

# Viability of glioblastoma stem cells is effectively reduced by diisothiocyanate-derived mercapturic acids

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**Abstract.** Glioblastoma is the most aggressive tumor of the central nervous system and is manifested by diffuse invasion of glioblastoma stem cells into the healthy tissue, chemoresistance and recurrence. Despite aggressive therapy, consisting of maximal surgical resection, radiotherapy and chemotherapy with temozolomide (Temodal®), life expectancy of patients with glioblastoma is typically less than 15 months. In general, natural isothiocyanates isolated from plants of the Cruciferae family are selectively cytotoxic to tumor cells. It has been demonstrated previously that diisothiocyanate-derived mercapturic acids are highly cytotoxic to colon cancer cells. In the present study, the application of diisothiocyanate-derived mercapturic acids led to a decrease in the viability of an established glioblastoma cell line, primary patient-derived sphere-cultured stem cell-enriched cell populations (SCs), and cells differentiated from SCs. Consequently, targeting glioblastoma cells by diisothiocyanate-derived mercapturic acids is a promising approach to restrict tumor cell growth and may be a novel therapeutic intervention for the treatment of glioblastoma.

## Introduction

A major drawback of cancer cell therapy is chemoresistance of cancer stem cells, resulting in repopulation of the tumor

niche even following a period of prolonged dormancy. Glioblastoma stem cells are responsible for the maintenance and phenotype of glioblastoma (astrocytoma grade IV or glioblastoma multiforme), the most common and aggressive tumor of the central nervous system, which is characterized by rapid cell proliferation and diffuse invasion into the healthy tissue (1). Following surgery, radiation and chemotherapy, the prognosis for patients diagnosed with glioblastoma remains poor. For instance, the postoperative median survival time of patients with glioblastoma is 6 months; radiotherapy increases the survival time of patients to 12 months, and radiotherapy in combination with the standard chemotherapeutic agent temozolomide (TMZ; Temodal®) increases the survival time by a further 2.6 months (total, 14.6 months) (2). Therefore, the elucidation of novel and more effective chemotherapeutics that interfere with glioblastoma stem cell proliferation, particularly invasion, is required.

Isothiocyanates (ITCs) are natural components of the Cruciferae family of plants (which includes radish, broccoli or mustard) that have intrinsic antitumor capacity as previously demonstrated (3,4). A major advantage of ITCs is that they selectively elicit an accumulation of reactive oxygen species (ROS), leading to apoptosis in transformed cells in contrast with wild-type cells, which are more resistant to ROS (5). Recently, Grzywa *et al* (6) observed that the application of diisothiocyanate-derived mercapturic acids was cytotoxic to a human adenocarcinoma cell line with a drug concentration yielding half-maximal response (EC<sub>50</sub>) of 2.02 μM. On the basis of these data, in the present study, various diisothiocyanate-derived mercapturic acids (J1-J4; Fig. 1) were investigated, and it was identified that J1-J4 selectively inhibited cell viability in glioblastoma cells and glioblastoma stem cells, indicating that these components are promising antitumor drugs in glioblastoma research.

## Materials and methods

**Diisothiocyanate-derived mercapturic acids.** Diisothiocyanate-derived mercapturic acids were synthesized as described previously (6). The diisothiocyanates (for compounds J3 and J4) were prepared from appropriate

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**Abbreviations:** ITCs, isothiocyanates; PCs, SC-derived differentiated/adherent glioblastoma cells; PBMCs, peripheral blood mononuclear cells; ROS, reactive oxygen species; SCs, sphere-cultured stem cell-enriched glioblastoma cell populations; TMZ, temozolomide

**Key words:** glioblastoma, isothiocyanates, MTT, diisothiocyanates, glioblastoma stem cells

diamine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and carbon disulfide (Sigma-Aldrich; Merck KGaA) using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (Iris Biotech GmbH, Marktredwitz, Germany) in the presence of triethylamine (Avantor Performance Materials Poland S.A., Gliwice, Poland). The diisothiocyanates (for compounds J1 and J2 commercially available from Sigma-Aldrich; Merck KGaA) and *N*-acetyl-L-cysteine (Sigma-Aldrich; Merck KGaA) were mixed with sodium hydrogen carbonate (Avantor Performance Materials Poland S.A.) to yield the final product (diisothiocyanate-derived mercapturic acid).

*Peripheral blood mononuclear cells (PBMCs).* Following Ficoll isolation of human PBMCs from heparinized blood (buffy coat), PBMCs were resuspended in Roswell Park Memorial Institute (RPMI-1,640 medium; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 1.5% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) and 1% penicillin (120 mg/ml)/streptomycin (120 mg/ml; Thermo Fisher Scientific, Inc.), and were cultured with J1-J4, TMZ or dimethylsulfoxide (DMSO) for 72 h at 37°C and 5% CO<sub>2</sub>. The use of PBMCs for *in vitro* studies was approved by the local Ethics committee of Ulm University, Ulm, Germany (no. 327/14).

*Glioblastoma cell line.* The human glioblastoma cell line U87-MG (U87) (American Type Culture Collection, Manassas, VA, USA), was cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1% penicillin (120 mg/ml)/streptomycin (120 mg/ml) at 37°C in a 5% CO<sub>2</sub> atmosphere.

*Sphere-cultured stem cell-enriched glioblastoma cell populations (SCs).* Astrocytoma grade IV tissue from 3 patients was obtained during surgery at the hospital in Ulm University Medical Center in Günzburg, Germany [nos. 35 (44 years, male; sample collected August 2009), 38 (75 years, male; sample collected, July 2010), and 40 (57 years, female; sample collected, July 2010)] was minced separately, washed in PBS and incubated with TrypLE™ Express (Gibco; Thermo Fisher Scientific, Inc.). Cells were filtered and cultured at 37°C in a 5% CO<sub>2</sub> atmosphere in DMEM/Ham's F-12 medium (Gibco; Thermo Fisher Scientific, Inc.) containing L-glutamine, 0.01% (v/v) epidermal growth factor (EGF; Biomol GmbH, Hamburg, Germany), 0.04% (v/v) fibroblast growth factor (FGF; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), 1% (v/v) B27 (Gibco; Thermo Fisher Scientific, Inc.), 2% fungizone (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin (120 mg/ml)/streptomycin (120 mg/ml; Thermo Fisher Scientific, Inc.) (7). These cells were defined as sphere-cultured stem cell-enriched glioblastoma cell populations (SCs; identified as SC35, SC38 and SC40 according to the patient number from which they derived). Stem cell and differentiation markers were expressed accordingly (8). To obtain adherent glioblastoma cells (PCs; identified as PC35, PC38 and PC40 according to the patient number from which they derived), SCs were kept at 37°C (5% CO<sub>2</sub> atmosphere) in DMEM supplemented with 10% FBS with 2 mM glutamine and 1% penicillin/streptomycin (120 mg/ml each; Thermo Fisher Scientific, Inc.) as SCs

differentiate into PCs when FBS is present in the culture medium. Use of SCs and patient samples was approved by the local ethics committee of Ulm University (#162/10).

*Determination of cellular metabolic activity (cell viability).* An MTT test was performed to assay the metabolic activity of the indicated cell populations as a measure of cell viability. The method is based on the reduction of the yellow tetrazolium compound MTT by metabolically active cells to an intracellular purple formazan, which is spectrophotometrically quantified. Adherent glioblastoma cells (U87, PC35, PC38 or PC40) were seeded in 96-well flat-bottomed tissue culture plates at 1.5x10<sup>4</sup> cells/ml in 100 µl DMEM containing 10% FBS and 1% penicillin/streptomycin. SCs (SC35, SC38 or SC40) were seeded in 96-well flat-bottomed tissue culture plates at 1.5x10<sup>4</sup> cells/ml in 90 µl DMEM/Ham's F-12 containing 0.01% EGF, 0.04% FGF, 1% B27, 2% fungizone and 1% penicillin/streptomycin. Freshly isolated PBMCs were seeded in 96-well flat-bottomed tissue culture plates at 5x10<sup>6</sup> cells/ml in 90 µl RPMI-1,640 medium containing 10% FBS, 1% penicillin/streptomycin, but lacking L-glutamine and phenol red. After 24 h of incubation, the medium was removed. Various concentrations (0.01, 0.1, 1, 10 and 100 µM) of J1, J2, J3, J4 or TMZ (DMSO served as a control) were prepared in DMEM containing 1.5% FBS and 1% penicillin/streptomycin, and added to U87, PC35, PC38 and PC40 cells (final volume, 100 µl); in the case of SCs or PBMCs, the medium was unchanged and the J1, J2, J3, J4 or TMZ was added directly (final volume, 100 µl). The cells were cultured for a further 3 days prior to removal of the medium. U87, PC35, PC38 and PC40 cells were incubated with 100 µl MTT working solution (Sigma-Aldrich; Merck KGaA), diluted 1:5 in RPMI-1640 medium without L-glutamine and phenol red. SC plates were centrifuged for 390 x g for 5 min at room temperature, the supernatant was removed, and cells were resuspended in 100 µl MTT working solution; 25 µl MTT working solution was added directly to PBMCs. Cells were incubated for 3 h at 37°C. Following incubation, formazan crystals were solubilized with 100 µl propan-2-ol. Cell viability was determined by measuring the optical density at 550 nm using a microplate spectrometer (Tecan Spectra Classic, Tecan Group Ltd., Männedorf, Switzerland).

*Microscopy images.* Images were captured using a PrimoVert microscope and AxioCam ICc1 camera (Zeiss AG, Oberkochen, Germany).

*Analysis of DNA content.* U87 cells were incubated with J1, J2, J3, J4 or TMZ, or a combination of TMZ with J1, J2, J3 or J4 for 144 h at 37°C (J1-J4 were used at their EC<sub>50</sub> values of J1, 250 nM; J2, 290 nM; J3, 2200 nM; J4, 500 nM; TMZ was used at 100 µM). The cell death readout used was DNA fragmentation (sub-G<sub>1</sub> population), a hallmark of apoptosis, as assessed by fluorescence-activated cell sorting using a FACScan instrument and CellQuest software 5.1 (BD Biosciences, Franklin Lakes, NJ, USA) analysis of DNA fragmentation of propidium iodide-stained nuclei as previously described (9). The specific (induced) DNA fragmentation was calculated as follows: 100x [experimental DNA fragmentation (%) - spontaneous DNA fragmentation (%)]/[100% - spontaneous DNA fragmentation

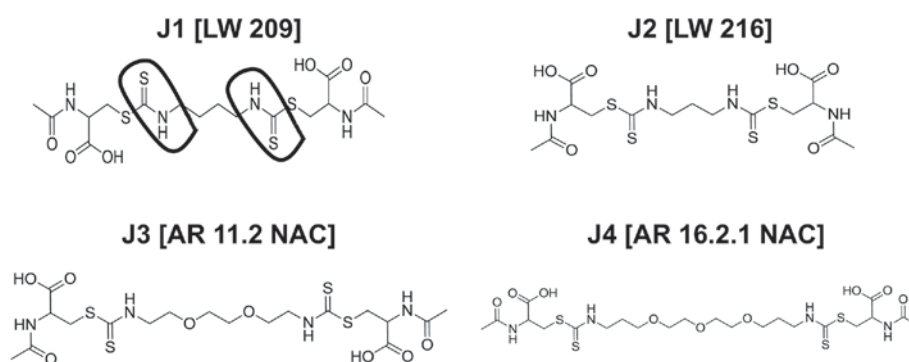


Figure 1. Chemical structures of diisothiocyanate-derived mercapturic acids. The diisothiocyanate structure is indicated in J1 by the thick line rings. AR, Agata Rudnicka; LW, Lukasz Winiarski; NAC, N-Acetyl-L-cysteine.

(%). For cell cycle distribution of live cells, only populations in clearly identifiable phases of the cell cycle, i.e.,  $G_{0/1}^-$ , S- or  $G_2/M$ -phase, were considered. For analysis of polyploidy, cells with DNA content exceeding that observed in the  $G_2/M$ -phase were compared with the total cell numbers obtained from the cell cycle analysis. For each analysis, ~10,000 cells were assayed.

**Alterations in cell number.** U87 cells were seeded and allowed to proliferate with or without J1, J2, J3, J4 or TMZ for the indicated times, prior to treatment with a trypsin/EDTA solution (Biochrom GmbH, Berlin, Germany) to suspend cells. The cell suspension was diluted 1:100 in CASYton solution (Innovatis AG, Reutlingen, Germany) and cell numbers were determined using a CASY® 1 DT cell counter (Innovatis AG).

**Statistical analysis.** Results are presented as the mean  $\pm$  standard error of the mean. Statistical analysis was assessed using an unpaired two-tailed Student's t-test and  $EC_{50}$  values were calculated using Prism (version 6; GraphPad Software, Inc., La Jolla, CA, USA).

## Results and Discussion

**Diisothiocyanate-derived mercapturic acids are cytotoxic to the glioblastoma cell line (U87).** To be viable for treatment of glioblastoma, diisothiocyanate-derived mercapturic acids in glioblastoma treatment are required to be able to traverse the blood-brain barrier (BBB). Therefore, compounds J1-J4 (Fig. 1) (6) were analyzed using online BBB Predictor software admetSAR (Immd.ecust.edu.cn:8000) (10). J1-J4 were identified to be theoretically able to cross the BBB, making delivery of such diisothiocyanate-derived mercapturic acids to the site of requirement possible.

It was determined whether diisothiocyanate-derived mercapturic acids are a potential therapeutic option for treatment of glioblastoma. U87 cells were incubated with various concentrations of J1, J2, J3, J4 or TMZ, and the metabolic activity of U87 cells was determined using an MTT assay. J1 and J2 were identified to markedly decreased U87 cell viability at a final concentration of 1  $\mu$ M with comparatively low  $EC_{50}$  values of 250 and 290 nM, respectively, in contrast with J3 (2.2  $\mu$ M) and J4 (500 nM) (Fig. 2). At  $<1 \mu$ M J1, J2, J3 or J4, there was no marked effect on PBMCs freshly isolated

from peripheral blood, and the  $EC_{50}$  values were ~10-fold higher than for U87 cells. Furthermore, U87 cells exhibited alterations in morphology when treated with J1, J2, J3 or J4; in particular, J1 and J2 caused alterations in the size and cell density of U87 cells at a final concentration of as low as 0.01  $\mu$ M (Fig. 3, microscopic images). Notably, the high concentration of TMZ used did not elicit a decrease in cell viability of  $<50\%$ , i.e., U87 cells exhibit increased sensitivity to diisothiocyanate-derived mercapturic acids than to the chemotherapeutic agent TMZ.

Furthermore, it was assessed whether  $EC_{50}$  values of J1 (250 nM), J2 (290 nM), J3 (2.2  $\mu$ M) or J4 (500 nM), or the combination with TMZ (100  $\mu$ M) were able to induce apoptosis in U87 cells by investigating the cell cycle distribution. Treatment with relatively low concentrations of diisothiocyanate-derived mercapturic acids caused a significantly prolonged negative effect on cell numbers for J1, J2 and J3 (Fig. 4A). Although only limited induction of apoptosis was observed upon single treatment with J1, J2, J3 or J4, it is noteworthy that robust cell death induced by TMZ, the standard chemotherapeutic agent used to treat glioblastoma, was dependent on high concentrations of the drug (Fig. 4B; see also Fig. 2). Furthermore, single treatment with J1, J2, J3 or J4 affects the cell cycle distribution and the nuclear integrity of U87 cells (Fig. 4C and D), possibly indicating that the cell cycle distribution of U87 cells is altered by J1. These results demonstrate that diisothiocyanate-derived mercapturic acids are cytotoxic to the glioblastoma cell line U87.

**Viability of glioblastoma stem cells is decreased by diisothiocyanate-derived mercapturic acids.** Further experiments were performed to address the question of whether the viability of primary patient-derived glioblastoma stem cells or primary glioblastoma cells is susceptible to diisothiocyanate-derived mercapturic acids. To this end, SCs from 3 different glioblastoma patients (SC35, SC38 and SC40) or SCs differentiated using FBS (PCs; PC35, PC38 and PC40) were incubated with J1, J2, J3 or J4, and cell viability was measured using an MTT assay. The cell viability of SCs or PCs was gradually decreased by increasing the concentration of J1, J2, J3 or J4 (Figs. 5 and 6). These compounds at a concentration of 1  $\mu$ M decreased the cell viability of PCs markedly in contrast with SCs which exhibited decreases in cell viability of 50% following treatment with J1, J2, J3 or J4 which may be due

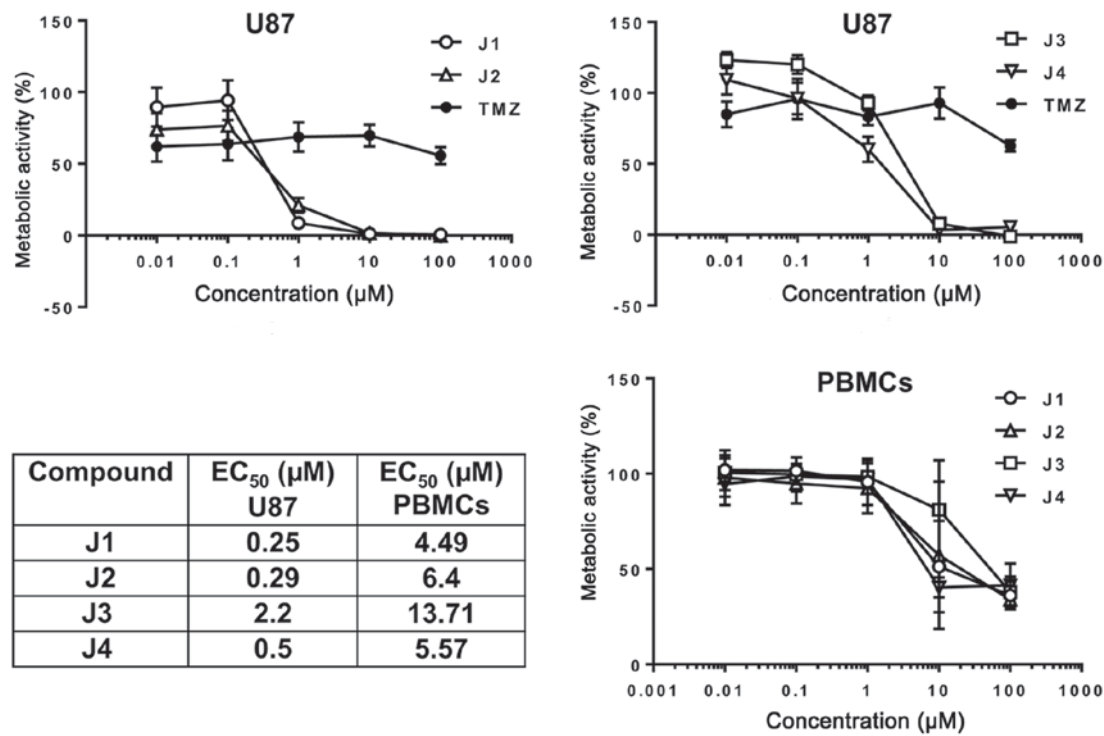


Figure 2. MTT assay to determine metabolic activity. The human glioblastoma cell line U87 or PBMCs were cultured in the presence or absence of J1, J2, J3, J4 or TMZ for 72 h and cell viability was measured using an MTT assay. The MTT assay was performed in triplicate with four independent experiments for U87 cells (n=4) and three independent experiments for PBMCs (n=3). PBMC, peripheral blood mononuclear cell; TMZ, temozolomide; EC<sub>50</sub>, drug concentration yielding half-maximal response.

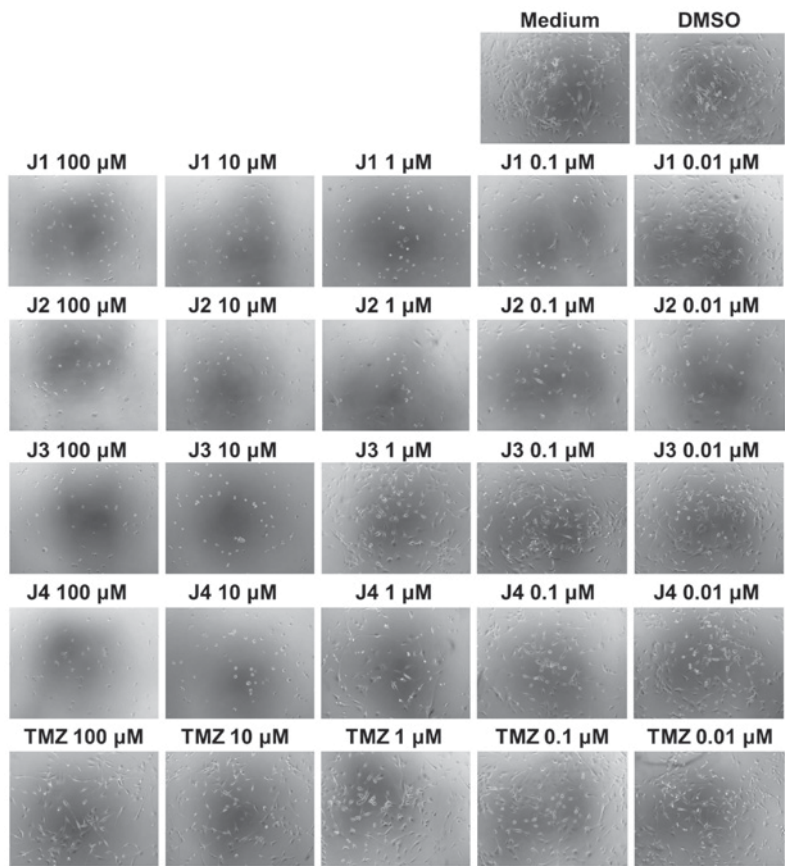


Figure 3. Microscopic images of U87 cells following treatment with medium, DMSO, J1, J2, J3, J4 or TMZ. U87 cells were treated with various concentrations of J1, J2, J3, J4 or TMZ. DMSO and medium treatments served as controls. After 72 h, images were captured under a light microscope. Magnification, x10. DMSO, dimethylsulfoxide; TMZ, temozolomide.



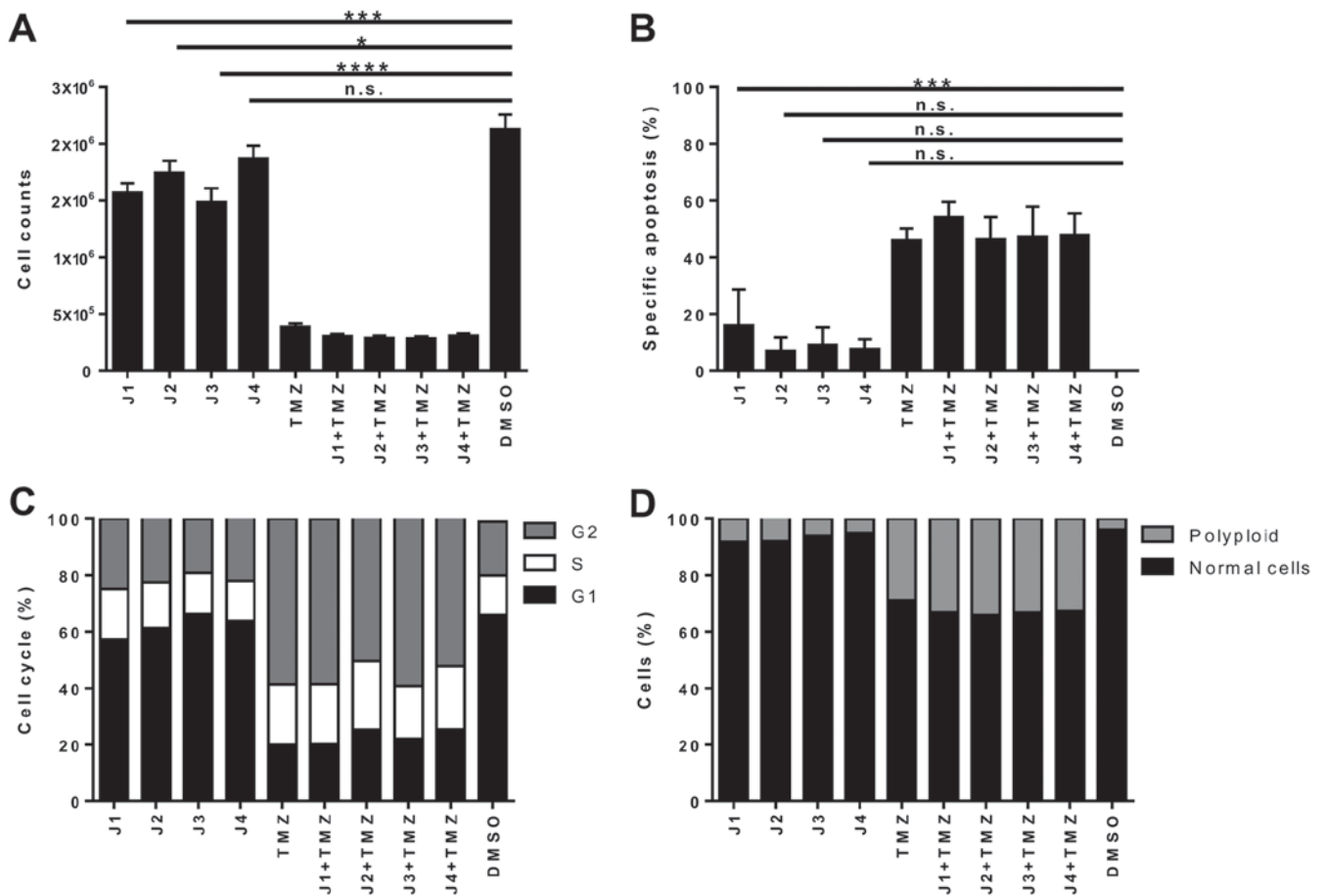


Figure 4. Apoptosis and cell distribution in U87 cells treated with J1-J4. U87 cells were incubated with J1, J2, J3, J4 or TMZ, or a combination of TMZ with J1, J2, J3 or J4, for 144 h. J1-J4 were used at their EC<sub>50</sub> values (J1, 250 nM; J2, 290 nM; J3, 2,200 nM; J4, 500 nM), and TMZ was used at 100  $\mu$ M. (A) Cell numbers were determined using a CASY® 1 DT cell counter. (B) DNA fragmentation, (C) cell cycle distribution of live cells, and (D) analysis of polyploidy, determined using propidium iodide staining and flow cytometry. Results are from three independent experiments. \*P $\leq$ 0.05; \*\*\*P $\leq$ 0.001; \*\*\*\*P $\leq$ 0.0001. n.s., not significant; TMZ, temozolomide; DMSO, dimethylsulfoxide; EC<sub>50</sub>, drug concentration yielding half-maximal response.

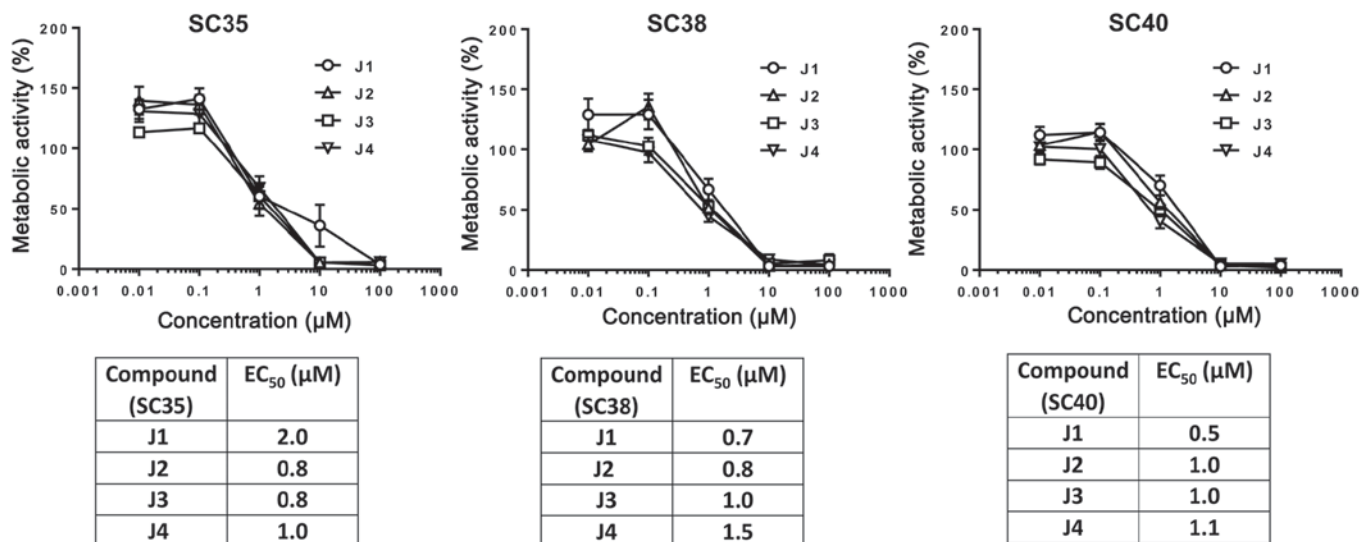


Figure 5. Cell viability of SCs. SC35, SC38 and SC40 cells were cultured in the presence or absence of J1, J2, J3 or J4 for 72 h, and the metabolic activity was determined using an MTT assay. Three independent experiments were performed (n=3). SC, sphere-cultured stem cell-enriched glioblastoma cell population; EC<sub>50</sub>, drug concentration yielding half-maximal response.

to distinct proliferation rates (11). Importantly, the viability of PCs and SCs may be decreased by diisothiocyanate-derived

mercapturic acids; however, SCs are more resistant to diisothiocyanate-derived mercapturic acids.

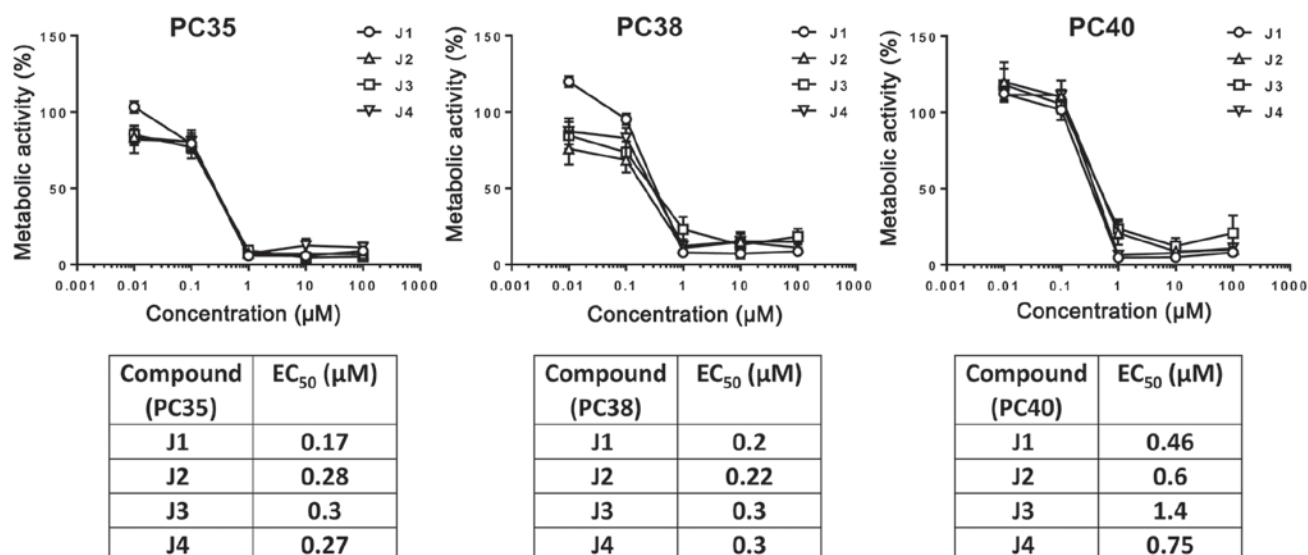


Figure 6. Cell viability of PCs. PC35, PC38 and PC40 cells were cultured in the presence or absence of J1, J2, J3 or J4 for 72 h, and the metabolic activity was determined using an MTT assay. Three independent experiments were performed (n=3). PC, SC-derived differentiated primary glioblastoma cell; EC<sub>50</sub>, drug concentration yielding half-maximal response.

The presence of invasive glioblastoma stem cells in glioblastoma (12) is thought to be the reason for poor survival prognosis. These cells contribute to recurrence and are highly resistant to typical treatments, which is partially due to the increased expression of the multidrug resistance of ATP-binding cassette transporter protein breakpoint cluster region pseudogene 1, DNA repair protein O-6-methylguanine-DNA methyltransferase and anti-apoptotic products in cluster of differentiation 133-expressing glioblastoma stem cells (13). This may explain, at least in part, why in the present study PCs are more sensitive to diisothiocyanate-derived mercapturic acids compared with SCs.

SCs exhibit increased therapy-resistance and are the reason for tumor recurrence (14); therefore, novel substances were sought which had been previously been identified to be selectively toxic to tumor cells. ITCs have been identified to exhibit a selective inhibitory capacity towards tumorigenesis (4). For instance, the ITC iberin induced apoptosis, inhibited tumor cell growth and was cytotoxic to the glioblastoma cell line SNB19 (15). Benzyl ITCs exhibited a decrease in proliferation, invasion and cell viability of U87 cells with an EC<sub>50</sub> of 12.6 μM (16), and a similar effect was identified by using the glioblastoma cell line GBM 8401 (EC<sub>50</sub>, 6 μM) (17,18). Furthermore, phenethyl ITC induced apoptosis in GBM 8401 cells at a final concentration of 8 μM (19). In the present study, it was demonstrated that the recently identified diisothiocyanate-derived mercapturic acids (6) are selectively cytotoxic to the glioblastoma cell line U87 (EC<sub>50</sub> for J1, 250 nM), differentiated glioblastoma cells and glioblastoma stem cells at much lower concentrations compared with ITCs.

In a further set of experiments, it was investigated whether J1-J4 may lower the threshold of intrinsic TMZ resistance in SCs. To address this, J1, J2, J3 or J4 (at EC<sub>50</sub>) were co-cultured (3 days) with 0.6 μg/ml (3.09 μM) TMZ which represents the concentration in the brain following treatment with TMZ (20). No sensitizing effect of J1-J4 for TMZ-induced apoptosis was identified when TMZ was used at physiologically relevant

concentrations (data not shown). Diisothiocyanate-derived mercapturic acids exert a more potent effect in comparison with TMZ, but do not exhibit a sensitizing effect for TMZ-mediated apoptosis.

The cell viability of PBMCs was not impaired by low concentrations of diisothiocyanate-derived mercapturic acids (Fig. 2), which is important since a chemopreventive mediator should activate an antitumor immune response and not inhibit the function of immune cells (21). Therefore, diisothiocyanate-derived mercapturic acids are potential therapeutic components to eliminate glioblastoma stem cells and may be considered for novel therapeutic treatments for glioblastoma.

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