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Dual Targeting NG2 and GD3A Using Mab-Zap Immunotoxin Results in Reduced Glioma Cell Viability *In Vitro*

SAMANTHA C. HIGGINS[1], HELEN L. FILLMORE[1], KEYOUMARS ASHKAN[2],
ARTHUR M. BUTT[3] and GEOFFREY J. PILKINGTON[1]

[1]*Cellular and Molecular Neuro-oncology Research Group and* [3]*Cellular Neurophysiology Research Group,*
Cellular and Molecular Medicine Research Division, Institute of Biomedical and Biomolecular Sciences,
School of Pharmacy and Biomedical Sciences, University of Portsmouth, Portsmouth, U.K.;
[2]*Department of Neurosurgery, King's College Hospital, London, U.K.*

Abstract. *Background: Effective treatments for glioblastoma multiforme (GBM) are lacking due, in part, to cellular heterogeneity. Consequently, single-target therapeutic strategies are unlikely to succeed. Simultaneous targeting of different neoplastic cell populations within the same tumour may, therefore, prove of value. Neuron-glia 2 (NG2), a transmembrane chondroitin sulphate proteoglycan, present on developing glial cells, and GD3^A, a ganglioside expressed on developing migratory glia, are re-expressed in GBM. Materials and Methods: The aims of this study were to conduct 'proof of concept' experiments in human GBM cell lines to show that proliferative high NG2-expressing cells and high GD3^A -expressing migratory cells could be effectively ablated using a Mab-Zap saporin immunotoxin system. Results: The combinatorial ablation of both NG2 and GD3^A-expressing cells resulted in significant reduction in GBM cell viability compared to single epitope targeting and controls ($p<0.0001$); non-neoplastic astrocytes were not affected. Conclusion: Multiple targeting of GBM sub-populations may, therefore, help inform novel therapeutic approaches.*

Glioblastoma multiforme (GBM) is the most common primary adult brain tumour and unfortunately, even with the current multimodality standard-of-care that includes maximal safe surgical resection, post-operative radiotherapy in parallel with temozolomide followed by adjuvant chemotherapy, the median survival is only 14 months (1). GBM is characterized by tumour heterogeneity, widespread tumour cell infiltration, angiogenesis, necrosis and proliferation (2). Due to tumour heterogeneity and the ability of tumour cells to adapt to changes in the microenvironment, the likelihood of a one targeted approach is low, especially when considering the molecular genotypic sub-groups of GBMs (3-6). Antigens that are associated with distinct and specific glioma cell biological functions may prove useful to deliver therapies to an area of tumour heterogeneity. In this report a 'proof of concept' was tested in human GBM cell lines in which two cell surface antigens known to be associated with distinct and overlapping GBM cell populations were targeted to deliver an immunotoxin. Neuron-glia 2 (NG2), also known as chondroitin sulphate proteoglycan 4 (CSPG4), has been well characterized in human adult brain tumours and its expression is highly correlated with increased tumour cell proliferation, grade of tumour, poor prognosis and angiogenesis (7-10). NG2 is associated with increasing histological grade of malignancy and proliferation rates in adult glioma with an aggressive molecular signature (11-13). NG2 is not detectable in normal quiescent vasculature and expression in neovasculature is limited to neovascular pericytes (9, 14). A study comparing proliferation, clonogenic and tumourigenic capacities of NG2-positive glioma cells with their negative counterparts revealed that NG2-positive cells had a higher level of proliferation, exhibited higher tumourigenic capabilities and over-expressed genes associated with aggressive tumourigenicity (13). Targeting NG2 by knockdown studies reduces tumour growth and angiogenesis in pre-clinical models of glioblastoma and melanoma. In the glioma model, targeting NG2 resulted in reductions in tumour growth and oedema levels, angiogenesis and normalised vascular function. Furthermore, it has been established that NG2 promotes tumour progression by multiple mechanisms and represents an accessible target for therapy (10).

Correspondance to: Dr. Samantha Higgins, Cellular & Molecular Neuro-oncology Group, Institute of Biomedical & Biomolecular Sciences, School of Pharmacy & Biomedical Sciences, White Swan Road, University of Portsmouth, Portsmouth PO1 2DT, UK. Tel: +44 02392842125, e-mail: samantha.higgins@port.ac.uk

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GD3, an oncofetal ganglioside, is crucial in cell migration during normal brain development. Upon brain maturation, GD3 accumulates and is responsible for the death of supernumerary progenitor cells during embryonic brain maturation (15). However, in neoplastic gliomas, GD3 is re-expressed and involved in cell invasion (16). In addition, modification of GD3 to $GD3^A$ by sialic acid acetylation is associated with cell survival (17). GD3 contains two sialic acid residues, sequentially attached to the galactose residue. The most common post-synthetic modification of GD3 is O-acetylation at the C9 position of its terminal sialic acid. The resulting 9-O-acetyl GD3 ($GD3^A$) is expressed in a developmentally regulated and tissue-specific manner and has been demonstrated in a variety of tumours (18, 19). O-acetylation of GD3 by tumour cells may represent an important strategy adopted to evade acute GD3 accumulation during cell stress and to enhance survival. In support of this, it was recently demonstrated that GBM cells undergo apoptosis when a gene therapy approach targets the deacetylation of $GD3^A$ (17).

Although there is some overlap of expression, NG2 and GD3 are associated with two distinct tumour cell populations (NG2, highly proliferative tumour cells and GD3/$GD3^A$, tumour cell survival and migration) (9). Herein, we examined the ability of an NG2 and a $GD3^A$ antibody to deliver a secondary antibody conjugated to the ribosome-inactivating protein saporin (Mab-Zap; Advanced Targeting Systems, San Diego, CA, USA) (Figure 1). Saporin is a ribosome-inactivating protein derived from the plant *Saponaria officinalis*, known to induce apoptosis and cell-cycle arrest. On antigen-antibody binding, the immunotoxin is internalized by the target cells and the enzymatic fragment of the toxin translocates to the cytosol, where it induces ribosomal damage, inhibits protein synthesis and causes cell death (20). This system has been used for targeting tumour antigens, including the receptor protein tyrosine phosphatase β in a model of GBM (21), the antigen tomoregulin (22) and prostate-specific membrane antigen (PMSA) in prostate cancer cell lines (23), as well as the CSPG4 (also known as NG2) antigen in a malignant mesothelioma model (24). Although the use of immuotoxins coupled with single tumour-specific ligands is not novel, we used this established method to demonstrate the feasibility of selectively targeting subpopulations of glioma. Given the functional importance of both NG2 and $GD3^A$ in GBM, this may serve as a promising dual target in which multiple sites within a highly heterogeneous tumour could be pursued.

Materials and Methods

Ethics statement. Biopsies from glioma patients were obtained under Ethics permissions LREC 00-173 or KCH 11-094 or 11/SC/0048 in accordance with the National Research Ethics Service (NRES) and the study was approved through ethics committees for the University of Portsmouth and King's College Hospital, London. All patients consented to the use of biopsy material for research purposes. Consent forms were read to and duly signed by participating patients prior to surgery.

Cell culture. Human-authenticated glioma-derived cell lines (25) established from primary cultures of biopsy-derived brain tumours (UP-007, UP-019 and UP-032; Table I) were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM – without pyruvate, with glucose, Glutamax and Phenol Red) (Gibco, Paisley, Renfrewshire, UK) and supplemented with 10% heat inactivated foetal bovine serum (FBS) (Sigma-Aldrich, Gillingham, Dorset, UK) and referred to as complete media. All cells were washed with Hank's balanced salt solution (HBSS) (Gibco) and harvested using TrpLE™ Express (Gibco). A primary astrocyte culture (UP-010), obtained from a patient undergoing a temporal lobe resection for epilepsy control, was also established.

Evaluation of NG2 and $GD3^A$ expression on human cell lines. The expression of NG2 and $GD3^A$ was quantitatively examined by flow cytometry. Cells were harvested and incubated with 100 µl pre-diluted primary antibody for 30 min at 40C. NG2 antibody (R&D Systems, Abingdon, Oxfordshire, UK) was used at a 1:1000 dilution (human NG2/MCSP, mouse IgG, catalogue # MAB2585). $GD3^A$ antibody (Novus Biologicals, Cambridge, Cambridgeshire, UK) was used at a 1:50 dilution (non-species specific OAcGD3, mouse IgG, catalogue # NB120-6218). Negative control samples were incubated with a mouse IgG isotype control antibody (catalogue # I-2000, Vector Labs, Peterborough, Cambridgeshire, UK) at the same concentration as primary antibodies. Samples were then incubated with 100 µl secondary Alexa Fluor 488 (Invitrogen, Paisley, Renfrewshire, UK) conjugated antibody for 15 min in the dark and at 4˚C. Samples were washed with buffer through centrifugation and the supernatant discarded. Cell pellets were re-suspended in PBS and 2% goat serum (GS), transferred to FACS tubes and analysed. Analysis was performed on a four-colour multi-parameter BD Biosciences FACS Calibur (BD Biosciences, Oxford, Oxfordshire, UK).

The expression of NG2 and $GD3^A$ was examined by double-labelled image cytometry. Cells were harvested and incubated with 100 µl pre-diluted NG2 primary antibody for 30 min at 4˚C. Negative control samples were incubated with a mouse IgG isotype control antibody at the same concentration as primary antibodies. This step was omitted from the negative control samples. Samples were washed with buffer and then incubated with 100 µl secondary Alexa Fluor 488 conjugated antibody for 15 min in the dark and at 4˚C. Samples were washed with buffer through centrifugation and the supernatant discarded. Samples were then incubated with the second primary antibody $GD3^A$ for 30 min at 4˚C, washed with buffer and then incubated with secondary Alexa Fluor 568 conjugated antibody for 15 min in the dark and at 4˚C. Samples were washed with buffer and the supernatant discarded. Cell pellets were re-suspended in PBS and 2% FBS, before analysis with a NucleoCounter NC3000 image cytometer (Chemometec, Allerød, Denmark).

Immunocytochemistry. Cells were grown to confluency on cover slips in duplicate and were fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich) for 2 min and stained with NG2 and $GD3^A$ antibodies described previously. Cells were incubated with primary NG2 antibody used at dilution 1:1000. Following three washes with

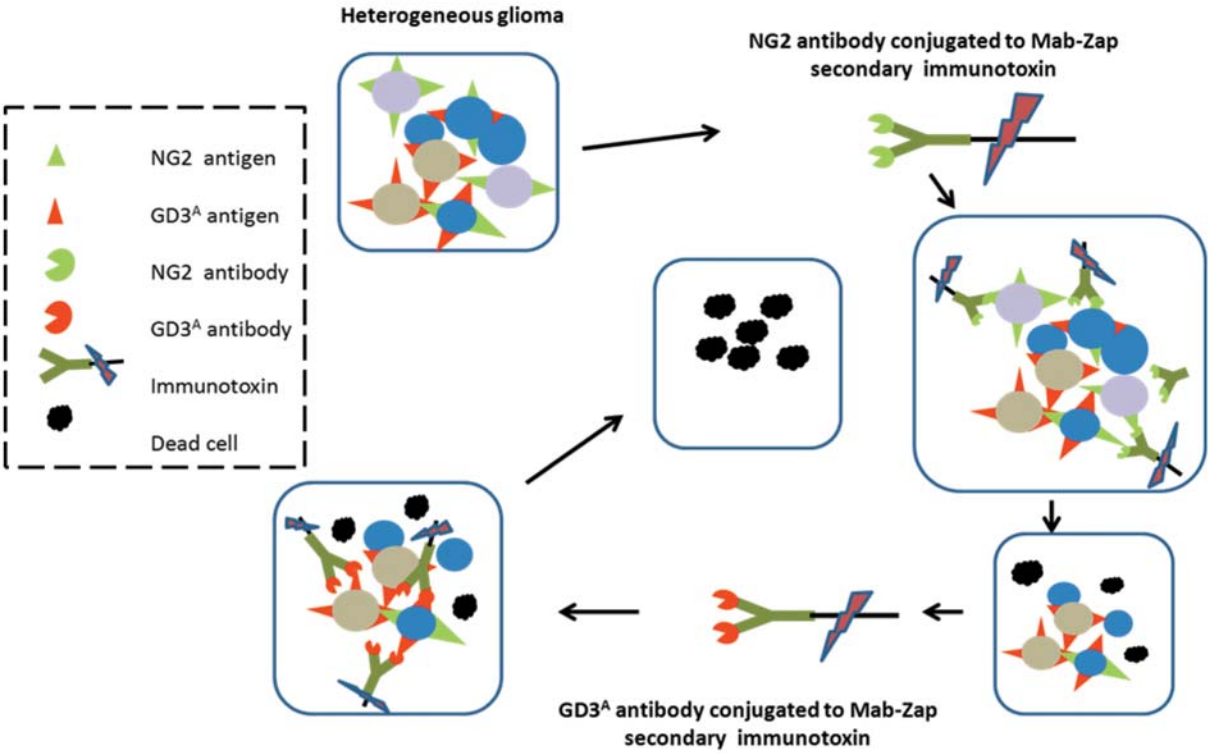

Figure 1. *A schematic diagram depicting the sequential and dual secondary Mab-Zap immunotoxin approach in which a heterogeneous population of cells are first treated with NG2 antibody-conjugated immunotoxin and then hit with a second treatment using GD3^A antibody-conjugated immunotoxin. The antibody-conjugated immunotoxin (saponin) binds to the cell surface antigen, is internalized and cell death occurs by inactivating ribosomes specifically killing cells expressing the antigens.*

Table I. *Human glioma and non-neoplastic astrocyte cell cultures and their histopathological grading.*

Cell lines	Age (years)	Gender No	Passage	Histopathological grading
UP-007	71	Male	25	GBM (Grade IV)
UP-019	68	Female	9	GBM (Grade IV)
UP-032	71	Male	9	Anaplastic Astrocytoma (AA Grade III)
UP-010	Unknown	Male	4	Temporal lobe resection-derived non-neoplastic astrocytes

GBM, Glioblastoma multiforme.

PBS, 1:500 of the secondary antibody anti-mouse Alexa Fluor 568 (Invitrogen) was added for thirty minutes in the dark followed by a final wash with PBS. The cells were then washed three times in PBS and re-probed with GD3^A primary antibody (1:200) and secondary anti-mouse Alexa Fluor 488 and, finally, nuclear counterstained with Hoechst Blue (10 μg/ml) (Sigma-Aldrich) for five seconds. The cells were washed three times in PBS, mounted and examined using a Zeiss Axioimager epifluorescence microscope with excitation and barrier filters for fluorescein isothiocyanate (FITC), Texas Red and 4',6-diamidino-2-phenylindole (DAPI) (Carl Zeiss Ltd., Cambridge, Cambridgeshire, UK). The primary antibody was omitted in the negative controls.

Dual targeted NG2/GD3 cell ablation in monolayer cultures using Mab-Zap immunotoxin. Mab-Zap (Advanced Targeting Systems, San Diego, CA, USA) is a chemical conjugate of affinity-purified goat anti-mouse IgG and Saporin. Saporin is a ribosome-inactivating protein with a molecular weight of 30 kDa from the seeds of the plant *Saponaria officinalis*. Second immunotoxins are conjugations of a secondary antibody to Saporin. This second immunotoxin uses the secondary antibody (affinity purified goat anti-mouse IgG) to "piggyback" onto the mouse IgG primary antibody. Once the Mab-Zap is internalised, Saporin breaks away from the targeting agent and inactivates ribosomes, which causes protein inhibition and, ultimately, cell death.

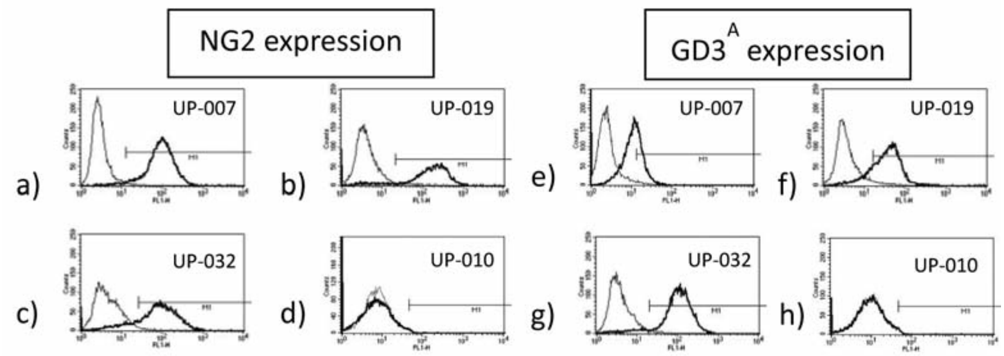

Figure 2. *Flow cytometric analysis of NG2 and GD3^{A} expression in glioma cells. Cells were incubated with either NG2 or GD3^{A} antibody and Alexa Fluor 488 (left-handed peaks) and measured using the FL1 channel. Expression was compared to an isotype control antibody (right-handed peaks). NG2 expression was measured as a) UP-007 97.61% positive; b) UP-019 95.9% positive; c) UP-032 96.36% positive d) UP-010 5.48% positive. GD3^{A} expression was measured as e) UP-007 47.27% positive; f) UP-019 21.18% positive; g) UP-032 45.13% positive h) UP-010 1.52% positive.*

Cells were harvested, counted using a ViCell XR Coulter Counter (Beckman Coulter UK, High Wycombe, Buckinghamshire, UK) and seeded into a 96-well plate at a density of 1×10^4 in a final volume of 100 µl complete clear DMEM supplemented with glutamax and 10% FBS. Cells were returned to the incubator and allowed to adhere overnight. Mab-Zap and primary antibodies (same as above) were made up fresh as per the treatment schedule and allowed to conjugate for 30 minutes at room temperature. Two 96-well plates were set up for each cell line per treatment; both plates were initially treated with either 1 µg/ml or 5 µg/ml NG2/Mab-Zap and incubated for 72 hours. After this time, the first plate was taken forward for the cell proliferation assay (see below) and the second plate had the NG2/Mab-Zap treatment removed and was then sequentially treated with either 1 µg/ml or 5 µg/ml GD3^{A} /Mab-Zap and incubated for a consecutive 72 hours, time after which these plates were then taken forward for the MTS assay. The negative controls included omission of immunotoxin treatment and inclusion of either primary antibody alone or Mab-Zab alone to investigate toxicity of both compounds unconjugated. 1M NaOH was added to the positive control wells.

MTS assay (CellTitre 96®AQ$_{ueous}$ non-radioactive cell proliferation assay). The CellTitre 96®AQ$_{ueous}$ non-radioactive cell proliferation assay (Promega, Southampton, Hampshire, UK) was utilised to determine cell viability of the human cell lines after treatment as per the manufacturer's guidelines. The absorbance was then read using the Labtech POLAR Star Optima plate reader at 490nm (BMG LABTECH Ltd., Aylesbury, Buckinghamshire, UK) and viability calculated from the ratio between control samples and test samples.

Statistical analysis. All data are expressed as mean values. All concentrations are expressed as the final concentrations to which cells were exposed. Statistical analysis was performed on the data using a one way analysis of variance (ANOVA) followed by the Tukey's multiple comparison post-test with a probability of <0.05 being regarded as significant. The software package GraphPad Prism 3.02 (GraphPad Software, San Diego, CA, USA) was used to calculate the statistical tests.

Results

Human glioma cell lines contain distinct NG2-positive cells and overlapping populations with cells positive for GD3^{A}. The expression profile of NG2 and GD3^{A} in GBM cell lines was determined using flow cytometry. Three primary established GBM cell lines (UP-007, UP-019 and UP-032) obtained from patient biopsies were examined for NG2 and GD3^{A} expression and compared to an established primary astrocyte culture (UP-010) obtained from a patient who had undergone a temporal lobe resection for intractable epilepsy. Compared to the astrocyte cell line, the GBM cell lines, UP-007, UP-019 and UP-032, contain high NG2 cell surface expression with over 90% of total cell population (97.6%, 95.9% and 96.4%, respectively) as examined by flow cytometry (Figures 2a-c). The control non-neoplastic human astrocytes contained approximately 5.48% NG2 positivity of the total population (Figure 2d).

GD3^{A} expression was also examined in the glioma cell lines and the astrocyte cell line. While the population of NG2-positive cells in the glioma cell lines were over 90%, GD3^{A}-positive cells were lower and varied between the cell lines (Figures 2e-g). The GBM cell lines, UP-007, UP-019 and UP-032, contain GD3^{A}-positive cells (47.3%, 21.2% and 43.1%, respectively, Figures 2e-g). The control non-neoplastic human astrocytes contained approximately 1.5% GD3^{A} positivity of the total population (Figure 2h). These results suggest some overlap with NG2 and GD3^{A} in the glioma cell lines. To examine this more closely, the UP-007 cell line was double-labelled with antibodies for both NG2 and GD3^{A} and subjected to immunocytochemistry and flow cytometric analysis. These data confirmed an overlap with cells positive for NG2 and GD3^{A}, as well as distinct and separate NG2- and GD3^{A}-positive populations as seen in UP-007 cells (Figure 3). There was co-expression of NG2 and GD3^{A} in

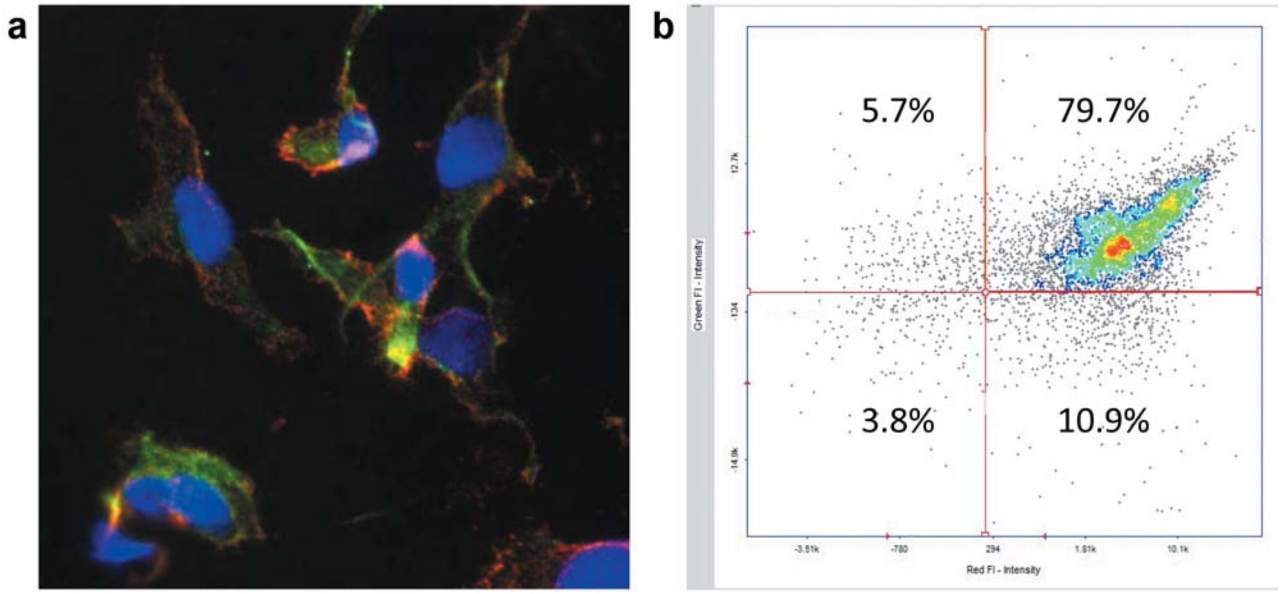

Figure 3. *Co-expression analysis in UP-007 cell line. a) Representative image of double labelling with NG2 and GD3^A. Red, NG2 and Green GD3^A. b) Cytometric dot plot of NG2 (green) and GD3^A (red) double labelling of UP-007. Less than four percent (3.8%) of cells in the bottom left quadrant are negative for both NG2 and GD3^A, 10.9% of cells in the bottom right quadrant are positive for NG2 only, 5.7% of cells in the top left quadrant are positive for GD3^A only and 79.7% of cells in the top right quadrant are positive for both NG2 and GD3^A.*

Table II. *Percentage cell viability after cell treatment with primary antibody alone and Mab-Zap alone as determined by the MTS assay.*

Cell line	No treatment	Primary antibody alone (1:1000)	Mab-Zap alone (1 μg/ml)	Mab-Zap alone (5 μg/ml)
UP-007	100%	99.43%	98.89%	97.66%
UP-019	100%	97.90%	97.10%	97.97%
UP-032	100%	98.21%	97.25%	98.26%
UP-010	100%	97.67%	98.56%	98.11%

No significance was found between treatment groups $p>0.005$.

79.7% of the cell population with two distinct populations (NG2-positive, 10.9% and GD3^A-positive, 5.7%).

Treatment of human glioma cells with NG2 and GD3^A immunotoxin. Glioma cell viability was reduced when cells were treated with NG2 immunotoxin alone when compared to non-neoplastic astrocytes (Figure 4a, $p<0.0004$). When glioma cell lines were sequentially treated with NG2 immunotoxin followed by GD3^A immunotoxin there was also a significant decrease in cell viability ($p<0.0001$), while no effect was seen in the non-neoplastic astrocyte cell line (Figure 4b). In both experimental paradigms, the effect of reducing viability was further enhanced with increasing the immunotoxin to 5 μg/ml from 1 μg/ml (Figure 4a and b, $p<0.0004$). UP-007 cell viability was reduced from approximately 30% (1 μg/ml NG2/GD3^A:Mab-Zap) to 5.0% (5 μg/ml NG2/GD3^A:Mab-Zap) ($p<0.0004$), for UP-019 28.88% to 6.81% ($p<0.0004$) and for UP-032 29.24% to 6.13% ($p<0.0004$) (Figure 4b). Glioma cell viability was not affected by treatment with primary antibody alone or with Mab-Zap immunotoxin alone (Table II) supporting the fact that both the antibody and the immunotoxin are needed for this effect on cell viability.

Discussion

As previously described in the literature, NG2 expression on glioma cells is indicative of increased histological malignancy (11); moreover, NG2-positive cells demonstrate higher levels of proliferation, higher tumourigenic capabilities and a more aggressive phenotype (13). High NG2 expression correlates with decreased survival outcomes by mediating resistance to chemotherapy (26) and radiotherapy (14). NG2 represents an attractive target for the secondary immunotoxin (ITX) approach to cancers.

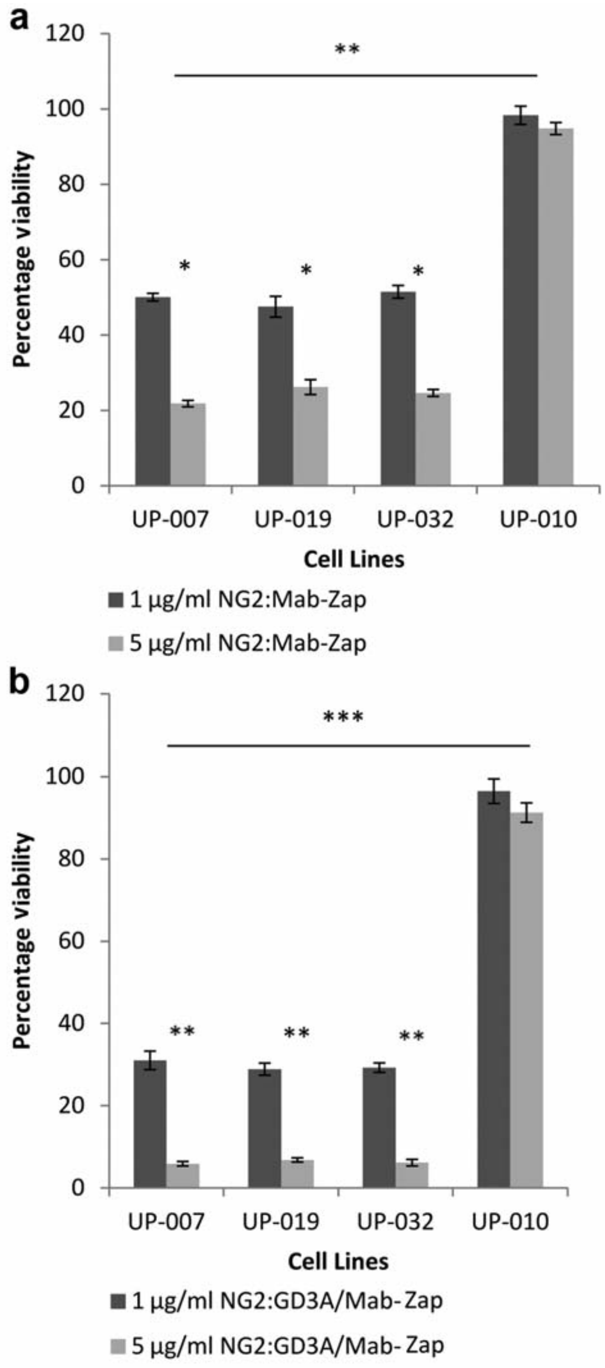

Figure 4. *Percent viability of three glioma cell lines and control astrocytes after exposure to a) 1 and 5 µg/ml NG2/Mab-Zap and b) 1 and 5 µg/ml NG2:GD3*^{A}*/Mab-Zap (72 h) and treatment with 5 µg/ml NG2:GD3*^{A}*/Mab-Zap (72 h) as measured by the MTS assay. Total exposure time 144 h and total toxin exposure 2 µg/ml and 10 µg/ml. Percentage viability was calculated from an untreated control. A significant reduction in cell viability is seen in glioma cell lines treated with the NG2 immunotoxin alone compared to the non-neoplastic astrocyte culture (*$p<0.0001$*). Glioma cell viability was significantly reduced following treatment with sequential treatment using 1 µg/ml NG2:GD3*^{A}*/Mab-Zap and 5 µg/ml NG2:GD3*^{A}*/Mab-Zap (*$p<0.0001$*). **$p<0.001$*; ***$p<0.0004$*; ****$p<0.0001$*.*

Antibody-based immunotherapy against NG2 expression in melanoma has been extensively investigated. Monoclonal antibodies (mAbs) have been used as the vehicle to selectively deliver radionucleotides and toxins to the tumour cells (27, 28). NG2 is expressed by the tumour vasculature, as well as in the tumour, therefore this form of targeted-therapy may offer several advantages over tumour-restricted therapies. Other than tumour-associated vascular pericytes and oligodendrocyte precursor cells (OPCs) in the brain, NG2 has restricted distribution in normal tissues (29).

The expression of NG2 is associated with proliferative activity in glioma, whereas pro-survival/anti-apoptosis, as well as invasion, is associated with the expression of GD3^{A}. Spatial distribution of NG2 and GD3 in tumour biopsies has been reported indicating that NG2 expression is confined to the main tumour mass and is reduced towards the tumour periphery, while GD3 expression is evident both in the main tumour mass and at the brain/tumour interface (7). Our results, utilising human glioma and astroctye cell lines, demonstrate the feasibility of targeting NG2-positive gliomas without affecting the viability of NG2-negative astrocytes. This involvement of NG2 in multiple aspects of tumour biology makes this proteoglycan an attractive candidate for future therapies against cancer. NG2 may provide a suitable target for cytotoxic therapy, particularly in harness with approaches aiming to target the shared glioma cell antigen GD3^{A}. Herein, we have conducted 'proof of principle' experiments and demonstrated the possibility of targeting two overlapping and distinct populations of cells with a secondary immunotoxin approach. This system demonstrated the ability to target different antigens with the sequential treatment of ablating NG2-positive cells and then GD3^{A}-positive cells, respectively. These results suggest the feasibility of selectively targeting subpopulations of glioma cells and may lead to improved therapeutics in which multiple sites, within a highly heterogeneous tumour, could be targeted.

Conflicts of Interest

Each Author declares that she or he has no conflict of interests.

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