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Inhibition of rat glioma cell migration and proliferation by a calix[8]arene scaffold exposing multiple GlcNAc and ureido functionalities

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Abstract

β1,4-Galactosyltransferases (β1,4-GalTase) exposed on the cell surface are involved in cell migration. Specifically, β1,4-GalTase V is highly expressed in glioma and promotes invasion, growth, and survival of glioma cells. A glycocalix[8]arene exposing *N*-acetylglucosamine (GlcNAc) residues (compound 1) inhibited rat C6 glioma cell migration as assessed in a scratch wound model. This effect was related to inhibition of focal adhesion kinase phosphorylation, measured by western blot analysis, and specifically observed in the area bordering the scratch wound. Compound 1 inhibited also C6 cell proliferation, an effect unrelated to its ability to interact with GalTase as it was mimicked by different calix[8]arene derivatives, all characterized by multivalency and ureido groups. Compound 1 did not induce apoptotic death, but caused a different distribution of C6 cells within the cell cycle. The results here reported identify compound 1 as a molecule able to exert inhibitory effects on C6 cell migration and proliferation, independently, because of distinct components in its structure.

Keywords: adhesion molecule, calixarene glycoconjugate, cell growth, galactosyl transferase.

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Cell migration is a major event during morphogenesis and development, but appears crucial for tumor spreading, metastasis, and invasiveness. It involves the interaction between cell surface components and molecules of the extracellular matrix or expressed by neighboring cells. In this regard, much attention has been given to cell adhesion molecules whose involvement in migration processes has been largely demonstrated (Hakomori 1996). More recently, however, increasing interest has been focused on the role of glycosyltransferases (Shur 1993; Guo *et al.* 2001; Sato *et al.* 2001). Although generally viewed as intracellular enzymes localized in the trans-Golgi complex and responsible for the synthesis of oligosaccharides, selected glycosyltransferases are expressed on the cell surface. Among these, galactosyltransferases (GalTase) such as β1-4 GalTase I, act as adhesion molecules and upon binding with terminal *N*-acetylglucosamine (GlcNAc) residues (Shur *et al.* 1998), mediate cell-matrix interaction (Begovac *et al.* 1991; Shur 1993; Shur *et al.* 1998) and migration (Appeddu and Shur 1994). The importance of β1-4 GalTase in tumor spreading and invasiveness is supported by its enhanced expression on the surface of metastatic cells (Penno *et al.* 1989; Passaniti and Hurt 1990), with a good correlation between β1-4 GalTase activity and the degree of *in vitro* invasiveness (Penno *et al.* 1989). In addition, modulation of the expression of surface β1-4 GalTase I is able to affect the invasive phenotype of murine melanoma cells *in vitro* as well as their metastatic potential *in vivo* (Johnson and Shur 1999).

Although malignant gliomas do not metastasize, they exhibit very high invasiveness and, interestingly, a specific

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Abbreviations used: β1,4-GalTase, β1,4-galactosyltransferases; Ara C, cytosine arabinoside; BrdU, bromodeoxyuridine; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; FAK, focal adhesion kinase; FCS, fetal calf serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline.

β1-4 GalTase (GalTase V; EC 2.41.38), is highly expressed in glioma (Xu *et al.* 2001; Jiang *et al.* 2006a) and its levels increase upon malignant transformation being correlated with the staging of the glioma tumor (Xu *et al.* 2001). β1-4 GalT V galactosylates GlcNAc residue(s) of oligosaccharides with the GlcNAcβ1 → 6 (GlcNAcβ1 → 2)Man branch to form highly branched *N*-linked oligosaccharides, which are markers of glioma and are involved in migration.

Targeting β1–4 GalTase function by competitive inhibitors may thus represent an alternative approach to control also glioma tumor migration and invasiveness.

In this context, we decided to investigate the potential of a synthetic multivalent glycocalix[8]arene 1 (Consoli *et al.* 2003) as perturbant of the migration of glioma cells, using rat C6 glioma cells as an experimental model.

Calix[n]arenes, synthetically versatile macrocycles characterized by [1_n] metacyclophane units (Gutsche 1998; Asfari *et al.* 2001), because of the low toxicity of their derivatives (Da Silva *et al.* 2004), have been widely exploited as molecular platforms for the design of fully synthetic multivalent constructs potentially useful for biomedical applications. Calixarene derivatives have been quoted for their enzyme inhibitor (Mecca *et al.* 2004; Francese *et al.* 2005; Consoli *et al.* 2007), anticoagulant and antithrombotic (Hwang *et al.* 1995), antiviral (Motornaya *et al.* 2006), and antimicrobial (Lamartine *et al.* 2002; Chen *et al.* 2006a; Mourer *et al.* 2006) properties. Recently, calixarene derivatives also proved promising candidates for applications in anticancer therapy. Appropriately functionalized calixarenes exhibit control of cell proliferation in various cell lines *in vitro* (Sun *et al.* 2005; Dings *et al.* 2006) as well as in animal models *in vivo* (Blaskovich *et al.* 2000; Sun *et al.* 2005; Dings *et al.* 2006). Calixarenes have also successfully been used as the backbone of growth factors binders (Blaskovich *et al.* 2000; Sun *et al.* 2005; Zhou *et al.* 2006) that, by preventing specifically platelet-derived growth factor tyrosine phosphorylation, exert antiangiogenic and anticancer activity (Blaskovich *et al.* 2000). A GlcNAc bearing calix[4]arene has been shown to activate natural killer (NK) cells thus contributing to natural killer (NK) cell-mediated antitumor immune response (Krěnec *et al.* 2007), and we have shaped an anticancer vaccine candidate built on a calix[4]arene scaffold that stimulates the production of anti-Tn antigen IgG in mice (Geraci *et al.* 2008).

The ability of glycocalixarene 1 to recognize and bind cognate carbohydrate protein receptors has already been reported. In particular, it has been demonstrated that compound 1 binds wheat germ agglutinin with high specificity and affinity so to inhibit human erythrocyte agglutination *in vitro* (Consoli *et al.* 2004).

We now report that treatment with 1 slows down C6 cell migration and has a significant inhibitory effect also on cell proliferation.

Materials and methods

Reagents and instruments

All chemical reagents and solvents for compound synthesis were obtained from commercial sources and were used without purification. Analytical TLCs were performed on 0.25 mm silica 60 coated aluminum foils with F-254 indicator (Merck, KgaA, Darmstadt, Germany). Preparative TLC were performed on silica gel 60 (230–400 mesh, E. Merck). ^{1}H NMR (400.13 MHz) and ^{13}C NMR (100.61 MHz) spectra were acquired on a Bruker Avance™ 400 spectrometer, BioSpin GmbH, Rheinstetten, Germany. Chemical shifts (δ) are expressed in parts per million. Spectra were referenced to the residual proton solvent peaks; coupling constant (*J*) values are given in Hz.

Synthesis, structural characterization, and sample preparation

GlcNAc-thioureido-calix[8]arene **1** *(compound* **1***) and octamino-calix[8]arene* **2**

Compounds 1 and 2 were prepared following the synthetic procedures reported in literature (Consoli *et al.* 2003). Compound 1 and the synthetic pathways of other compounds used are reported in Fig. 1 and Scheme 1.

Butyl-ureido-calix[8]arene **(3)**

To a solution of compound 2 (104 mg, 0.08 mmol) in 8 mL of dry $CHCl_3$, butyl-isocyanate (180 μL, 1.6 mmol) was added. The reaction mixture was stirred and refluxed for 12 h. The solvent was removed under vacuum and pure compound 3 was obtained in 85% yield (145 mg) after purification by preparative TLC (MeOH/CH_2Cl_2, v : v/5 : 95). ^{1}H NMR (dimethylsulfoxide; DMSO-d_6, 297 K) δ 0.70 (t, 24 H, J = 7.2 Hz, 8 × $OCH_2CH_2CH_3$), 0.84 (t, 24 H, J = 7.1 Hz, 8 × $NCH_2CH_2CH_2CH_3$), 1.11–1.33 (overlapped m, 48 H, 8 × $OCH_2CH_2CH_3$, 8 × $NCH_2CH_2CH_2CH_3$, 8 × $NCH_2CH_2CH_2CH_3$), 1.46 (m, 16 H, J = 6.8 Hz, 8 × $NCH_2CH_2CH_2CH_3$), 2.99 (br t, 16 H, 8 × $OCH_2CH_2CH_3$), 3.80 (br s, 16 H,

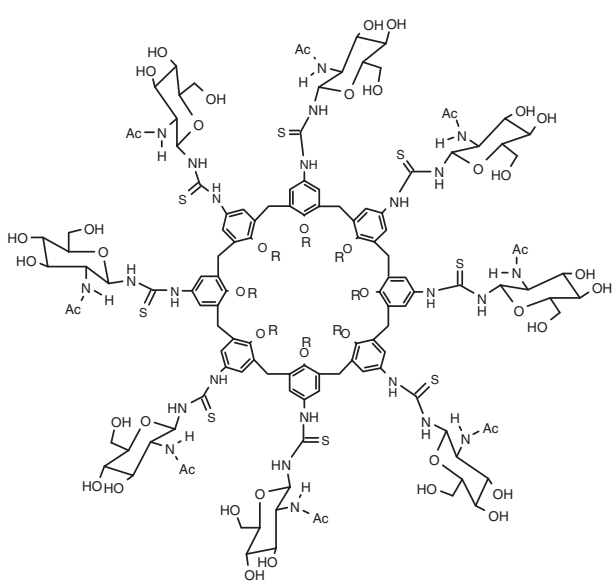
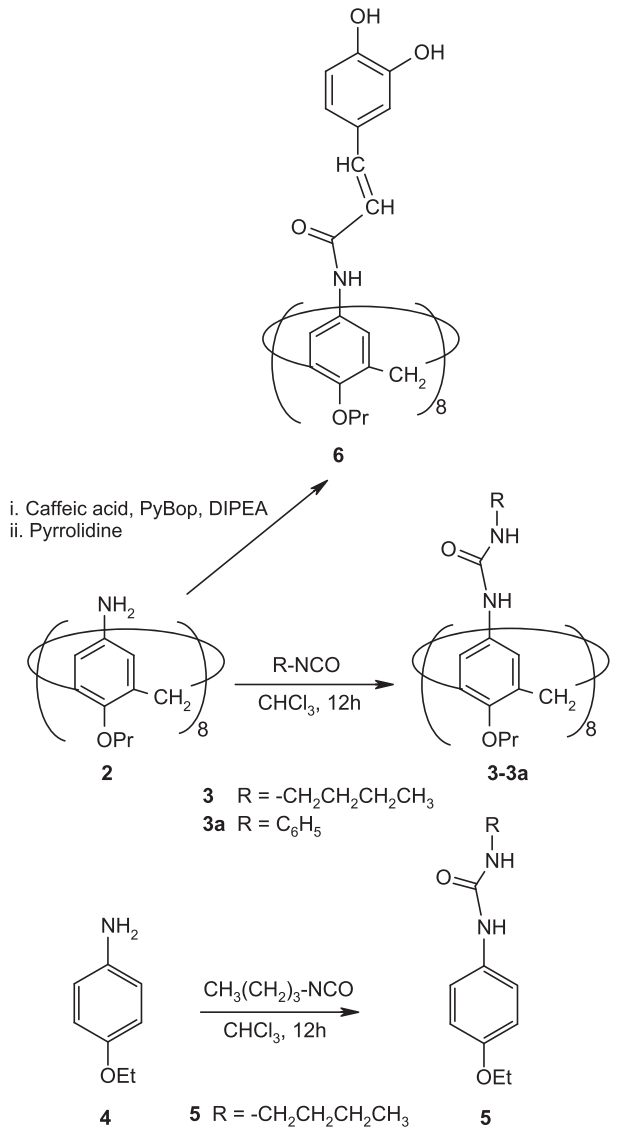


Fig. 1 Molecular structure of compound **1**.



Scheme 1 Synthetic procedures to obtain compounds **3–6**. [Correction added after online publication 15 October 2008: Revised Scheme 1 and caption inserted.]

8 × ArCH_2Ar), 5.86 (s, 8 H, 8 × NH-CH_2), 6.95 (s, 16 H, 16 × ArH), 8.07 (s, 8 H, 8 × Ar-NH).

Phenyl-ureido-calix[8]arene (3a)

To a solution of compound 2 (100 mg, 0.08 mmol) in 13 mL of dry $CHCl_3$, phenyl-isocyanate (73 µL, 0.67 mmol) was added. The reaction mixture was stirred and refluxed for 8 h. The solvent was removed under vacuum and pure compound 3 was obtained in 85% yield (143 mg) after crystallization MeOH/CH_2Cl_2. ^{1}H NMR (DMSO-d_6, 297 K) δ 0.71 (t, 24 H, J = 7.2 Hz, 8 × $OCH_2CH_2CH_3$), 1.48 (br m, 16 H, 8 × $OCH_2CH_2CH_3$), 3.43 (br t, 16 H, 8 × $OCH_2CH_2CH_3$), 3.88 (br s, 16 H, 8 × ArCH_2Ar), 6.90 (t, 8 H, J = 7.3 Hz, 8 × ArH), 7.08 (s, 16 H, 16 × ArH), 7.20 (t, 8 H, J = 7.7 Hz, 8 × ArH), 7.35 (d, 8 H, J = 8.3 Hz, 8 × ArH), 8.40 (s, 8 H, 8 × Ar-NH), 8.41 (s, 8 H, 8 × Ar-NH).

Butyl-ureido-p-phenetidine (5)

p-Phenetidine 4 (200 µL, 1.55 mmol) was dissolved in 5 mL of dry $CHCl_3$, then butyl-isothiocyanate (200 µL, 1.77 mmol) was added. The reaction mixture was stirred at 23°C for 12 h. After removal of the solvent under vacuum, pure compound 5 was obtained by crystallization in 88% yield. ^{1}H NMR (MeOD, 297 K) δ 1.08 (t, 3 H, J = 7.3 Hz, OCH_2CH_3), 1.45–1.54 (overlapped m, 7 H, $NCH_2CH_2CH_2CH_3$), 1.61 (m, 2 H, J = 7.2 Hz, $NCH_2CH_2CH_2CH_3$), 4.11 (q, 2 H, J = 6.9 Hz, OCH_2CH_3), 6.94 (d, 2 H, J = 8.6 Hz, 2 × ArH), 7.32 (d, 2 H, J = 8.6 Hz, 2 × ArH).

Caffeoyl-calix[8]arene (6)

A solution of caffeic acid (89.7 mg, 0.34 mmol), benzotriazol-1-yl-oxytripyrrolldinophosphoniumhexafluorophosphate (PyBOP) (177 mg, 0.34 mmol), and N, N-Diisopropylethylamine (DIPEA) (50 µL) in dry Dimethylformamide (DMF) (2.5 mL) was stirred for 5 min at 0°C. Octa-aminocalix[8]arene 2 (50 mg, 0.038 mmol) was added and the solution was stirred for 30 min at 0°C and for 18 h at 23°C. The reaction mixture was poured into 15 mL of 1 N HCl. The insoluble material was collected by filtration, washed with water and methanol, dried and purified by TLC on silica gel (97 : 3 CH_2Cl_2/MeOH) to give the amide intermediate in 20% yield. Amide derivative (25 mg) was dissolved in pyrrolidine (100 µL) at 23°C. After 1 h, a solution of 1 N HCl (10 mL) was added and the insoluble material was collected by filtration and dried to give de-acetylated compounds 6. (MeOD, 297 K) δ 0.78 (br t, 24 H, 8 × $OCH_2CH_2CH_3$), 1.57 (br m, 16 H, 8 × $OCH_2CH_2CH_3$), 3.55 (br t, 16 H, 8 × $OCH_2CH_2CH_3$), 4.00 (br s, 16 H, 8 × $ArCH_2Ar$), 6.44 (d, 8 H, J = 15.6 Hz, 8 × CH), 6.70 (d, 8 H, J = 8.3 Hz, 8 × ArH), 6.85 (d, 8 H, J = 8.3 Hz, 8 × ArH), 6.97 (s, 8 H, ArH), 7.33 (s, 16 H, ArH), 7.41 (d, 8 H, J = 15.6 Hz, 8 × CH).

Stock solutions of the synthesized compounds were prepared in DMSO and stored at 4°C. Subsequent dilutions were made in Dulbecco's modified Eagle's medium (DMEM) just before treatment.

Cell culture

C6 cells were cultured in a monolayer in 25-cm^2 plastic flasks. Cells were maintained at 5% CO_2 and 37°C in DMEM supplemented with 1% fetal calf serum (FCS) and 1 : 200 penicillin/streptomycin (all from Invitrogen, Milan, Italy). All plastics were from Falcon, Becton Dickinson Labware Europe, Milan, Italy.

Cell proliferation assays

C6 cell proliferation was measured with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) colorimetric assay based on the conversion of MTT into blue formazan by mitochondrial activity of viable cells. C6 cells were placed in 96- and 24-well culture plates; after 24 h cells were treated with the tested compounds for 48 h.

Bromodeoxyuridine incorporation assay

Cells were seeded at a density of 1 × 10^5 cells/well, grown for 24 h in 1% FCS, then treated for additional 24 h with compound 1 in the presence of 10 µM bromodeoxyuridine (BrdU) (Sigma-Aldrich). After fixation in 2% *p*-formaldehyde, cells were incubated with anti-BrdU antibody (1 : 2000; Chemicon, Temecula, CA, USA) overnight and a byotinilated secondary anti-mouse (1 : 200) for 1 h.

Diaminobenzidine was then used for visualization. Immunopositive cells were counted in five, randomly selected, fields per well.

Hoechst staining for nuclear fragmentation

Cells were seeded in 35-mm dish and allowed to grow overnight in complete medium. On the following day, cells were treated with the tested compounds for 48 h. After fixation with 4% formaldehyde for 30 min on ice, and permeabilization with 0.1% Triton X-100 for 5 min, cells were incubated with 0.8 μg/mL Hoechst (Sigma-Aldrich), at 37°C for 30 min. After repeated washing in H_2O, cells were visualized with a fluorescent microscope and observed with a 20× objective.

Cell cycle analysis

Cell cycle distribution was evaluated by flow cytometric analysis of the DNA content following staining with propidium iodide. Briefly, cells exponentially grown to approximately 70% confluence were exposed to compound 1 (10 μM) for 48 h, harvested, washed briefly in ice-cold phosphate-buffered saline (PBS), and fixed in 70% ethanol. After repeated washings, cells were incubated with 100 μg/mL Rnase A (Sigma-Aldrich) for 60 min at 37°C and DNA was stained with 50 μg/mL propidium iodide (Sigma-Aldrich). Cell debris was gated out based on light scatter evaluation and analysis was restricted to cells with diploid, tetraploid, and hypodiploid DNA content. Cell cycle distribution was analyzed by the Multicycle AV software program (Phoenix Flow System, San Diego, CA, USA).

Cell migration assay

Scratch wound closure assay

C6 cells were plated in 24-well cultures plates and grown in complete medium for 24 h; when indicated, cells were pre-treated with cytosine arabinoside (Ara C; 5 μM; Sigma-Aldrich) for 4 h. Monolayers were scratched using a 200 μL sterile plastic pipette tip as previously described (Lind *et al.* 2006), washed three times and then cultured in DMEM–FCS 1% in the presence or absence of the tested compounds. Scratch wound closure was monitored by phase microscopy using a 10× objective at 0, 18, 24, and 48 h.

Transwell migration assay

In parallel, cell migration was evaluated using a six-well Transwell chamber (Corning, Lowell, MA, USA). For this purpose, cells were plated in the upper chamber of a 8-μm pore size insert in the six-well plate and allowed to migrate toward a 10% FCS containing medium present in the lower chamber. Cells were incubated for 24 h, then the non-migrating cells of the upper chamber were removed with the aid of a cotton swab and the cells that had migrated to the lower surface of the membrane were stained with hematoxylin and counted.

Western blot

For assessment of protein expression, cells were washed in cold PBS, harvested with trypsin and placed in lysis buffer for 60 min at 4°C. Protein concentrations were quantified using by biofotometric analysis. Equal amounts of proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and electro-transferred onto a nitrocellulose membrane (Hybond ECL; Amersham, Milan, Italy). Non-specific antibody binding was blocked by incubation with 1% non-fat dry milk in PBS–Tween (0.1%) for 4 h at 37°C. Blots were then probed with rabbit anti-phospho-focal adhesion kinase (FAK) (pTyr397), 1 : 1000 (Sigma-Aldrich), overnight at 4°C. After repeated washing, membranes were incubated with a 1 : 7000 dilution of horseradish peroxidase-linked anti-rabbit antibody (Santa Cruz Biotech. Inc, Santa Cruz, CA, USA) at 23°C for 1 h. Specific bands were visualized by Supersignal West Pico Chemiluminescence detection system (Pierce, Milan, Italy). After stripping, blots were re-probed with rabbit anti-FAK antibody (1 : 600; Santa Cruz Biotech. Inc) to ensure equal loading and transfer of proteins.

Immunocytochemistry

Cells were fixed with 2% *p*-formaldehyde for 30 min on ice and permeabilized with 0.1% Triton X-100 for 10 min at 4°C. Non-specific immunostaining was prevented by blocking for 30 min with 3% bovine serum albumin at 37°C prior to overnight incubation at 4°C with rabbit anti-pFAK antibody (1 : 500; Sigma-Aldrich). For diaminobenzidine-based detection, cells were incubated for 1 h with biotinylated anti-rabbit secondary antibody (1 : 200), and staining was revealed with the ABC detection system (all from Vector Laboratories, Burlingame, CA, USA).

Statistical analysis

Data were analyzed by one-way ANOVA followed by Newman–Keuls test for significance or by Student's *t*-test as indicated in figure legends. A value of $p < 0.05$ was considered significant.

Results

The ability of compound 1 to affect glioma cell migration was tested by means of a scratch-wound model in which a subconfluent C6 cell monolayer was scratched with a pipette tip and treated, immediately after, with compound 1 (10 μM). Images were taken from the same field at time zero and after 18, 24, and 48 h (Fig. 2b); the edge of the cell-free area was outlined and total free areas for each condition were measured by the aid of a specific software. Treatment with 1 slowed down wound closure compared with control, an effect that became more pronounced and significant after 24 h ($p < 0.05$) (Fig. 2a). GlcNAc *per se*, was able to modify cell migration, but a slight first significant effect was detectable only at very high concentrations (starting at 80 μM; not shown) and after prolonged incubation time (not before 48 h; not shown), whereas the analogous butyl-ureido-calix[8]arene derivative 3 lacking GlcNAc residues, did not exhibit any activity (Fig. 2a). Similar results were obtained using a transwell cell migration assay that confirmed the ability of compound 1 to inhibit cell migration as shown by the lower number of cells migrated toward a serum-rich medium used as chemoattractant (8.2 + 0.6 and 6.5 + 0.3 cells counted in five different fields/well in control and compound 1-treated cultures, respectively).

Interestingly, multiple scratches on the C6 cell layer induced enhanced FAK phosphorylation after 2 h, a time that may be coincident with starting of migration toward the

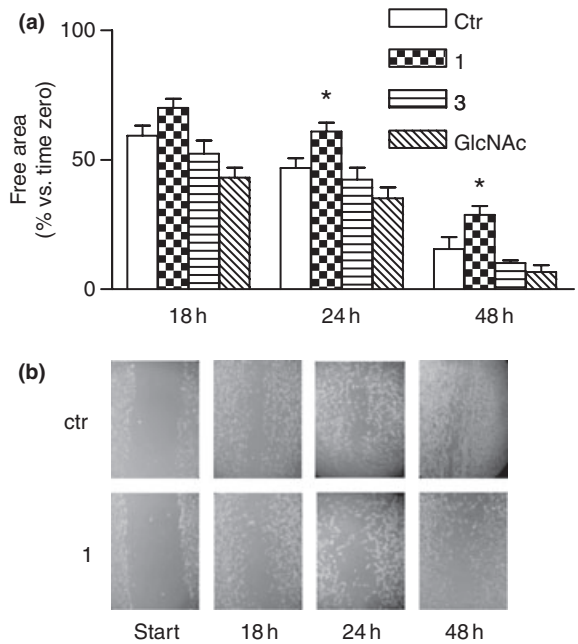


Fig. 2 Treatment with compound **1** reduced scratch-wound closure in C6 glioma cells. A subconfluent C6 cell monolayer was scratched with a pipette tip and treated with compound **1**, butyl-ureido-calix[8]arene (compound **3**), and GlcNAc all at the concentration of 10 μM (a). Cells were examined by phase contrast microscopy and wound areas were measured at 0, 18, 24, and 48 h after scratching. Representative photomicrographs of control and compound **1**-treated cells at the indicated time points is shown in (b). Bars represent mean ± SE of three to five independent experiments each run in triplicates; $^*p < 0.05$ versus control by Student's *t*-test.

cell-free area (Fig. 3a, left panel). Treatment with 1 (10 μM) for 2 h reduced FAK phosphorylation, as measured by western blot analysis (Fig. 3a, right panel) and immunocytochemistry (Fig. 3c), whereas no effect was detected at 30 min (Fig. 3a and b, right panel). Specificity of this effect was proved by the fact that derivative 3 did not modify the level of FAK phosphorylation (Fig. 3a).

To evaluate specifically the contribution of the migratory component in the effect of compound 1 and to exclude a concomitant activity on cell proliferation, cells were pretreated with 5 μM of the anti-mitotic compound Ara C for 4 h prior to scratching, and then with compound 1 in the presence or absence of Ara C. Such treatment produced complete blockade of cell proliferation as revealed by MTT assay [0.904 + 0.075 and 0.955 + 0.042 (optical density values), respectively, at 6 and 48 h following treatment with 5 μM Ara C].

Measurements of cell-free areas were carried out after 18, 24, and 48 h of treatment. Combined treatment with compound 1 (10 μM) and Ara C (5 μM) significantly slowed down and inhibited closure of the gap compared with treatment with Ara C alone ($p < 0.05$), confirming a direct effect of 1 on cell migration (Fig. 4).

As cells exposed to Ara C alone exhibited a slower repairing process compared with control, the possibility that 1 exerted an action also on cell proliferation was tested. Treatment with compound 1 (1–10 μM) reduced the growth of C6 glioma cells as detected by the MTT proliferation assay (Fig. 5a) and BrdU incorporation (34 ± 3.1, 22 ± 1.8, and 23 ± 2.6 immunopositive cells/field for control, compound 1- and 3-treated C6 cells, respectively). Inhibition of cell proliferation by compound 1 was not accompanied by induction of apoptotic cell death as demonstrated by the lack of nuclear fragmentation following Hoechst staining (Fig. 5b) and assessment of the pre-diploid population at cell cycle analysis (about 4% of the total cell population under both basal and compound 1-treated conditions), but rather to modifications of cell proliferation as shown by changes in the distribution of cells during different phases of cell cycle. Specifically, an accumulation of cells in S phase was observed (Fig. 5c), suggesting modifications of cell cycle progression with cells loosing the ability to complete their duplication.

To establish the specificity of compound 1 effect, related compounds of our library (2, 3, 3a, 5, and 6, Scheme 1) were tested. Unexpectedly, butyl-ureido-calix[8]arene derivative 3 that was devoid of any activity on cell migration, mimicked 1 in reducing C6 cell proliferation (Fig. 5d), pointing out that GlcNAc moieties are not responsible for the observed inhibitory effect.

Accordingly, increasing concentrations (1–80 μM) of GlcNAc sugar alone did not show any activity (Fig. 5d). Amino-calix[8]arene 2 lacking both the sugar and the ureido moieties also failed to affect C6 cell growth (Fig. 5d), suggesting that the calixarene skeleton alone does not elicit any inhibitory activity. In contrast, similarly to butyl-ureido-calix[8]arene derivative 3, the purposely synthesized phenyl-ureido-calix[8]arene 3a (Fig. 5d) affected cell proliferation, giving clear proof that the ureido groups are involved in this process. Accordingly, caffeoyl-calix[8]arene derivative 6, lacking the ureido portion but provided with additional polyphenolic moieties, known for antiproliferative properties, also failed to modify cell proliferation (Fig. 5d). In addition, compound 5, representing the monovalent non-cyclic analogous of 3, did not show any effect (Fig. 5d) underlining the importance of multiple ureido units arranged onto a calix[8]arene skeleton for the observed inhibitory effect on C6 cell proliferation.

Discussion

Glioma is the most common of primary brain tumors. They are characterized by high invasiveness and often drug resistance that can make chemotherapy unsuccessful (DeAngelis 2001). The expression of β1,4-GalTase V, one of several members of the GalTase family, is increased in the process of glioma development (Xu *et al.* 2001), and its ectopic expression promotes the invasion, growth and

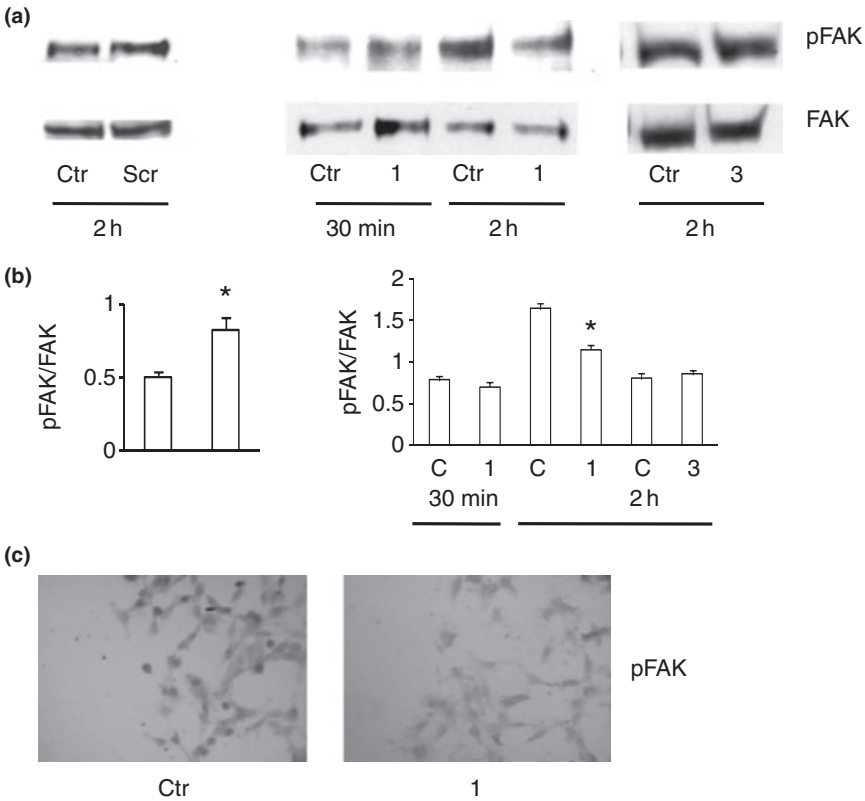


Fig. 3 Treatment with compound 1 reduced FAK phosphorylation. The C6 cell layer was scratched (multiple scratches) and treated with GlcNAc-glyco-calix[8]arene (compound **1**; 10 μM) for 30 min and 2 h. Western blot analysis (a) shows an increased FAK phosphorylation 2 h after scratching (scr; left panel), that was reduced by treatment with compound **1** only at 2 h (middle panel). Compound **3** was ineffective (right panel). Bands are from one blot representative of three other experiments. Densitometric analysis (b) quantified pFAK versus FAK in control (Ctr) and compound **1**- and **3**-treated C6 cultures 2 h after scratching. pFAK as detected by immunocytochemistry along the wound scratch border of vehicle- (Ctr) and compound **1**-treated cells is shown in (c). Bars are mean ± SE of three independent determinations; $^*p < 0.05$ versus control as by Student's *t*-test.

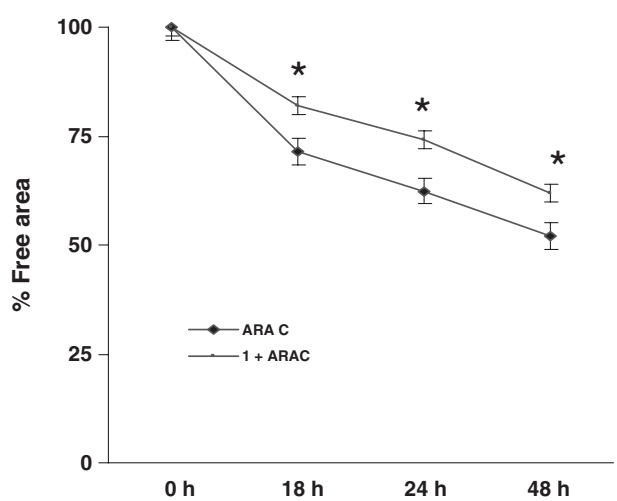


Fig. 4 Compound **1** slowed down wound closure in C6 glioma cells in the presence of Ara-C. C6 cells were treated with 5 μM Ara-C for 4 h prior to scratching and then exposed again to Ara-C in the presence (squares) or absence (diamonds) of compound **1** (10 μM). Wound closure was examined at 18, 24, and 48 h. Data represent mean ± SE of three experiments each run in triplicate; $^*p < 0.05$ versus control by two-way ANOVA and Newman–Keuls multiple comparison test.

survival of glioma cells (Jiang *et al.* 2006a). GalTase has also been detected on the surface of migrating cells (Shur *et al.* 1998), where it mediates cell migration on basal lamina matrices by associating with the cytoskeleton and binding to *N*-linked oligosaccharides in the E8 domain of laminin. GalTase V galactosylates the GlcNAcβ1 → 6Man arm (Sato *et al.* 1998) of the highly branched *N*-glycans. This represents a main feature for malignant transformation (Asada *et al.* 1997) and is characteristic of glioma, playing a major role in glioma invasiveness (Yamamoto *et al.* 2000). Accordingly, knocking-down GalTase V results in inhibition of *N*-glycans expression in SHG44 glioma (Jiang *et al.* 2006a) as well as in SH-SY5Y neuroblastoma cells (Sato and Furukawa 2004). GalTase V is thus increasingly emerging as a novel target in glioma treatment.

In this regard, modulation of GalTase V expression and function has been related to enhanced sensitivity to the apoptotic effect of antitumoral agents such as etoposide (Jiang *et al.* 2006a,b) a drug widely used for the treatment of malignant glioma. In addition, knocking-down GalTase V produces enhanced expression of cell surface integrin β1 resulting in increased adhesion to fibronectin (Chen *et al.* 2006b), and suppression of tumor development and metastatic potential in experimental animals (Johnson and Shur 1999; Jiang *et al.* 2006b).

Most of the studies to date available have relied on modulation of the expression of β1,4-GalTase by molecular tools. Thus, the possibility to disrupt GalTase-mediated C6 glioma cell migration because of an interaction with GalTase on the cell surface appeared valuable and was tested using

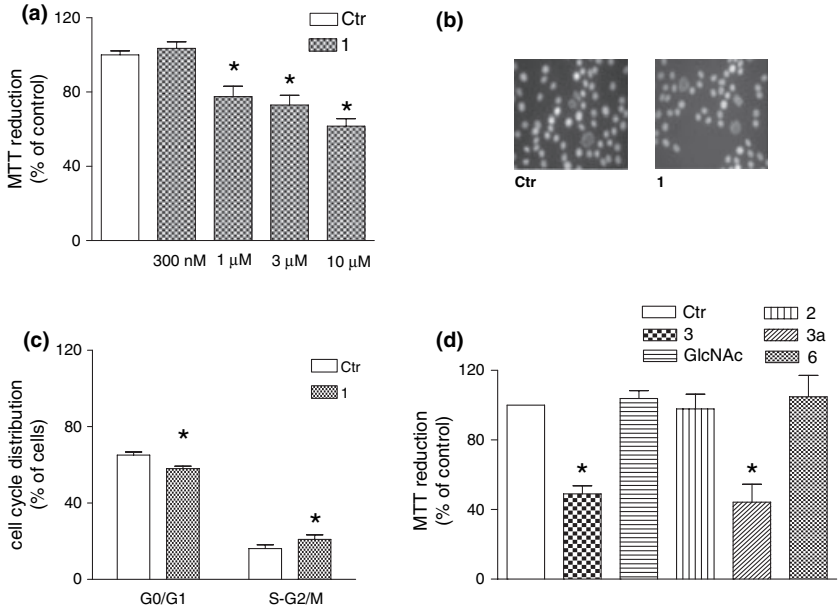


Fig. 5 Treatment with compound **1** reduced cell proliferation and modified cell cycle distribution. Treatment for 48 h with compound **1** (1–10 μM) reduced C6 cell proliferation as detected by MTT assay (a); in (b), Hoechst staining in vehicle- (Ctr) and compound **1**-treated C6 cells. Few cells with DNA fragmentation are indicated both in control and compound **1**-treated cultures. Changes in cell cycle distribution induced by treatment for 48 h with 10 μM compound **1** are shown in (c). In (d), modifications of cell proliferation, as measured by the MTT assay, induced by different compounds related to **1**: butyl-thiureido-calix[8]arene (**3**), phenyl-thiureido-calix[8]arene (**3a**), GlcNAc, amino-calix[8]arene (**2**), and caffeic acid-calix[8]arene (**6**) All compounds were tested at a concentration of 10 μM. Data represent mean ± SE of three experiments each run in duplicate (cell cycle) and triplicate (MTT assay); *$p < 0.05$ versus control by one-way ANOVA followed by Newman–Keuls multiple comparison test.

compound 1, a macrocycle molecule exposing multiple copies of GlcNAc onto the surface of a calix[8]arene scaffold.

Our results, showing that compound 1 exerted an inhibitory effect on C6 cell migration, confirmed previous findings that reported reduced migration of human SHG44 glioma cells observed following inhibition of GalTase V expression by transfection with a GalTase V antisense cDNA construct (Jiang *et al.* 2006b).

In addition, the role of distinct functional groups of compound 1, i.e. the sugar moieties and the calixarene scaffold was clearly highlighted. GlcNAc residues competing with the natural substrate for GalTase V appeared necessary to recognize and bind the enzyme. On the other hand, a cluster and multivalent effect could account for the marked inhibitory activity of compound 1 when compared with GlcNAc alone that was effective only at very high concentrations and at later time points. This is consistent with the hypothesis that compound 1 as well as other multivalent ligands can elicit binding interactions, precluded to monovalent ligands, which determine enhanced receptor affinity and a different or more efficient cell response. In addition, a cluster of weakly binding monovalent ligands when properly orientated can generate enhancements in association constants with cognate receptor sites (Kiessling *et al.* 2006). Calix[8]arene skeleton appears to be a valid platform for the arrangement of multiple GlcNAc moieties in a spatial organization suitable to elicit GalTase recognition.

As the function of GalTase in promoting migration requires association with glycoside ligands in the extracellular matrix on one side and the cytoskeleton on the other side, the ability of compound 1 to affect intracellular signaling was tested by evaluating the activation of FAK under conditions of scratch-promoted cell migration. In other cellular systems, a strict interaction between GalTase and FAK has been reported (Wassler and Shur 2000; Ji *et al.* 2003; de la Cruz *et al.* 2004) and clustering of GalTase with multivalent ligands results in a transient FAK phosphorylation and disassembly of actin stress fibers (Wassler and Shur 2000). In our experimental conditions, compound 1, by binding surface GalTase inhibited migration-induced FAK phosphorylation, identifying a first step in intracellular signaling mediating its inhibitory effect on cell migration. These results are consistent with the rapid and transient FAK phosphorylation observed in fibroblasts, in which clustering of cell surface GalTase was induced by either multivalent polymers of GlcNAc or anti-GalTase antibodies (Wassler and Shur 2000). The ability of our compound to reduce FAK activation may be related to its activity as a competitive substrate for GalTase. Structure and conformational flexibility of 1 (Fig. 6) may favor strong

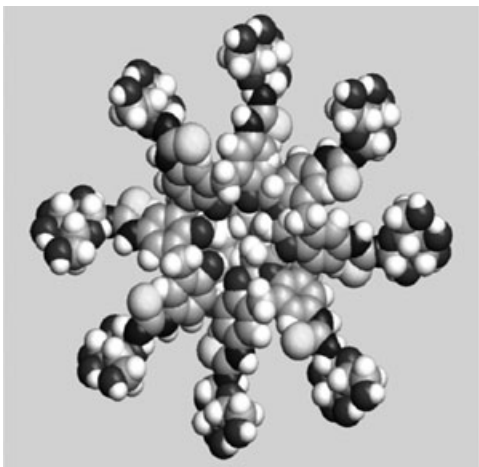


Fig. 6 Space-filling CPK computer model of compound 1 (MacroModel 7.1). The model is built on a calix[8]arene skeleton in a pleated loop conformation.

and stable binding to the enzyme blocking its function as adhesion molecule.

In contrast, inhibition of cell proliferation appeared independent of interactions with cell surface GalTase as it was not mimicked by even high concentrations of GlcNAc, but rather observed after treatment with compounds lacking GlcNAc residues. Surprisingly, all compounds with a macrocyclic structure and presenting a ureido group induced inhibition of cell proliferation focusing on the combination of multiple ureido units arranged onto a calix[8]arene skeleton as a crucial point for the observed inhibitory effect on C6 cell proliferation. The mechanism responsible for this effect cannot be identified from the present results. However, it is plausible that ureido NH protons with a strong hydrogen-bond donor capability and the aromatic calixarene moieties, can establish multipoint hydrogen bonded patterns and hydrophobic interactions with complementary acceptor groups on their specific target. The nature of this target in unknown at present and will be the issue of future studies. Interestingly enough, this effect shows a certain specificity as it was observed in other, but not in all cell types tested (data not shown).

Evidence has been provided that molecules arranged onto a calixarene scaffold can exert antitumoral activity also when administered peripherally (Blaskovich *et al.* 2000). In this context, design of synthetic molecules able to bind to a protein surface and to disrupt protein-protein or protein-carbohydrate interactions represents an emerging strategy (antiadhesion therapy) to control migration and proliferation of tumor cells. Compound 1 may represent such an example. Multivalent ligand 1, in which multiple copies of NAcGlc are anchored to a suitable platform that drives shape, flexibility, size, valency, and spatial organization of the recognition elements, mimics in fact the natural carbohydrate array on matrix surface and acts as an inhibitor of cell-surface adhesion molecules.

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