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Original article

Activity of novel quinoxaline-derived chalcones on *in vitro* glioma cell proliferation

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ABSTRACT

Gliomas are the most common and devastating tumors of the central nervous system (CNS). Many pieces of evidence point out the relevance of natural compounds for cancer therapy and prevention, including chalcones. In the present study, eight synthetic quinoxaline-derived chalcones, structurally based on the selective PI3Kγ inhibitor AS605240, were evaluated for anti-proliferative activity and viability inhibition using glioma cell lines from human and rat origin (U-138 MG and C6, respectively), at different time-periods of incubation and concentrations. The results revealed that four chalcones (compounds **1**, **6**, **7** and **8**), which present methoxy groups at A-ring, displayed higher efficacies and potencies, being able to inhibit either cell proliferation or viability, in a time- and concentration-dependent manner, with an efficacy that was greater than that seen for the positive control compound AS605240. Flow cytometry analysis demonstrated that incubation of C6 cells with compound **6** led to G1 phase arrest, likely indicating an interference with apoptosis. Furthermore, compound **6** was able to visibly inhibit AKT activation, allied to the stimulation of ERK MAP-kinase. The chalcones tested herein, especially those displaying a methoxy substituent, might well represent promising molecules for the adjuvant treatment of glioma progression.

1. Introduction

Gliomas comprise several types of primary brain tumors, accounting for approximately 50% of all neoplasms of the central nervous system (CNS) [1–3]. Glioblastoma multiforme (GBM) is the most common, aggressive and deadly malignant glioma in adults [4–6]. This kind of brain tumor is characterized by marked cell proliferation and invasiveness with a rapid progression, presenting a high grade of recurrence [2,7–9]. Therefore, the prognosis for the patients with these tumors is poor. The mean survival is around one year [8,10,11] and few patients survive beyond five years [12]. Treatment options for patients with GBM are very limited, or in most situations they are ineffective. Despite the increasing advances in radiotherapy, chemotherapy, and surgical techniques, the survival rate for these patients remains low [13]. Therefore, there is an urgent need for novel and effective therapies for treating these tumors. In this regard, molecules based on natural products represent very interesting therapeutic alternatives.

Extensive research over the past decades has identified numerous dietary and botanical natural compounds with clear anti-cancer effects. They might also present synergistic beneficial effects when used in combination with known chemotherapeutic drugs [14,15]. Previous studies have shown that the simple chemical structure and the uncomplicated process of synthesis make plant-derived polyphenols an attractive scaffold for the development of new compounds [16,17].

Chalcones are a group of natural precursors of flavonoid and isoflavonoids synthesis in high plants [18–21], and they are cancer

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preventive components found in human diet rich in fruits and vegetables [20,22]. Concerning the chemical structural level, these compounds are open-chained molecules, in which the two aromatic rings are joined by a three-carbon α,β-unsaturated carbonyl system (1,3-diphenyl-2-propen-1-one) [16,23,24]. Several studies have shown that chalcones display a wide variety of biological and pharmacological proprieties that include antiproliferative and anti-cancer activities [25–27]. Clinical trials have shown that these compounds reach reasonable plasma concentrations, they are not associated to marked toxicity [28] and flavonoids and their derivatives are able to cross the brain blood barrier [29,30].

A large number of chemical structures have been tested, and it was reported that hydroxy-derivatives of chalcones display marked anti-proliferative effects on cancer cells. These groups are likely necessary for the inhibitory effects of glutathione S-transferase, what is associated to cancer cell sensitization for chemotherapy agents [15,31]. Other chemical modifications of chalcones have been investigated, such as hydrogenation and bromination across the carbon–carbon double bond, but they failed in potentiate the anti-tumor effects of chalcones [16]. Recently, it was demonstrated that quinoxaline derivatives display a broad spectrum of biological activities. Of relevance, some of these compounds have been described as potential candidates for the treatment of cancer and disorders associated with angiogenesis [32].

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is implicated in a variety of cellular processes including cell growth, proliferation, and survival, which is frequently deregulated in cancer, including glioma [33–35]. The alteration of this pathway through mutation of its coding genes increases the activation status of the signaling and can thus lead to cellular transformation [33] and cancer development [36]. Given the frequency of deregulated PI3K signaling, the modulation of this pathway might have therapeutic potential in glioma and other cancer types. AS605240 [5-(quinoxalin-6-ylmethylene)thiazolidine-2,4-dione] is a low-toxicity and selective inhibitor of the PI3Kγ isoform, which is able to suppress inflammation in different experimental models [37], and further studies are currently testing its effects on tumor cells [38], considering the involvement of this signaling pathway in cancer development. Then, in the present study, we have examined the *in vitro* effects of eight novel synthetic quinoxaline-derived chalcones, structurally based on the selective PI3Kγ inhibitor AS605240 (Fig. 1), on viability, proliferation, cell cycle, and signaling pathways, by using human U-138 MG and rat C6 glioma cell lineages.

ERK1 and ERK2 are members from the family of MAP-kinases (mitogen-activated protein kinases), and are activated by various growth factors, inducing the transition from the quiescent state into the cell cycle. ERK signaling pathways are also involved in cell proliferation, differentiation, actin cytoskeleton reorganization, and cell migration. Moreover, ERKs are also involved in the stress response and cell death [39–42]. Therefore, this is another relevant signaling pathway to be investigated in cancer research.

2. Chemistry

The quinoxaline-6-carbaldehyde (**12**) was synthesized from 3,4-diaminobenzoic acid (**9**) as previously described [43] and presented in Fig. 2, with yield of 80%. Eight chalcones (**1**–**8**) were prepared by aldolic condensation between quinoxaline-6-carbaldehyde (**12**) and corresponding acetophenones, in methanol and KOH 50% w/v, under magnetic agitation and room temperature. The novel chalcones were obtained with yields between 41% and 93%, and the structures were confirmed by chemical identification data: ^{1}H NMR, ^{13}C NMR, IR and elementary analysis. ^{1}H NMR spectra revealed that all structures were geometrically pure and configured *E* ($J_{H\alpha-H\beta} = \sim 16.0$ Hz).

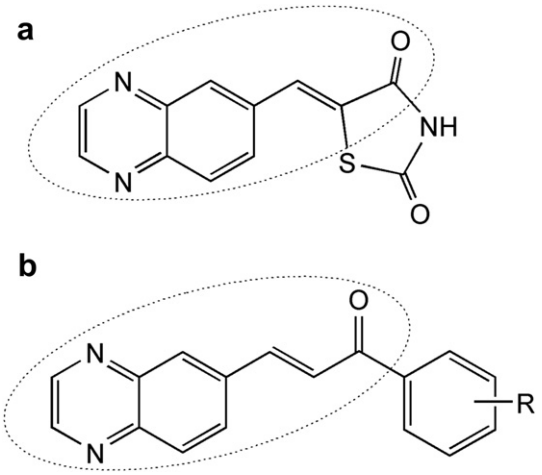

Fig. 1. PI3Kγ inhibitor AS605240 (a) and chalcones proposed in this research (b).

3. Biological results and discussion

As a first approach, we performed a screening using both U-138 MG and C6 glioma cell lines, in order to assess the inhibitory effects of the chalcones. This was carried out following 48 h of incubation, at concentrations between 0.1 and 10 μg/mL. All tested chalcones produced a significant decrease of C6 rat glioma cell line viability, at 5 μg/mL (Table 1). The estimated IC_{50} values (accompanied by the confidence interval) for the rat cells ranged from 2.66 (2.09–3.38) μg/ml to 9.19 (8.93–9.48) μg/ml. Concerning the U-138 MG glioma cells, with exception of the compound **5**, all other compounds produced a significant inhibition of cell viability at 5 μg/mL. However, for the human cell line, it was not possible to calculate the IC_{50} values, as the calculated percentages of inhibition did not exceed 50% (Table 1). As demonstrated in the present study for the tested chalcones, Zamin et al. (2009) also showed that rat C6 and human U-138 glioma cell lines displayed a different sensitivity to the treatment with the compounds resveratrol and quercetin, two well-studied natural polyphenols [29].

From this experimental set, it was possible to observe that four of tested chalcones (compounds **1**, **6**, **7** and **8**) displayed higher efficacies and potencies. This is especially notable if we compare with the low inhibitory rates obtained for reference chemotherapy drugs, such as cisplatin and doxorubicin [44]. Of note, these four chalcones present a similar chemical structure, having methoxy groups in the ring A. In fact, previous studies demonstrated that the activity of chalcones is largely dependent on the presence and position of the substituted groups added in both rings A and B. It has been reported that dimethoxylation and trimethoxylation are highly beneficial for the anticancer activity [17,45–47]. In our studies, chalcone **6**, dimethoxylated in positions 2 and 5 at A-ring, displays the best activity.

As a next step, we assayed the compounds **1**, **6**, **7** and **8** in concentrations ranging from 0.1 to 10 μg/mL, at different periods of incubation (24, 48 and 72 h), by using a hemocytometer. Cell counting assay revealed marked inhibitory effects on the proliferation of either human or rat cell lines (Table 2). For this assay, the estimated IC_{50} values ranged from 2.29 (2.03–2.58) μg/ml to 2.67 (2.55–2.80) μg/mL, indicating a high potency for these compounds (Table 2). Of high interest, the chemotherapy drug doxorubicin, incubated during 48 h, at 2-μg/mL concentration, produced an

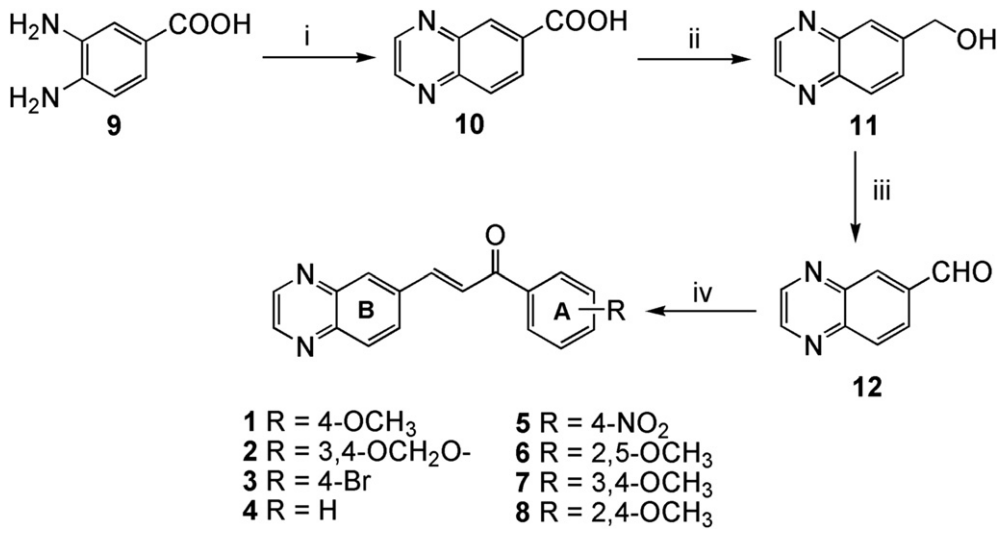

Fig. 2. Synthesis of compounds. (i) glyoxal, acetic acid, CH_3CH_2OH, reflux; (ii) $LiAlH_4$, THF; (iii) CCP, dichloromethane; (iv) corresponding acetophenones, CH_3OH, KOH 50% w/v, magnetic stirring, 24 h, r.t.

inhibition of 27 ± 3% and 31 ± 4%, in C6 and U-138 MG, cells, respectively (results not shown).

The concentration-dependent effects of compounds **1**, **6**, **7** and **8**, at both the MTT and the cell counting assays are depicted in the Figs. 3–6. From these figures, it is possible to observe that compounds **1**, **6**, **7** and **8** presented anti-tumor-like effects in concentrations as low as 0.1–0.5 μg/mL. Furthermore, it is feasible to observe that maximal inhibitions for these four chalcones were observed between 48 h and 72 h of treatment, whereas the compounds **1**, **6**, **7** and **8** failed to significantly alter cell viability and proliferation when incubated for 24 h. This assembly of results revealed a concentration-related profile of inhibition for the tested chalcones on either the cell viability or proliferation. In addition, the effects of these four chalcones were found to be time-dependent, being maximal between 48 and 72 h following *in vitro* treatment.

AS605240 is a well-characterized selective PI3Kγ inhibitor, which blocks human recombinant PI3Kγ in an ATP-competitive manner, displaying marked anti-inflammatory effects in several animal disease models [37,48]. The chalcones derived from quinoxaline-6-carbaldehyde analyzed in this work are structurally based on this selective PI3Kγ inhibitor. Therefore, some experiments were conducted with the aim of comparing the effects of quinoxaline-derived chalcones to those displayed by AS605240. The glioma cells were treated with AS605240 at the concentrations of 100 nM, 10 μM and 30 μM [37], during 48 h, and subsequently evaluated in the MTT and cell counting assays. The results show that AS605240 failed to significantly affect C6 and U-138 MG cell viability and proliferation at the concentration of 100 nM, whereas this compound visibly reduced these parameters at 10 and 30 μM (Fig. 7). The percentages of inhibition for the concentration of 30 μM were 35 ± 2% and 73 ± 4% for C6; and 10 ± 3% and 10 ± 0.2% for U-138 MG, considering the viability and the proliferation, correspondingly (Fig. 8). It is worth mentioning that the concentration of 5 μg/ml of the compound **6** (which corresponds to 16 μM) displayed inhibitions of 50 ± 7% for C6 viability and 85 ± 8% for C6 proliferation; and 36 ± 1% for U-138 MG viability and 87 ± 4% for proliferation of this cell line. This series of results suggest a greater efficacy to compound **6**, in comparison to the reference compound AS605240, especially concerning the cell lineage U-138 MG.

Previous literature data demonstrated that natural and synthetic chalcones are able to induce cell cycle arrest and apoptosis in different cancer cell lines [17,26]. To examine the possible mechanisms responsible for the inhibitory effects displayed by the quinoxaline-derived chalcones in our study, we have used flow cytometry analysis. For this purpose, we have selected the compound **6**, which presented the more favorable profile in our experiments. The results demonstrate that incubation of compound **6** caused an accumulation of cells in sub-G1/G1 phases in C6 cells at 6 and 12 h (results not shown). When assessed after 24 h of incubation, compound **6** (2.5 μg/ml) visibly increased the cell population of the sub-G1/G1 phases from 57.9 to 66.4, when compared with the control group (Fig. 8). It is feasible to suggest that part of

Table 1
Effects of quinoxaline-derived chalcones on the viability of rat and human glioma cell lines, according to assessment by MTT assay.

	Glioma cell line			
	Rat C6		Human U−138 MG	
Chalcones	I_{max} (%)[a]	IC_{50} (μg/ml)[b]	I_{max} (%)[a]	IC50 (μg/ml)[b]
1	53 ± 3	4.42 (4.03–4.84)	34 ± 3	–
2	55 ± 5	8.39 (7.66–9.20)	15 ± 5	–
3	56 ± 6	9.19 (8.93–9.48)	16 ± 5	–
4	60 ± 6	4.63 (3.40–6.30)	21 ± 7	–
5	26 ± 6	–	9 ± 2	–
6	50 ± 7	2.66 (2.09–3.38)	36 ± 1	–
7	51 ± 7	4.16 (3.51–4.93)	33 ± 3	–
8	52 ± 7	4.60 (4.07–5.20)	42 ± 2	–

[a] The maximal percentages of inhibition were calculated at the concentration of 5 μg/ml.
[b] The compounds were tested at concentrations ranging between 1 and 10 μg/ml.

Table 2
Effects of quinoxaline-derived chalcones **1**, **6**, **7** and **8** on the proliferation of rat and human glioma cell lines, as assessed by cell counting in a hemocytometer.

	Glioma cell line			
	Rat C6		Human U-138 MG	
Chalcones	I_{max} (%)[a]	IC_{50} (μg/ml)[b]	I_{max} (%)[a]	IC50 (μg/ml)[b]
1	90 ± 4	2.19 (1.85–2.59)	84 ± 2	2.67 (2.55–2.80)
6	85 ± 8	1.35 (1.10–1.65)	87 ± 4	2.64 (2.46–2.84)
7	82 ± 6	1.00 (0.21–4.65)	84 ± 3	2.29 (2.03–2.58)
8	67 ± 7	1.53 (1.21–1.95)	86 ± 3	2.48 (2.17–2.79)

[a] The maximal percentages of inhibition were calculated at the concentration of 5 μg/ml.
[b] The compounds were tested at concentrations ranging between 1 and 10 μg/ml.

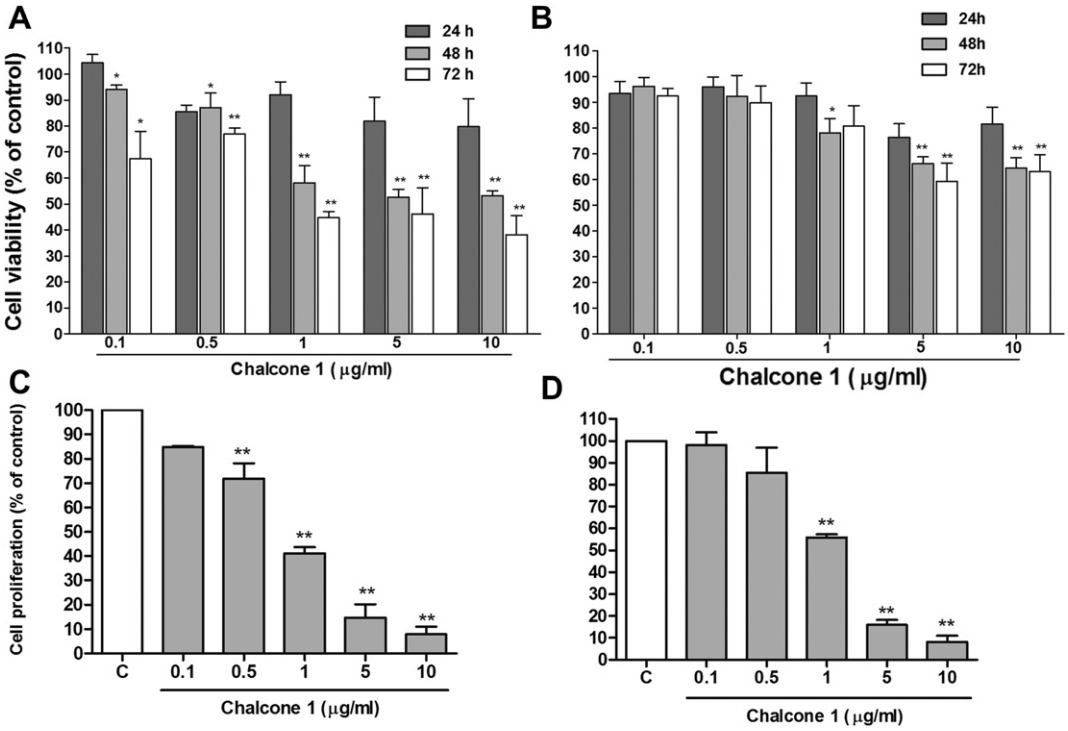

Fig. 3. Effects of compound **1** on glioma cell viability and proliferation. The viability of rat glioma cells C6 (A) and human glioma cells U-138 MG (B) were assessed by MTT assay. Cell viability is provided as the percentage of control group viability. Proliferation of C6 (C) and U-138 MG (D) cells was assessed by cell counting in a hemocytometer. Each column represents the mean of 3 independent experiments performed in triplicate and the lines indicate the standard error means. Data were analyzed by oneway ANOVA, followed by multiple comparisons post-hoc test (Tukey test). *Significantly different from the control group *($P < 0.05$); ** ($P < 0.01$).

the decrease in glioma cell viability caused by **6** is likely associated with cell cycle arrest at the G1 phase.

It has been demonstrated that alterations of the PI3K/Akt activation might lead to cellular transformation [33] and cancer development [46]. In tumor cells, PI3K plays an important role in tumor initiation, growth and proliferation. Inhibition of PI3K may thus have the potential to inhibit formation of secondary-site metastases [49–52]. To examine the possible modulation of PI3Kγ, we performed flow cytometry experiments with compound **6** and the selective PI3Kγ inhibitor AS605240, as the positive control drug. As expected, both the compound **6** and AS605240 were able to reduce AKT activation, according to assessment at 15 and 30 min. Of note, differently from AS605240, the compound **6** led to increased activation of ERK 1/2 MAP-kinase, indicating additional mechanisms of action for this compound (Fig. 9). Nevertheless, we can infer that inhibition of PI3K signaling may be related with the reduction of cell proliferation observed in our previous results and can be one of the mechanisms involved in the anti-tumor actions of chalcone **6**. Furthermore, the ability of compound **6** in positively modulating ERK 1/2 activation might possibly accounts for cell death, likely by inducing apoptosis. In fact, previous studies suggest that apoptosis might be induced by the disruption of MAP-kinase signal transduction. The duration and intensity of ERK activation appear to be important in determining the cell fate (growth, survival or apoptosis), indicating that ERK may have a dual role in the regulation of cell survival and death [53,54]. According to Werlen and col. (2003), depending on the affinity of the ligand, the ERK pathway is activated before p38 and JNK, resulting in cell apoptosis [55]. In support of this, our results showed that compound **6** led to marked ERK 1/2 activation, which was accompanied by increased granulosity of the cells (data not shown).

It is obviously necessary to investigate the ability of these chalcones to cross the blood–brain barrier, as cerebral sites need to be reached. Of interest, previous studies have shown that flavonoids and their derivatives were able to cross this barrier [29,30]. Further *in vitro* and *in vivo* studies should be performed for verify this matter. Thus, the synthetic quinoxaline-derived compounds **1**, **6**, **7** and **8** might well represent promising molecules for the adjuvant treatment of glioma progression.

4. Conclusions

The present results show for the first time the activity of synthetic quinoxaline-derived chalcones in two different glioma cell lines. These results clearly suggest that compounds **1**, **6**, **7** and **8** might represent promising molecules for the treatment of glioma progression, although the *in vivo* efficacy of these chalcones remains to be confirmed in future studies. Nevertheless, these chalcones might represent potential useful alternatives for treating gliomas, even when used alone or in combination with currently available chemotherapy agents.

5. Materials and methods

5.1. Preparation of the compounds

All reagents used were obtained commercially (Sigma–Aldrich), except the quinoxaline-6-carbaldehyde, which was obtained as previously described [43] by condensation between 3,4-diaminobenzoic acid (**9**) and glyoxal at reflux with ethanol and acetic acid, generating the quinoxalinecarboxylic acid (**10**) with yield of 25%. Compound **10** was then reduced to its primary alcohol (**11**) with $LiAlH_4$ in THF, which was after oxidized to aldehyde (**12**)

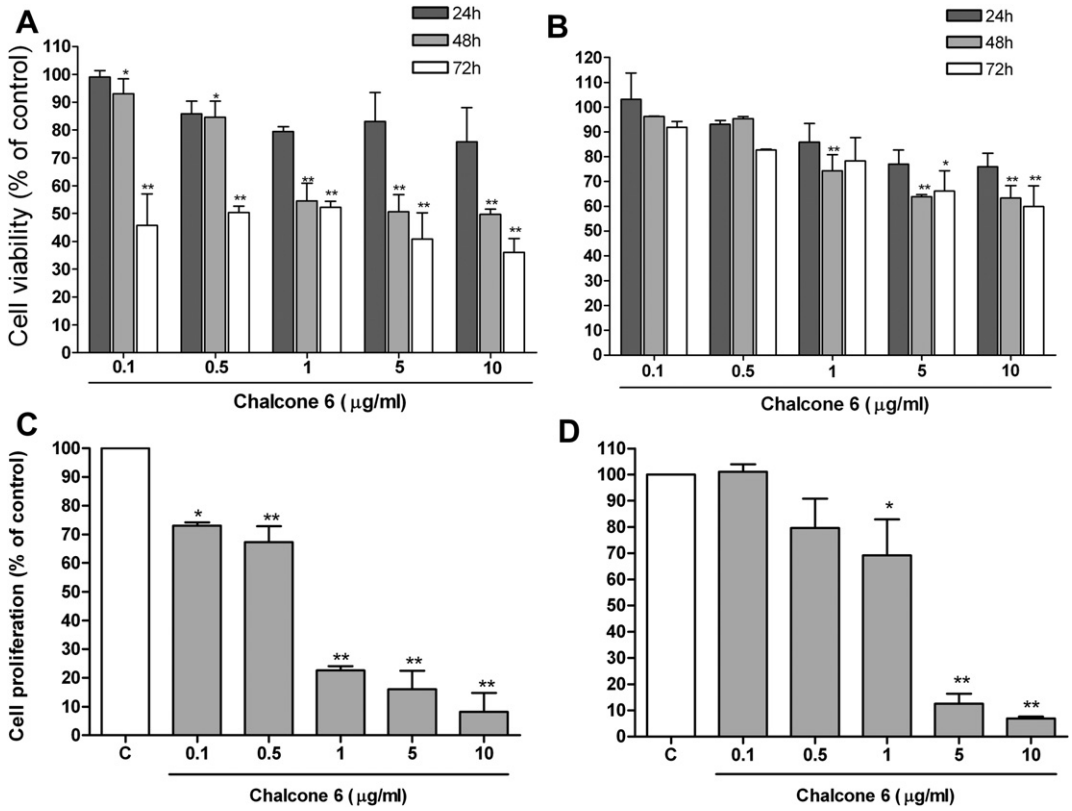

Fig. 4. Effects of compound **6** on glioma cell viability and proliferation. The viability of rat glioma cells C6 (A) and human glioma cells U-138 MG (B) were assessed by MTT assay. Cell viability is provided as the percentage of control group viability. Proliferation of C6 (C) and U-138 MG (D) cells was assessed by cell counting in a hemocytometer. Each column represents the mean of 3 independent experiments performed in triplicate and the lines indicate the standard error means. Data were analyzed by oneway ANOVA, followed by multiple comparisons post-hoc test (Tukey test). *Significantly different from the control group *($P < 0.05$); ** ($P < 0.01$).

with pyridine–chlorochromate in dichloromethane (Fig. 2), with yield of 80%. The novel chalcones (**1**–**8**)were prepared by magnetic stirring with acetophenone (1 mmol), methanol (30 ml), KOH 50% w/v (5 ml) and quinoxaline-6-carbaldehyde (**12**) (1 mmol), at room temperature for 24 h. Distilled water and chloridric acid 10% were added in the reaction for total precipitation of the compounds. The compounds were then obtained by vacuum filtration and later recrystallized in dichloromethane/hexane. The purified chalcones were obtained with yields between 41% and 93%.

5.2. Physico–chemical data of the compounds

The structures were identified using melting points (m.p.), infrared spectroscopy (IR), ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) and elementary analysis. Melting points were determined with a Microquimica MGAPF-301 apparatus and are uncorrected. IR spectra were recorded with an Abb Bomen FTLA 2000 spectrometer on KBr disks. Elementary analyses were obtained with a CHNS EA 1110. Percentages of C and H were in agreement with the product formula (within 0.4% of theoretical values to C). NMR (^{1}H and ^{13}C NMR) were recorded on Varian Oxford AS-400 (400 MHz), using tetramethylsilane as internal standard. ^{1}H NMR spectra revealed that all the structures were geometrically pure and configured *E* ($J_{H\alpha\text{-}H\beta} = \sim 16.0$ Hz).

5.2.1. (2E)-1-(4-methoxyphenyl)-3-(quinoxalin-6-yl)prop-2-en-1-one (*1*)

Cream solid, m.p. 160–161 °C; ^{1}H NMR ($CDCl_3$) δ 3.92 (s, 3H, OCH_3), 7.02 (d, 2H, $J = 8.0$ Hz, H3′, H5′), 7.76 (d, 1H, $J = 16.0$ Hz, Hα), 8.00 (d, 1H, $J = 16.0$ Hz, Hβ), 8.09 (m, 1H, H3), 8.10 (d, 2H, $J = 8.0$ Hz, H2′, H6′), 8.16 (d, 1H, $J = 8.0$ Hz, H4), 8.33 (s, 1H, H1), 8.88 (dd, 2H, $J = 8.0/1.0$ Hz, H6, H7). ^{13}C NMR ($CDCl_3$) δ 55.34 (OCH_3), 114.15 (C3′, C5′), 124.76 (Cα), 128.82 (C1′), 130.20 (C3), 130.75 (C1), 131.17 (C2′, C6′, C4), 137.24 (C2), 141.71 (Cβ, C4a), 144.02 (C8a), 146.28 (C6), 146.53 (C7), 164.04 (C4′), 187.27 (C=O). IR ν_{max}/cm^{-1} 1654 (C=O), 1599 (C=C), 1259, 1017 (C–O), 3447 (C–N), 1504, 1416, 1367, 1223, 1181, 817, 610 (Ar) (KBr). Anal. Calcd for $C_{18}H_{14}N_2O_2$: C 74.47, H 4.86, N 9.65. Found: C 74.65, H 4.94, N 10.73. Yield: 68%.

5.2.2. (2E)-1-(1,3-benzodioxol-5-yl)-3-(quinoxalin-6-yl)prop-2-en-1-one (*2*)

Yellow solid, m.p. 225–226 °C; ^{1}H NMR (DMSO-d_6) δ 6.16 (s, 2H, $-OCH_2O-$), 7.09 (d, 1H, $J = 8.0$ Hz, H5′), 7.70 (s, 1H, H2′), 7.91 (d, 1H, $J = 8.0$ Hz, H6′), 7.92 (d, 1H, $J = 16.0$ Hz, Hα), 8.12 (d, 1H, $J = 8.0$ Hz, H3), 8.14 (d, 1H, $J = 16.0$ Hz, Hβ), 8.43 (d, 1H, $J = 8.0$ Hz, H4), 8.53 (s, 1H, H1), 8.95 (d, 2H, $J = 8.0$ Hz, H6, H7). ^{13}C NMR (DMSO-d_6) δ 102.85 ($-OCH_2O-$), 108.71 (C2′), 108.93 (C5′), 125.25 (Cα), 126.16 (C6′), 129.89 (C3), 130.26 (C1), 131.26 (C4), 132.74 (C1′), 137.34 (C2), 142.60 (Cβ), 143.13 (C4a), 143.84 (C8a), 146.95 (C6), 147.16 (C7), 148.78 (C3′), 152.50 (C4′), 187.56 (C=O). IR ν_{max}/cm^{-1} 1651 (C=O), 1595 (C=C), 1255, 1034 (C–O), 3440 (C–N), 3048, 2909, 1496, 1444, 1367, 1314, 1114, 969, 925, 793, 651 (Ar) (KBr). Anal. Calcd for $C_{18}H_{12}N_2O_3$: C 71.05, H 3.97, N 9.21. Found: C 71.76, H 4.04, N 10.94. Yield: 88%.

5.2.3. (2E)-1-(4-bromophenyl)-3-(quinoxalin-6-yl)prop-2-en-1-one (*3*)

Cream solid, m.p. 131–132 °C; ^{1}H NMR ($CDCl_3$) δ 7.69 (dd, 2H, $J = 8.0/1.0$ Hz, H3′, H5′), 7.70 (d, 1H, $J = 16.0$ Hz, Hα), 7.95 (d, 2H, $J = 8.0$ Hz, H2′, H6′), 8.02 (d, 1H, $J = 16.0$ Hz, Hβ), 8.08 (m, 1H, H3), 8.17 (d, 1H, $J = 8.0$ Hz, H4), 8.34 (s, 1H, H1), 8.89

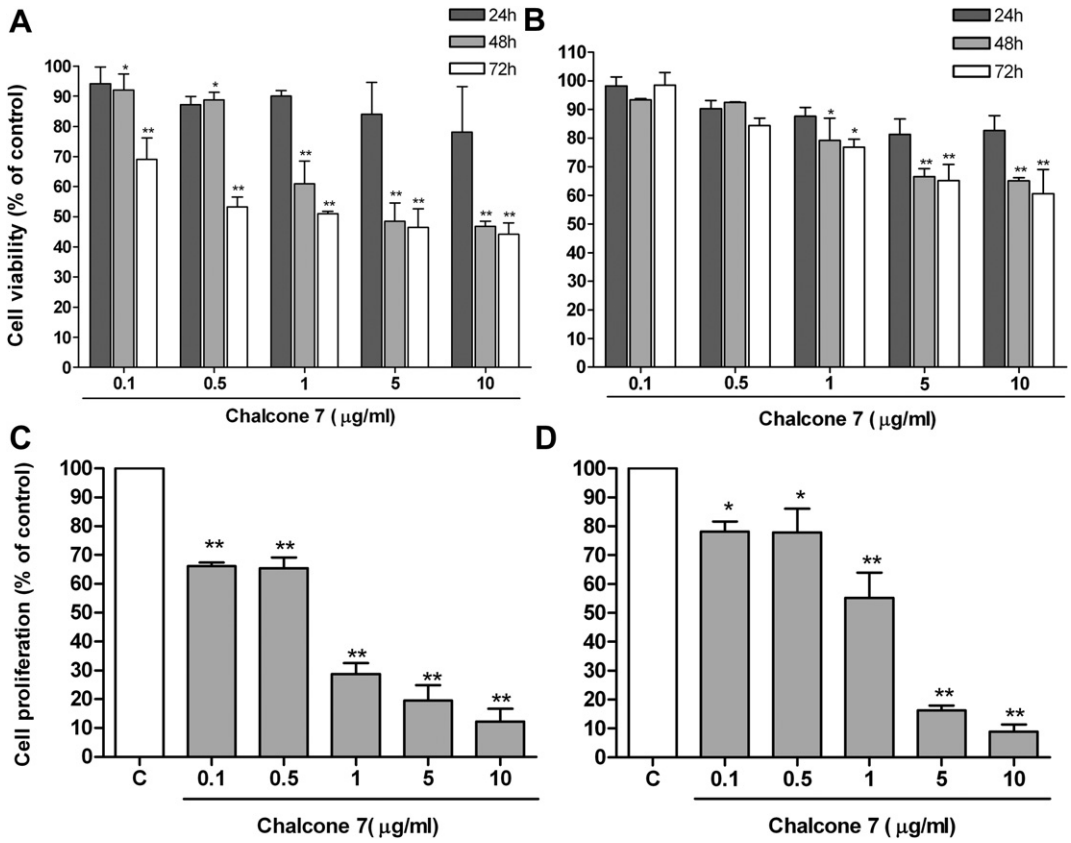

Fig. 5. Effects of compound **7** on glioma cell viability and proliferation. The viability of rat glioma cells C6 (A) and human glioma cells U-138 MG (B) were assessed by MTT assay. Cell viability is provided as the percentage of control group viability. Proliferation of C6 (C) and U-138 MG (D) cells was assessed by cell counting in a hemocytometer. Each column represents the mean of 3 independent experiments performed in triplicate and the lines indicate the standard error means. Data were analyzed by oneway ANOVA, followed by multiple comparisons post-hoc test (Tukey test). *Significantly different from the control group *($P < 0.05$); ** ($P < 0.01$).

(dd, 2H, J = 8.0/1.0 Hz, H6, H7). ^{13}C NMR ($CDCl_3$) δ 124.35 (Cα), 128.82 (C3), 129.46 (C4′), 130.27 (C2′, C6′), 130.73 (C3′, C5′), 131.17 (C1), 132.03 (C1′), 132.18 (C4), 137.14 (C2), 143.15 (Cβ, C4a), 144.14 (C8a), 146.46 (C6), 146.62 (C7), 188.31 (C═O). IR ν_{max}/cm^{-1} 1656 (C═O), 1589 (C═C), 1279, 1026 (C–O), 3447 (C–N), 1070 (C-Br), 3000, 1493, 1393, 1370, 1218, 1179, 1000, 866, 820, 516 (Ar) (KBr). Anal. Calcd for $C_{17}H_{11}BrN_2O$: C 60.20, H 3.27, N 8.26. Found: C 59.79, H 3.24, N 8.91. Yield: 72%.

*5.2.4. (2E)-1-phenyl-3-(quinoxalin-6-yl)-2-propen-1-one (**4**)*

Cream solid, m.p. 158–159 °C; ^{1}H NMR ($CDCl_3$) δ 7.60 (t, 2H, J = 8.0 Hz, H3′, H5′), 7.69 (t, 1H, J = 8.0 Hz, H4′), 8.03 (d, 1H, J = 16.0 Hz, Hα), 8.14 (d, 1H, J = 8.0 Hz, H10), 8.16 (d, 1H, J = 16.0 Hz, Hβ), 8.24 (d, 2H, J = 8.0 Hz, H2′, H6′), 8.40 (dd, 1H, J = 8.0/1.0 Hz, H9′), 8.48 (s, 1H, H2), 8.95 (dd, 2H, J = 8.0/1.0 Hz, H5, H6). ^{13}C NMR ($CDCl_3$) δ 124.77 (Cα), 128.81 (C3′, C5′), 128.97 (C2′, C6′, C10), 130.25 (C2), 131.01 (C9), 133.28 (C4′), 137.05 (C1′), 138.22 (C1), 142.57 (Cβ, C8), 143.34 (C3), 146.37 (C6), 146.56 (C5), 189.12 (C═O). IR ν_{max}/cm^{-1} 1656 (C═O), 1601 (C═C), 1279, 1018 (C–O), 3421 (C–N), 3053, 1497, 1440, 1367, 1308, 1216, 984, 864, 828, 770, 692, 659 (Ar) (KBr). Anal. Calcd for $C_{17}H_{12}N_2O$: C 78.44, H 4.65, N 10.76. Found: C 81.01, H 4.69, N 13.65. Yield: 41%.

*5.2.5. (2E)-1-(4-nitrophenyl)-3-(quinoxalin-6-yl)prop-2-en-1-one (**5**)*

Ocher solid, m.p. 235–236 °C; ^{1}H NMR ($CDCl_3$) δ 7.67 (d, 1H, J = 16.0 Hz, Hα), 8.02 (s, 1H, H1), 8.06 (d, 1H, J = 8.0 Hz, H3), 8.17 (d, 1H, J = 8.0 Hz, H4), 8.19 (d, 2H, J = 8.0 Hz, H2′, H6′), 8.36 (d, 2H, J = 8.0 Hz, H3′, H5′), 8.37 (d, 1H, J = 16.0 Hz, Hβ), 8.88 (d, 2H, J = 8.0 Hz, H6, H7). ^{13}C NMR ($CDCl_3$) δ 123.80 (Cα), 124.24 (C3′, C5′), 128.54 (C3), 129.75 (C2′, C6′), 130.71 (C1), 131.37 (C4), 136.20 (C2), 140.32 (C4a), 143.32 (C8a), 145.08 (C1′, Cβ), 146.23 (C6), 147.08 (C7), 153.96 (C4′), 188.72 (C═O). IR ν_{max}/cm^{-1} 1661 (C═O), 1588 (C═C), 1281, 1030 (C–O), 3418 (C–N), 1516, 1348, 847 (NO_2), 3107, 1315, 1215, 1105, 985, 826, 755, 706, 515 (Ar) (KBr). Anal. Calcd for $C_{17}H_{17}N_3O_3$: C 66.88, H 3.63, N 13.76. Found: C 65.63, H 3.36, N 13.08. Yield: 77%.

*5.2.6. (2E)-1-(2,5-dimethoxyphenyl)-3-(quinoxalin-6-yl)prop-2-en-1-one (**6**)*

Yellow solid, m.p. 142–144 °C; ^{1}H NMR ($CDCl_3$) δ 3.83 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 6.98 (d, 1H, J = 8.0 Hz, H3′), 7.08 (d, 1H, J = 8.0 Hz, H4′), 7.27 (s, 1H, H6′), 7.68 (d, 1H, J = 16.0 Hz, Hα), 7.86 (d, 1H, J = 16.0 Hz, Hβ), 8.03 (dd, 1H, J = 8.0/1.0 Hz, H3), 8.13 (d, 1H, J = 8.0 Hz, H4), 8.28 (s, 1H, H1), 8.87 (dd, 2H, J = 8.0/1.0 Hz, H6, H7). ^{13}C NMR ($CDCl_3$) δ 56.11 (OCH_3), 56.72 (OCH_3), 113.60 (C3′), 114.69 (C6′), 120.19 (C4′), 128.92 (Cα), 129.57 (C3), 129.36 (C1′), 130.32 (C1), 130.42 (C4), 137.32 (C2), 141.09 (Cβ, C4a), 143.40 (C8a), 145.64 (C6), 145.93 (C7), 153.15 (C5′), 153.93 (C2′), 191.83 (C═O). IR ν_{max}/cm^{-1} 1659 (C═O), 1588 (C═C), 1276, 1027 (C–O), 3444 (C–N), 3042, 2929, 2832, 1497, 1456, 1407, 1367, 1323, 1222, 1159, 977, 861, 818, 713 (Ar) (KBr). Anal. Calcd for $C_{19}H_{16}N_2O_3$: C 71.24, H 5.03, N 8.74. Found: C 70.56, H 4.41, N 8.60. Yield: 56%.

*5.2.7. (2E)-1-(3,4-dimethoxyphenyl)-3-(quinoxalin-6-yl)prop-2-en-1-one (**7**)*

Yellow solid, m.p. 143–144 °C; ^{1}H NMR ($CDCl_3$) δ 4.00 (s, 6H, OCH_3), 6.97 (d, 1H, J = 8.0 Hz, H5′), 7.67 (s, 1H, H2′), 7.74 (dd, 1H, J = 8.0/1.0 Hz, H6′), 7.77 (d, 1H, J = 16.0 Hz, Hα), 8.01 (d, 1H, J = 16.0 Hz, Hβ), 8.09 (dd, 1H, J = 8.0/1.0 Hz, H3), 8.16 (d, 1H, J = 8.0 Hz, H4), 8.34 (s, 1H, H1), 8.88 (dd, 2H, J = 8.0/1.0 Hz, H6, H76). ^{13}C NMR ($CDCl_3$) δ 56.11 (OCH_3), 56.17 (OCH_3), 110.03 (C5′),

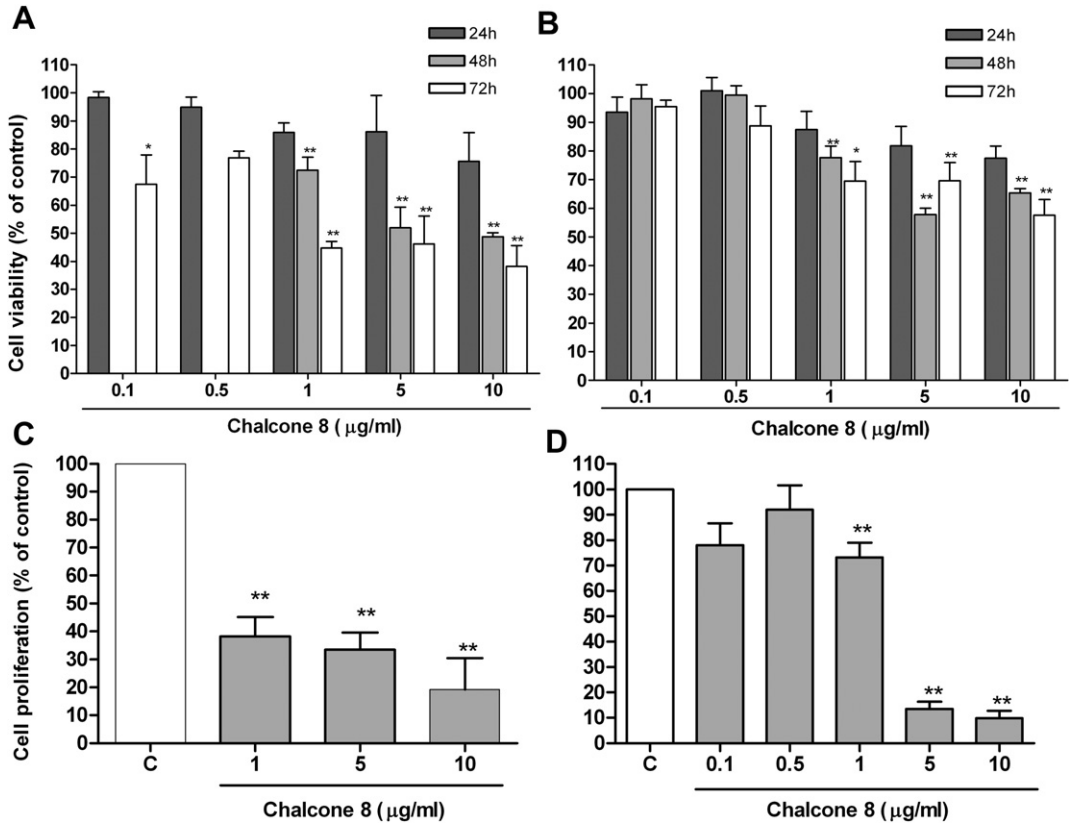

Fig. 6. Effects of compound **8** on glioma cell viability and proliferation. The viability of rat glioma cells C6 (A) and human glioma cells U-138 MG (B) were assessed by MTT assay. Cell viability is provided as the percentage of control group viability. Proliferation of C6 (C) and U-138 MG (D) cells was assessed by cell counting in a hemocytometer. Each column represents the mean of 3 independent experiments performed in triplicate and the lines indicate the standard error means. Data were analyzed by oneway ANOVA, followed by multiple comparisons post-hoc test (Tukey test). *Significantly different from the control group *($P < 0.05$); ** ($P < 0.01$).

110.75 (C2′), 123.24 (C6′), 124.19 (Cα), 128.64 (C3), 130.18 (C1′, C1, C4), 136.88 (C2), 142.10 (Cβ), 143.18 (C4a), 143.90 (C8a), 145.52 (C6), 145.80 (C7), 149.40 (C3′), 153.60 (C4′), 187.24 (C═O). IR ν_{max}/cm^{-1} 1654 (C═O), 1581 (C═C), 1281, 1022 (C–O), 3487 (C–N), 1518, 1453, 1419, 1358, 1316, 1161, 984, 918, 776, 718, 636 (Ar) (KBr). Anal. Calcd for $C_{19}H_{16}N_2O_3$: C 71.24, H 5.03, N 8.74. Found: C 70.98, H 4.33, N 8.11. Yield: 51%.

5.2.8. (2E)-1-(2,4-dimethoxyphenyl)-3-(quinoxalin-6-yl)prop-2-en-1-one (*8*)

Cream solid, m.p. 119–120 °C; ^{1}H NMR ($CDCl_3$) δ 3.90 (s, 3H, OCH_3), 3.96 (s, 3H, OCH_3), 6.53 (s, 1H, H3′), 6.60 (dd, 1H, J = 8.0/1.0 Hz, H5′), 7.77 (d, 1H, J = 16.0 Hz, Hα), 7.84 (d, 1H, J = 8.0 Hz, H6′), 7.88 (d, 1H, J = 16.0 Hz, Hβ), 8.04 (dd, 1H, J = 8.0/1.0 Hz, H3), 8.12 (d, 1H, J = 8.0 Hz, H4), 8.28 (s, 1H, H1), 8.86 (dd, 2H, J = 8.0/1.0 Hz, H6, H7). ^{13}C NMR ($CDCl_3$) δ 55.62 (OCH_3), 55.85 (OCH_3), 98.62 (C3′), 105.45 (C1′), 109.77 (C5′), 128.84 (Cα), 129.77 (C3), 129.85 (C1), 130.01 (C4), 133.23 (C6′), 137.46 (C2), 139.84 (Cβ), 143.21 (C4a), 143.75 (C8a), 145.28 (C6), 145.65 (C7), 160.69 (C2′), 164.63 (C4′), 189.71 (C═O). IR ν_{max}/cm^{-1} 1651 (C═O), 1609 (C═C), 1274, 1026 (C–O), 3447 (C–N), 2967, 1500, 1473, 1422, 1367, 1323, 1214, 1128, 974, 865, 821, 632 (Ar) (KBr). Anal. Calcd for $C_{19}H_{16}N2O_3$: C 71.24, H 5.03, N 8.74. Found: C 70.80, H 4.77, N 8.29. Yield: 93%.

5.3. Pharmacology

5.3.1. General cell culture procedures

U-138 MG human and C6 rat GBM cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA). The cells were grown in culture flasks in Dulbecco's Modified Eagle's medium (DMEM), supplemented with 15% and 5% fetal bovine serum (FBS), for U-138 MG and C6 lines, respectively. Culture cells were maintained at 37 °C, a minimum relative humidity of 95%, at atmosphere of 5% CO_2, and were allowed to reach confluence.

5.3.2. Cell viability assay

Glioma cell lines were seeded at 1×10^3 cells/well in DMEM/5% FBS for C6 line, or DMEM/15% FBS for U-138 MG in 96-well plates. They were then exposed to increasing concentrations (0.1, 0.5, 1, 5 and 10 μg/ml) of the eight quinoxaline-derived chlacones, for 24 h, 48 h or 72 h. Parallel control experiments were carried out with the addition of 5% and 15% FBS (cell viability control) or DMSO (vehicle control, 0.01%), in the absence of chalcones. The glioma cell lines were also treated with the PI3Kγ inhibitor, AS605240, at the concentrations of 100 nM, 10 μM and 30 μM, for 48 h.

Cell viability was evaluated by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [56]. This assay measures the activity of cellular dehidrogenases (mainly from mitochondria) and, indirectly, the cell viability, even of the spontaneously detached cells in the culture medium. The method is based on the reduction of a tetrazolium bromide salt (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]). It provides a quantitative measure of the number of metabolically viable cells. The results were expressed as the percentage of cell viability in relation to the control.

5.3.3. Cell counting

U-138 MG and C6 cells were seeded at 1×10^4 and 5×10^3 cells/well, respectively, in appropriate medium. Both lineages were

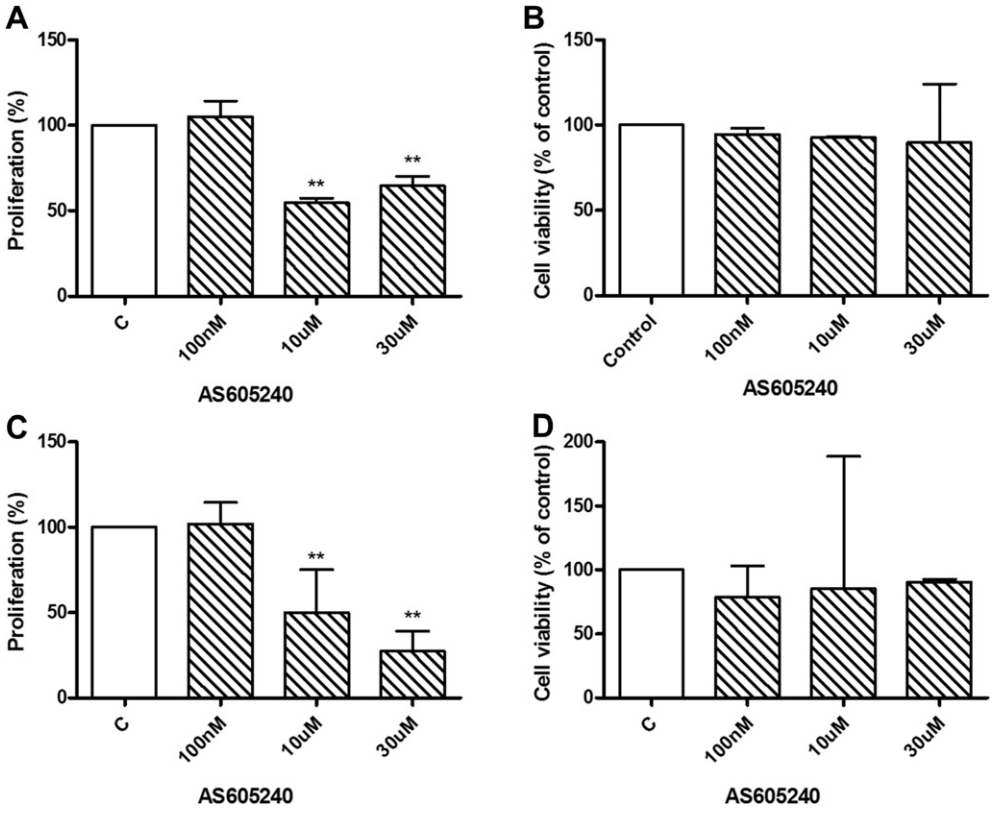

Fig. 7. Effects of AS605240 on glioma cell viability and proliferation. The viability of rat glioma cells C6 (A) and human glioma cells U-138 MG (B) were assessed by MTT assay. Cell viability is provided as the percentage of control group viability. Proliferation of C6 (C) and U-138 MG (D) cells was assessed by cell counting in a hemocytometer. Each column represents the mean of 3 independent experiments performed in triplicate and the lines indicate the standard error means. Data were analyzed by oneway ANOVA, followed by multiple comparisons post-hoc test (Tukey test). *Significantly different from the control group *($P < 0.05$); ** ($P < 0.01$).

seeded in 24-well plates and allowed to grow for 24 h. The medium was changed prior to treatment with the different chalcones (0.1, 0.5, 1, 5 and 10 μg/ml) and also with AS605240 (100 nM, 10 μM and 30 μM). After 48 h of treatment the medium was removed, the cells were washed with calcium- and magnesium-free phosphate-buffered saline (CMF), and 200 μl of 0.5% trypsin/EDTA solution (Gibco, BRL) was added to detach the cells. After that, the cells were counted in a Neubauer chamber.

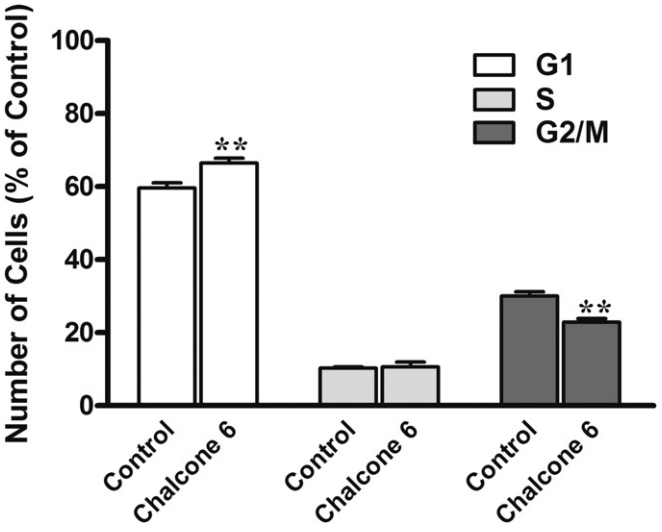

Fig. 8. Effect of compound **6** treatment on cell cycle distribution. C6 cells were treated for 6, 12 and 24 h with dimethylsulfoxide (control) or chalcone **6** (2.5 μg/mL), collected, fixed, stained with PSSI solution and subjected to flow cytometry cell cycle analysis. Values are the relative number of cells in the sub-G1/G1, S and G2/M phases of cell cycle. Each column represents the mean of 6 independent experiments performed in triplicate and the lines indicate the standard error means. Data were analyzed by oneway ANOVA, followed by Bonferroni's post-hoc test. *Significantly different from the control group *($P < 0.05$); ** ($P < 0.01$).

5.3.4. Cell cycle analysis

The effects of compound **6** on C6 cell cycle phase distribution was assessed using flow cytometry. The cells (3×10^5 cells per well) were cultured in triplicate in 6-well plates. After 6, 12 or 24 h of incubation, the cells were treated with vehicle (0.1% DMSO) or compound **6** (2.5 μg/ml). Cells were harvested, fixed with 70% ethanol and stained with PSSI solution (Triton, RNAse, Propidium Iodide and PBS). Data acquisition and analysis were performed on flow cytometer (Guava EasyCyte 8HT Flow Cytometry System, Millipore Corporation, MA, USA), and data from 10,000 cells were collected for each data file. Cell cycle analysis was performed with CellQuest software (Becton Dickinson, Canada, Inc.).

5.3.5. PI3K and ERK 1/2 MAP-kinase analysis

C6 rat glioma cells (2×10^5 cells per well) were plated in 6-well plates, with 5% FBS DMEM medium and allowed to grow for 24 h. The cells remained incubated for more 24 h with DMEM 0.5%, to stop the cell cycle. Cells were treated with the compound **6** (5 μg/ml), AS605240 (30 μM), or vehicle (0.1% DMSO), in the presence of FBS 5% (used to induce protein activation). After 15 min and 30 min of incubation, the cells were detached with 0.5% trypsin/EDTA solution (Gibco, BRL), and then incubated with Phosflow Fix Buffer (BD) for 10 min, and permeabilizated with Phosflow Perm Buffer III (BD). The antibodies for AKT and ERK 1/2 were incubated during 30 min. Data acquisition and analysis was performed on flow cytometer (Facscanto) and FlowJo 7.6.3software.

	AKT (% activation)	
	15 min	30 min
Negative Control	0	0
DMEM FBS 5%	438	486
AS605240	31	41
Compound 6	60	46

	ERK1/2 (% activation)	
	15 min	30 min
Negative Control	0	0
DMEM FBS 5%	363	681
AS605240	243	244
Compound 6	585	991

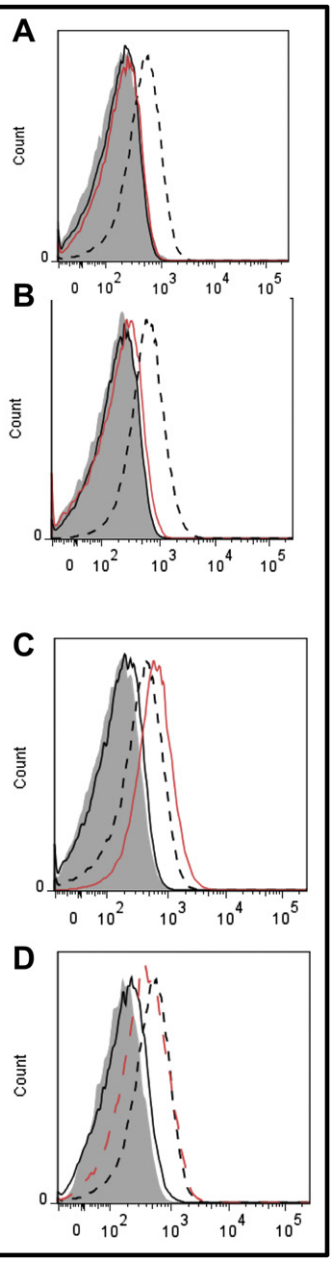

Fig. 9. Effects of compound **6** (5 μg/ml, red line) and AS605240 (30 μM, black line) on AKT (A and B) and ERK 1/2 MAP-kinase (C and D) activation, following addition of cell culture medium supplemented with 5% FBS. Cells were treated with compound **6** (5 μg/ml) or AS605240 (30 μM) during 15 (A and C) and 30 min (*B* and D). The antibodies for AKT and ERK 1/2 were incubated during 30 min and the cells were subjected to flow cytometry cell cycle analysis. The gray line represents the negative control, and the dotted line indicates the positive control with 5% FBS plus DMSO 0.01%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5.3.6. Statistical analysis

The percentage of inhibition is presented as mean ± standard error mean. The estimated IC_{50} values are provided as the geometric means accompanied by the 95% confidence limit. Data were analyzed by oneway analysis of variance (ANOVA), followed by Tukey's or Bonferroni's tests, depending on the experimental protocol, using the software GraphPad Prism® 4.02. *P* values less than 0.05 ($P < 0.05$) were considered as indicative of significance.

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