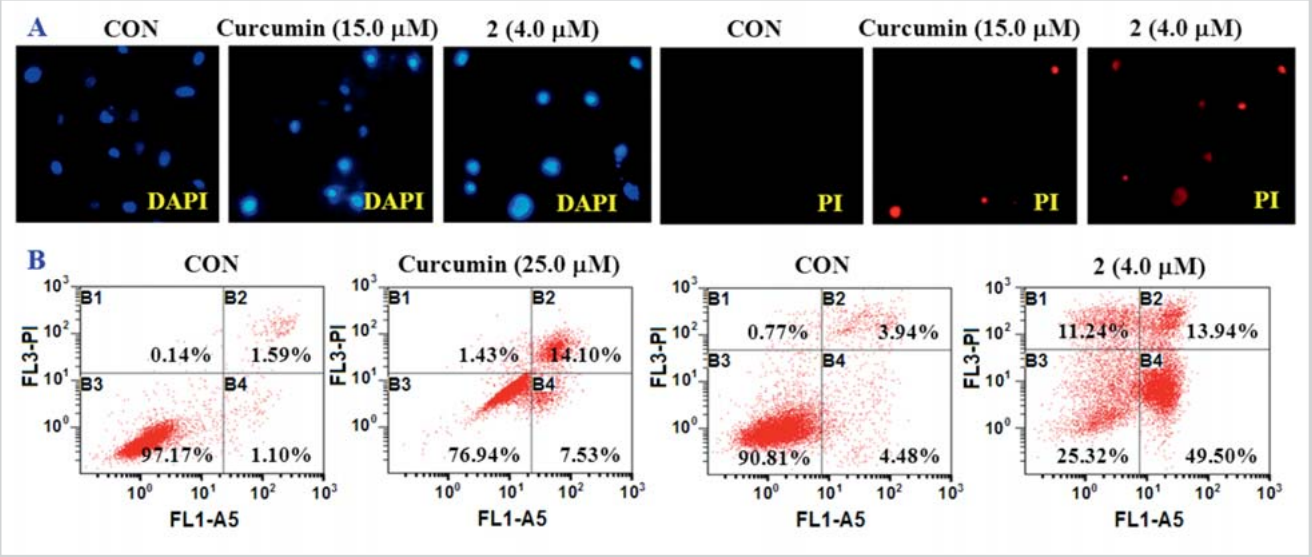

Fig. 3 **A** Saponin **2** induced apoptosis and necrosis in U87-MG cells. U87-MG cells were treated with compound **2** (4.0 µM) for 24 h and then doubly stained with DAPI and PI. Apoptotic cells showed bright blue nuclear condensation and necrotic cells displayed red fluorescence. **B** Dot-plot represents the double staining analysis with annexin V-FITC/PI in U87-MG cells. U87-MG cells were treated with saponin **2** (4.0 µM) for 24 h, stained with annexin-V FITC and PI, and then analyzed by flow cytometry (B1: necrotic cells; B2: late apoptotic cells; B3: glioma cells; B4: early apoptotic cells). Curcumin was used as a positive control. (Color figure available online only.)

caused a 20.23% increase in total apoptotic and necrotic cells compared to CON (**Fig. 3B**). The effects of **2** on proapoptotic genes (BCL-2/BCL-Xl-associated death promoter, BAD, and BCL-2-associated X protein, BAX) and antiapoptotic genes (B-cell lymphoma 2, BCL-2, and B-cell lymphoma-extra large, BCL-XL) were further investigated. However, saponin **2** did not regulate the expression levels of both pro- and antiapoptotic genes as analyzed by Western blot analysis (**Fig. 41S**, Supporting Information). These data suggested that the apoptosis induced by **2** in U87-MG cells might not relate to these analytic apoptotic genes. Thus, the mechanisms of apoptosis induced by sulfated saponin **2** in glioma cells need to be explored further.

The effects of sulfated saponin **2** on the expression levels of several glioma metabolic enzymes including HK2 [19], PFKFB3 [20], PKM2 [21] of glycolysis, and GLS [22] of glutaminolysis were investigated by Western blot analysis. HK2, PFKFB3, PKM2, and GLS are highly expressed in cancer cells, which preferentially rely on these specific enzymes [9, 19–23]. 2-Deoxy-D-glucose (2DG), an inhibitor of hexokinase and a well-studied anti-glioma agent [24, 25], was used as the positive control. As shown in **Fig. 4A**, compound **2** (4.0 µM) significantly reduced the expression levels of HK2, PFKFB3, PKM2, and GLS in U87-MG cells after 48 h treatment. The control 2DG also lowered the expression levels of these metabolic regulators.

To understand whether compound **2** selectively targets these glioma metabolic regulators or not, the effects of **2** on the expression levels of these metabolic enzymes in normal human astrocytes were also investigated. The results (**Fig. 4B**) showed that compound **2** (4.0 µM) had no significant effects on the expression levels of HK2, PFKFB3, PKM2, and GLS in human astrocytes after 48 h treatment. The effects of **2** on the expression levels of aconitase 2 (ACO2), ATP synthase beta (ATPB), pyruvate dehydrogenase beta (PDHB), and cytochrome C (CytoC) were further investigated. As presented in **Fig. 4C**, compound **2** (4.0 µM) also showed no influence on these levels. ACO2, ATPB, PDHB, and CytoC are important regulators in the processes of the tricarboxylic acid cycle and oxidative phosphorylation [25], which are the metabolic processes of glucose in normal cells. It is known that tumors display increased rates of glucose uptake (enhanced glycolysis) compared with normal tissues and that even in the presence of oxygen, tumors metabolize glucose via oxygen-independent aerobic glycolysis to produce a high level of lactate rather than via the more efficient but oxygen-dependent process of oxidative phosphorylation to generate ATP (the main carrier of cellular energy) [26]. Taken together, the results from this study suggest that selectively targeting multiple glioma metabolic regulators of glycolysis and glutaminolysis might be one of the anti-glioma mechanisms of saponin **2**.

Sulfated saponins are the major and most interesting bioactive metabolites found in many sea cucumbers. Although this type of saponin has been proven to have potent cytotoxicity against many different tumor cells [1], to the best of our knowledge,

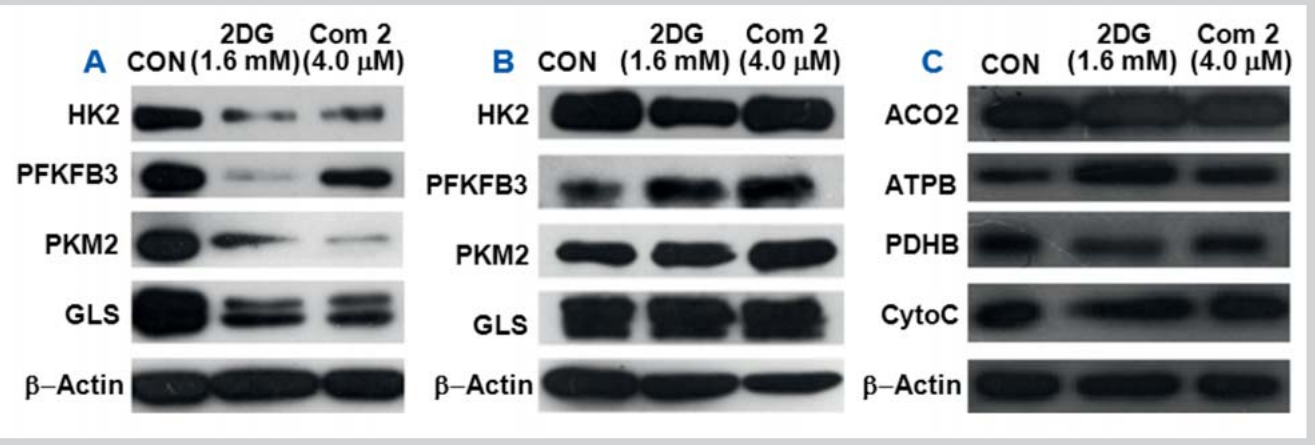

Fig. 4 **A** Saponin **2** reduced the expression levels of HK2, PFKFB3, PKM2, and GLS in U87-MG cells (HK2: hexokinase 2; PFKFB3: 6-phosphofructo-2-kinase/2, 6-bisphosphatase 3; PKM2: pyruvate kinase M2; GLS: glutaminase; *β*-actin: internal control). **B** Saponin **2** had no significant effect on the expression levels of HK2, PFKFB3, PKM2, and GLS in human astrocytes. **C** Saponin **2** had no effect on the expression levels of ACO2, ATPB, PDHB, and CytoC in U87-MG cells. U87-MG cells or human astrocytes were treated with **2** (4.0 μM) or 2DG (1.6 mM) for 48 h. Protein extracted from the cells was subjected to Western blot analysis. 2DG was used as a positive control. (Color figure available online only.)

there were rarely studies examining whether this class of saponins has activity against glioma cells. Furthermore, the mechanisms of their activity against tumors are largely unclear. This study demonstrated, for the first time, that sulfated saponins **1**–**4** from *H. moebii* have potent activity suppressing the proliferation of glioma cells. Also, for the first time, this study uncovered that sulfated saponin **2** could have a unique antitumor mechanism by selectively targeting multiple glioma metabolic regulators of glycolysis and glutaminolysis. Therefore, it is worthwhile to further pursue whether the new sulfated saponin **2** has antiglioma activity in animal models.

Materials and Methods

General experimental procedures

Optical rotations were measured on a JASCO DIP-370 digital polarimeter. IR spectrum was carried out on an AVATAR 370 FT-IR spectrometer (Thermo Nicolet). NMR spectra were recorded on a Bruker AV III-500 instrument at 500 MHz for ^{1}H and 125 MHz for ^{13}C using standard pulse programs and acquisition parameters. Chemical shifts were reported in δ (ppm) referencing to the NMR solvent pyridine-d_5. HRESIMS data were acquired on an AB Sciex Triple TOF 5600 spectrometer. GC analysis was conducted on an Agilent 6890 N gas chromatograph system using a DB-624 capillary column (30 m × 0.53 mm, 3.0 μm, Agilent Technologies). N_2 (4.0 mL/min) was used as the carrier gas and FID as the detector. The detector and injection port temperatures were set at 260 °C and 250 °C, respectively. The column temperature was 100 °C in 5 min, raised 20 °C/min to 260 °C, and then kept at 260 °C for 10 min. A fluorescence microscope (Nikon SMZ1000) was used to detect apoptosis and necrosis in glioma cells doubly stained by DAPI (4,6-diamidino-2-phenylindole) and PI (propidium iodide). Flow cytometry (Beckman Coulter, FC500MCL) was used for quantitation of apoptotic and necrotic cells. Octadecyl-functionalized silica gel (ODS, Cosmosil 75C18-Prep) and Diaion HP-20 (Mitsubishi Chemical) were applied for column chromatography. TLC analysis was conducted on silica gel 60 RP-18 F254S aluminum TLC plates (Merck). D-Xylose, D-quinovose, and 2DG (> 98%) were purchased from Sigma-Aldrich, TMZ (> 95.0%) from BePharm Ltd., and curcumin (≥ 98.0%) from Yuanye Bio-Technology Co. Ltd. The annexin V apoptosis detection kit was obtained from Invitrogen and the bicinchoninic acid (BCA) kit from Thermo Scientific. Rat glioma C6 cells and human glioma U251, U87-MG, and SHG-44 cells were purchased from the Cell Bank of the Chinese Academy of Sciences. Human astrocytes (cat. No. 1800) were obtained from ScienCell. Antibodies of HK2 (cat. ab104836, Abcam), PFKFB3 (cat. ab113107, Abcam), PKM2 (cat. 6989–1, Epitomics), GLS (cat. 7485–1, Epitomics), ATPB (cat. ab14730, Abcam), ACO2 (cat. 5806–1, Epitomics), PDHB (cat. 7339–1, Epitomics), CytoC (cat. 1896–1, Epitomics), BAD (cat. 1541–1, Epitomics), BAX (cat. 1063–1, Epitomics), BCL-2 (cat. 2870, Cell Signaling Technology), BCL-Xl (cat. 1018–1, Epitomics), and *β*-actin (cat. ab6276, Abcam) were used for Western blot analysis.

Sea cucumber material

Fresh sea cucumbers from the species *H. moebii* were collected from the Turtle Islet in the South China Sea close to Shanwei City, Guangdong Province, China, in October 2012 and then frozen for later use. A voucher sample (SW-A18) was authenticated by one of the authors (Z. Zhang) and deposited in the Laboratory of Institute of Marine Biology, Ocean College, Zhejiang University, China.

Extraction and isolation

Ten frozen whole bodies of *H. moebii* specimens were cut into small pieces (5 × 5 mm) and percolated with MeOH five times (first with 2000 mL for 12 h, then 1000 mL for 4 h for four times). The combined MeOH solution was concentrated under reduced pressure to 1000 mL and then partitioned successively with hexane and BuOH to afford cyclohexane and n-BuOH fractions (HMB, 15.0 g). HMB was applied to a column (60 × 5 cm) of Diaion HP-20 (300 mL) washing in turn with 10% and 80% MeOH. The dried 80% MeOH residue (2.5 g) was further separated by an ODS (100 g) column (50 × 3.5 cm) eluting with 30% and 70% MeOH, respectively. The fractions (each 50 mL) from the 70% MeOH elution were analyzed by TLC (MeOH/H_2O, 7: 3 v/v, sprayed with 10% H_2SO_4 and then heated at 105 °C). The fractions containing the same compounds were combined and then dried under reduce pressure to give **3** (196.0 mg, R_f 0.43) from fractions 12 and

13, **4** (7.6 mg, R_f 0.29) from 15, and a mixture (30 mg) of **1** (R_f 0.57) and **2** (R_f 0.51) from fractions 9 and 10. The mixture of **1** and **2** was further separated by an ODS (30 g) column (35 × 2.5 cm) eluting with 60% MeOH (each fraction 30 mL) to give **1** (15.6 mg, fraction 13) and **2** (6.3 mg, fraction 16). Compounds **1**–**4** had a purity of 94.7–97.8% as determined by HPLC for NMR analysis and the activity assay.

*Compound **2***: white amorphous powder; $[\alpha]_D^{21}$ +16.7 (*c* 0.10, MeOH); IR λ_{max} (KBr) 3423, 2948, 1741, 1633, 1463, 1384, 1258, 1181, 1067, 1008 cm^{-1}; ^{13}C NMR and ^1H NMR data (in pyridine-d_5), see **Table 1**; HRESIMS: *m/z* = 917.3842 $[M - Na]^-$ (calcd. for $C_{43}H_{65}O_{19}S$, 917.3841).

Alkaline hydrolysis of compound 3

Compound **3** (40 mg) was refluxed with 10 mL 0.8 N NaOH at 80 °C for 4 h. After cooling, the hydrolysate was neutralized with 1 N HCl and then extracted with BuOH (10 mL × 3 times). The organic layers were combined and then evaporated under reduced pressure to dryness. The residue was separated by an ODS (50 g) column eluting with 85% MeOH using TLC ($MeOH/H_2O$, 9 : 1) to furnish compound **3B** (10.8 mg, R_f 0.62).

*Compound **3B***: white amorphous powder; molecular formula $C_{41}H_{62}O_{13}$; $[\alpha]_D^{21}$ +11.5 (*c* 0.10, MeOH); IR λ_{max} (KBr) 3416, 2947, 1740, 1633, 1445, 1384, 1208, 1165, 1069, 1010 cm^{-1}; ^{13}C and ^1H NMR data (in pyridine-d_5), see **Table 1**; HRESIMS: *m/z* 761.4115 $[M - H]^-$ (calcd. for $C_{41}H_{61}O_{13}$, 761.4112).

Acidic hydrolysis of compounds 2, 3, and 3B

Compound **2** (2.0 mg) was hydrolyzed in 2 mL 1 N HCl (dioxane-water, 1 : 1) at 80 °C for 2 h in a water bath. The reaction mixture was neutralized with 0.8 N NaOH and then applied to a column of Diaion HP-20 (15 mL) eluting with 50 mL H_2O. The H_2O elution was evaporated under reduced pressure to give a residue. This residue was first treated with 10 mg hydroxylamine hydrochloride in 2 mL pyridine at 90 °C for 30 min in a water bath. After cooling, it was mixed with 2 mL acetic anhydride at 90 °C for 1 h in a water bath. Then, the reaction products were concentrated *in vacuo* to dryness and finally dissolved in 2 mL chloroform. The sugar acetyl derivatives were identified as D-quinovose (t_R 15.132 min) and D-xylose (t_R 15.391 min) by GC analysis with standard D-quinovose (t_R 15.129 min) and D-xylose (t_R 15.392 min) as references. Compound **3** (50 mg) was hydrolyzed using the same method for compound **2**, and the hydrolytic mixture was extracted with $CHCl_3$ three times (each 3 mL). The $CHCl_3$ solution was then washed with water and concentrated to dryness. The dried residue was subjected to an ODS (50 g) column eluting with 90% MeOH using TLC ($MeOH/H_2O$, 9 : 1) for the detection of each fraction to furnish **3A** (9.6 mg, R_f 0.55). In the same way, acidic hydrolysis of **3B** also produced **3A**.

Tumor cell culture

Rat glioma C6 and human glioma U251 cells were cultured in DMEM (Gibco) with 10% FBS (PAA Laboratories, Inc.), human glioma U87-MG cells in MEM (Gibco), human glioma SHG-44 cells in RPMI-1640 (Gibco), and human astrocytes in astrocyte medium (AM, ScienCell, cat. No. 1801). All cells were incubated at 37 °C in a humidified 5% CO_2 incubator. Cells after the third generation were used for the experiment.

Sulforhodamine B (SRB) assay

The SRB assay, as described in previous reports [4,6], was used to determine the activity of the isolated compounds suppressing the proliferation of glioma U87-MG, U251, SHG-44, and C6 cells. TMZ was used as a positive control.

Detection of apoptosis and necrosis in glioma cells

Apoptosis and necrosis induced by the tested compounds in U87-MG cells were detected by DAPI and PI double staining. Annexin V-FITC/PI double staining was applied for the quantification of apoptotic and necrotic cells using an annexin V apoptosis detection kit. Curcumin was used as a positive control. Detailed methods can be found in previous publications [4,6].

Protein sample preparation

Human glioblastoma U87-MG cells were cultured in modified Eagle's medium (Gibco) with 10% FBS (PAA Laboratories, Inc.) at 37 °C in a humidified 5% CO_2 incubator. Cells (1.5×10^7) were plated in 10-cm culture dishes and treated with the tested compounds after cell adhesion for 24 h. After 48 h treatment, the cells were washed twice with icy PBS and then lysed with 200 µL icy lysis buffer (20 mM Tri-HCl, pH = 7.5, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM EDTA, 1% NP40, 50 mM NaF) for 15 min. The lysates were centrifuged at 11 200 rpm for 15 min at 4 °C to afford the supernatant (protein sample).

Determination of protein concentration

The protein concentration of each protein sample was determined using a BCA assay kit. A mixture of BCA reagents A and B (50 : 1) was prepared as the working reagent (WR). A series of known concentrations of BCA (standard solution) was prepared by diluting with bovine serum albumin (BSA). Ten µL of each standard or sample solution was mixed with 200 µL of WR, and then incubated at 37 °C for 30 min. The absorbance of each incubated solution was measured at 562 nm with a microplate reader (Bio-Tech). The standard linear regression equation between absorbance and concentration was determined. The protein concentration of each sample was calculated from the equation.

Western blot analysis

Equal amounts of proteins (15 µg) were fractionated by 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (PVDF) (pore size 0.2 µm), which were then blocked with 5% nonfat milk in 0.1% Tween 20-TBS (TBST) for 2 h at room temperature. The membranes were incubated with primary antibodies against HK2, PFKFB3, PKM2, GLS, ACO2, ATPB, PDHB, CytoC, BAD, BAX, BCL-2, and BCL-XL at 4 °C overnight, washed with TBST, and then probed with the secondary antibodies conjugated with horseradish peroxidase (HRP) at room temperature for 2 h. After being repeatedly washed with TBST, immunoreactivity was detected by using enhanced chemiluminescence reagents (Beyotime), and the films were scanned. The expression levels of HK2, PFKFB3, PKM2, GLS, ACO2, ATPB, PDHB, CytoC, BAD, BAX, BCL-2, and BCL-XL in U87-MG cells treated with the tested compounds were compared with those in untreated U87-MG cells. 2DG, an inhibitor of hexokinase and a well-studied anti-glioma agent, was used as a positive control. Normalization of the results was insured by running parallel Western blots with mouse monoclonal β-actin.

Supporting information

^{1}H NMR, ^{13}C NMR, HMQC, COSY, HMBC, and HRESIMS spectra of compounds **2** and **3B**, ^{13}C NMR and ^{1}H NMR data of the known compounds, as well as ^{1}H-^{1}H COSY and key NOE correlations of compound **2** are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

References

1 *Kim SK, Himaya SW.* Triterpene glycosides from sea cucumbers and their biological activities. Adv Food Nutr Res 2012; 65: 297–319
2 *Guan HS, Wang SG.* Chinese Marine Materia Medica (Vol. III). Shanghai: Shanghai Scientific and Technical Publishers; 1999: 561–598
3 *Bordbar S, Anwar F, Saari N.* High-value components and bioactives from sea cucumbers for functional foods – a review. Mar Drugs 2011; 9: 1761–1805
4 *Xin W, Ye X, Yu S, Lian XY, Zhang Z.* New capoamycin-type antibiotics and polyene acids from marine *Streptomyces fradiae* PTZ0025. Mar Drugs 2012; 10: 2388–2402
5 *Guo X, Shen L, Tong YH, Zhang J, Wu G, He Q, Yu SR, Ye XW, Zou LB, Zhang ZZ, Lian XY.* Antitumor activity of caffeic acid 3,4-dihydroxyphenethyl ester and its pharmacokinetic and metabolic properties. Phytomedicine 2013; 20: 904–912
6 *Yu S, Ye X, Xin W, Xu K, Lian XY, Zhang Z.* Fatsioside A, a rare baccharane-type glycoside inhibiting the growth of glioma cells from the fruits of *Fatsia japonica*. Planta Med 2014; 80: 315–320
7 *Yu S, Ye X, Chen L, Lian XY, Zhang Z.* Polyoxygenated 24,28-epoxyergosterols inhibiting the proliferation of glioma cells from sea anemone *Anthopleura midori*. Steroids 2014; 88: 19–25
8 *Wolf A, Agnihotri S, Guha A.* Targeting metabolic remodeling in glioblastoma multiforme. Oncotarget 2010; 1: 552–577
9 *Ru P, Williams TM, Chakravarti A, Guo D.* Tumor metabolism of malignant gliomas. Cancers 2013; 5: 1469–1484
10 *Kitagawa I, Nishino T, Kobayashi M, Matsuno T, Akutsu H, Kyogoku Y.* Marine natural products. VIII. Bioactive triterpene-oligoglycosides from the sea cucumber *Holothuria leucospilota* Brandt (2). Structure of holothurin A. Chem Pharm Bull 1981; 29: 1951–1956
11 *Kitagawa I, Nishino T, Kobayashi M, Matsuno T, Akutsu H, Kyogoku Y.* Marine natural products. VII. Bioactive triterpene-oligoglycosides from the sea cucumber *Holothuria leucospilota* Brandt. Structure of holothurin B. Chem Pharm Bull 1981; 29: 1942–1950
12 *Kobayashi M, Hori M, Kan K, Yasuzawa T, Matsui M, Suzuki S, Kitagawa I.* Marine natural products. XXVII. Distribution of lanostane-type triterpene oligoglycosides in ten kinds of Okinawan sea cucumbers. Chem Pharm Bull 1991; 39: 2282–2287
13 *Yuan WH, Yi YH, Li L, Liu BS, Zhang HW, Sun P.* Two triterpene glycosides from the sea cucumber *Bohadschia marmorata* Jaeger. Chin Chem Lett 2008; 19: 457–460
14 *Wu LJ.* Natural medicinal chemistry. Beijing: People's Medical Publishing House; 2011: 98–100
15 *Patil SA, Hosni-Ahmed A, Jones TS, Patil R, Pfeffer LM, Miller DD.* Novel approaches to glioma drug design and drug screening. Expert Opin Drug Discov 2013; 8: 1135–1151
16 *Qi XC, Xie DJ, Yan QF, Wang YR, Zhu YX, Qian C, Yang SX.* LRIG1 dictates the chemo-sensitivity of temozolomide (TMZ) in U251 glioblastoma cells via down-regulation of EGFR/topoisomerase-2/Bcl-2. Biochem Biophys Res Commun 2013; 437: 565–572
17 *Low SY, Ho YK, Too HP, Yap CT, Ng WH.* MicroRNA as potential modulators in chemoresistant high-grade gliomas. J Clin Neurosci 2014; 21: 395–400
18 *Aggarwal BB, Kumar A, Bharti AC.* Anticancer potential of curcumin: preclinical and clinical studies. Anticancer Res 2003; 23: 363–398
19 *Wolf A, Agnihotri S, Micallef J, Mukherjee J, Sabha N, Cairns R, Hawkins C, Guha A.* Hexokinase 2 is a key mediator of aerobic glycolysis and promotes tumor growth in human glioblastoma multiforme. J Exp Med 2011; 208: 313–326
20 *Kessler R, Bleichert F, Warnke JP, Eschrich K.* 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3) is up-regulated in high-grade astrocytomas. J Neurooncol 2008; 86: 257–264
21 *Yang W, Xia Y, Cao Y, Zheng Y, Bu W, Zhang L, You MJ, Koh MY, Cote G, Aldape K, Li Y, Verma IM, Chiao PJ, Lu Z.* EGFR-induced and PKCε monoubiquitylation-dependent NF-κB activation upregulates PKM2 expression and promotes tumorigenesis. Mol Cell 2012; 48: 771–784
22 *Daye D, Wellen KE.* Metabolic reprogramming in cancer: unraveling the role of glutamine in tumorigenesis. Semin Cell Dev Biol 2012; 23: 362–369
23 *Vander Heiden MG.* Targeting cancer metabolism: a therapeutic window opens. Nat Rev Drug Discov 2011; 10: 671–684
24 *Dwarakanath BS, Singh D, Banerji AK, Sarin R, Venkataramana NK, Jalali R, Vishwanath PN, Mohanti BK, Tripathi RP, Kalia VK, Jain V.* Clinical studies for improving radiotherapy with 2-deoxy-D-glucose: present status and future prospects. J Cancer Res Ther 2009; 5 (Suppl. 1): S21–S26
25 *Galluzzi L, Kepp O, Vander Heiden MG, Kroemer G.* Metabolic targets for cancer therapy. Nat Rev Drug Discov 2013; 12: 829–846
26 *Jones NP, Schulze A.* Targeting cancer metabolism–aiming at a tumour's sweet-spot. Drug Discov Today 2012; 17: 232–241