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Targeting glucosylceramide synthase induction of cell surface globotriaosylceramide (Gb3) in acquired cisplatin-resistance of lung cancer and malignant pleural mesothelioma cells

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Abstract

Background: Acquired resistance to cisplatin treatment is a caveat when treating patients with non-small cell lung cancer (NSCLC) and malignant pleural mesothelioma (MPM). Ceramide increases in response to chemotherapy, leading to proliferation arrest and apoptosis. However, a tumour stress activation of glucosylceramide synthase (GCS) follows to eliminate ceramide by formation of glycosphingolipids (GSLs) such as globotriaosylceramide (Gb3), the functional receptor of verotoxin-1. Ceramide elimination enhances cell proliferation and apoptosis blockade, thus stimulating tumor progression. GSLs transactivate multidrug resistance 1/P-glycoprotein (MDR1) and multidrug resistance-associated protein 1 (MRP1) expression which further prevents ceramide accumulation and stimulates drug efflux. We investigated the expression of Gb3, MDR1 and MRP1 in NSCLC and MPM cells with acquired cisplatin resistance, and if GCS activity or MDR1 pump inhibitors would reduce their expression and reverse cisplatin-resistance.

Methods: Cell surface expression of Gb3, MDR1 and MRP1 and intracellular expression of MDR1 and MRP1 was analyzed by flow cytometry and confocal microscopy on P31 MPM and H1299 NSCLC cells and subline cells with acquired cisplatin resistance. The effect of GCS inhibitor PPMP and MDR1 pump inhibitor cyclosporin A for 72 h on expression and cisplatin cytotoxicity was tested.

Results: The cisplatin-resistant cells expressed increased cell surface Gb3. Cell surface Gb3 expression of resistant cells was annihilated by PPMP whereas cyclosporin A decreased Gb3 and MDR1 expression in H1299 cells. No decrease of MDR1 by PPMP was noted in using flow cytometry, whereas a decrease of MDR1 in H1299 and H1299res was indicated with confocal microscopy. No certain co-localization of Gb3 and MDR1 was noted. PPMP, but not cyclosporin A, potentiated cisplatin cytotoxicity in all cells.

Conclusions: Cell surface Gb3 expression is a likely tumour biomarker for acquired cisplatin resistance.
of NSCLC and MPM cells. Tumour cell resistance to MDR1 inhibitors of cell surface MDR1 and Gb3 could explain the aggressiveness of NSCLC and MPM. Therapy with GCS activity inhibitors or toxin targeting of the Gb3 receptor may substantially reduce acquired cisplatin drug resistance of NSCLC and MPM cells.

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### 1. Introduction

Non-small cell lung cancer (NSCLC) and malignant pleural mesothelioma (MPM) are aggressive malignant tumors associated with dismal patient survival. Chemotherapy with cisplatin is frequently used for treatment of advanced stages, usually leading to partial tumor responses or disease stabilization with a slight increase in patient survival time. Cisplatin acts by forming platinum-DNA adducts which hinders rapidly dividing cells from duplicating their DNA for mitosis and activates apoptosis [1,2]. However, a main limitation to the clinical usefulness of cisplatin treatment is the high incidence of acquired drug-resistance [3,4]. Circumventing cisplatin resistance is therefore of prime importance in these tumor diseases.

One major mechanism for cisplatin resistance is reduction of intracellular platinum accumulation [5,6] by either reduced uptake [7] or increased efflux by trans-membrane pumps such as multi-drug resistance 1/P-glycoprotein (MDR1), which was the first ATP-binding cassette (ABC) protein demonstrated to confer resistance to cancer chemotherapeutics [8–10]. Overexpression of MDR1 enhances DNA damage repair and reduces apoptosis induction [11,12]. Other transporter proteins such as multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP) have in addition been described to confer cisplatin resistance. MRP1 also belongs to the ABC transporter super-family and readily extrude glutathione-S-cisplatin conjugates from the cells and is associated with cisplatin resistance [13].

The glycosphingolipids (GSLs) are a subtype of glycolipids that are synthesized by glycosylation of ceramide. Ceramide increases in response to chemotherapy, leading to proliferation arrest and apoptosis. However, a tumour cell reaction follows to eliminate cell surface MDR1 and Gb3 expression or inhibition of GCS activity has not been examined in NSCLC/MPM. Our previous studies suggested an interrelationship between cell multidrug-resistance and plasma membrane Gb3 expression in cultured NSCLC and MPM cells with or without acquired cisplatin resistance. The studies also suggested that cisplatin preferentially eradicates MPM cells with low Gb3 expression. The GCS activity inhibitor DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), or a subtoxic concentration of the Gb3-receptor binding bacterial toxin verotoxin-1, resensitized Gb3-overexpressing cisplatin-resistant NSCLC and MPM subline cells to cisplatin [27].

We hypothesized that Gb3, MDR1 and possibly MRP1 would be co-expressed and co-localized in MPM and NSCLC, and that GCS activity inhibition would confer inhibition not only of Gb3, but also of the expression of MDR1 and/or MRP1 proteins and re-sensitize resistant tumour cells to cisplatin cytotoxicity. To test this hypothesis, we used flow cytometry and confocal microscopy to analyze cell-surface as well as intracellular expression of Gb3, MDR1 and MRP1 and to study if inhibitors of GCS or MDR1 activity would affect their expression in cultured NSCLC and MPM cells with or without acquired cisplatin resistance and re-sensitize the resistant cells to cisplatin.

### 2. Materials and methods

#### 2.1. Cell lines and cell culture

Two human cell lines were used: the MPM cell line P31 [28] and the NSCLC cell line H1299 (American Type Culture Collection, CRL-5803, (Manassas, VA, USA) and their corresponding cell sublines with acquired-cisplatin resistance (P31res and H1299res). The cisplatin-resistant cell sublines were created by culturing the parental cells in medium with gradually increasing concentrations of cisplatin; the LD50 concentration after 72 h incubation with cisplatin needed for the acquired cisplatin-resistant sublines was approx. 4 times as high as for the parental cell line. The cells were maintained under standard cell culture conditions, grown as monolayer culture in Eagle’s MEM in Earl’s salt (Gibco Ltd, Paisley, Scotland, UK) supplemented by 10% fetal bovine serum (Biochrom KG, Berlin, Germany) and 200 mmol/L L-glutamine. They were maintained at 37°C in a humidified atmosphere containing 5% CO2. The maintenance concentration...
of cisplatin was 1.2 mg/L for the P31res and 2.0 mg/L for the H1299res cell sublines.

DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) (Sigma-Aldrich, St Louis, MO, USA), a chemical inhibitor of glucosylceramide synthase activity, and cyclosporin A (Sigma-Aldrich), an inhibitor of MDR1 pump activity, were used to deplete Gb3 expression by culturing cells with 2 μmol/L PPMP or 10 μmol/L cyclosporin A for 72 h, respectively.

2.2. Flow cytometry analysis

P31, P31 res, H1299 and H1299res cells were trypsinized, suspended in PBS and washed with PBS twice. Fixed and permeabilised cells for total, mainly intracellular, expression, and un-fixed and non-permeabilised cells for cell surface expression were obtained by incubating with or without 2% formaldehyde for 1 h in room temperature, followed by one wash with 2% BSA in PBS, with or without permeabilisation in 0.5% Triton X-100 and 0.1% sodium citrate at 4°C for 10 min, and finally washed twice with PBS-BSA. Cells were then stained with rat anti-Gb3 IgM 1:20 (Beckman Coulter Inc., Brea, CA, USA), mouse anti-MDR1 IgG2A 1:50 (Chemicon Internat., Temecula, MA, USA) or mouse anti-MRP1 IgG2A 1:40 (R&D Systems Inc., Minneapolis, MN, USA) primary antibodies. To distinguish from non-specific binding, cells were stained with corresponding rat IgM and mouse IgG2A isotype antibodies (Invitrogen, Carlsbad, CA, USA). Subsequently, cell pellets were re-suspended and incubated with Alexa Fluor 488 or 647–labeled goat anti-rat IgM (1:50, Invitrogen) and goat anti-mouse IgG2A (Invitrogen) secondary antibodies (1:100) for 1 h at 4°C. After washing the cells and centrifuging for 10 min, cells were analyzed with a FACScan flow cytometer (Becton Dickinson Immunotech Systems, San Jose, CA, USA) on channels FL4 or FL1 and data was processed using the BD CellQuest® software (Becton Dickinson). After gating out debris and cell aggregates, the data was dot plotted. The analysis for MDR1 and MRP1 was made separately, which is why there is more data on Gb3 than MDR1 and MRP1.

2.3. Confocal microscopy analysis of expression and inhibition of Gb3, MDR1 and MRP1

The four cell line/subline cells were grown on glass cover-slips for a total of three days, the total number of cells added to each slide were 20,000 for P31, 60,000 for P31res, 10,000 for H1299 and 120,000 for H1299res. The seeding density for cells growing with cyclosporin A and PPMP were doubled to achieve similar confluence. Incubation with isotype or primary antibodies (Gb3, MDR1, MRP1 – see flow cytometry) at room temperature for 1 h was followed by incubation with Alexa Fluor®-labeled secondary antibodies (see flow cytometry) for another hour at room temperature. Photobleaching was prevented by using ProLong® Gold Anti-fade DAPI (Invitrogen, Carlsbad, CA, USA) containing 4', 6-diamidino-2-phenylindole (DAPI) for nuclear staining. Images were acquired using ZEN 2010 imaging software on a LSM 710 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

Quantitation of the fluorescence intensity of the confocal microscope images was performed using the Imaris software (Bitplane, Switzerland). This method allows for determination of fluorescence intensity in small well-defined volumes (VOI) in single cells, permitting the comparison of different cells for the same proteins. VOI represents the number of voxels in the x, y- and z-dimension, where size and resolution depends on the magnification, the numerical aperture of the objective lens and the number of layers defined at the time of confocal data acquisition. The fluorescence threshold was set against the fluorescence of isotype antibodies so that only cells with elevated protein expression were quantified.

2.4. Cell viability assay

A fluorometric method utilizing fluorescein diacetate, FDA, (Amersham International, Amersham, UK) was used to quantify viable cells and determine cisplatin (Bristol-Myers Squibb, New York, NY, USA) sensitivity of all H1299 and P31 cell line/subline cells in combination with PPMP or cyclosporin A in vitro. Cells (3–10 × 10^6) were plated with 100 μL medium in the wells of 96-well microtiter plates. The plates were first incubated at 37°C for 24 h with culture medium only, then medium was replaced with fresh medium, containing LD50 concentrations of cisplatin and/or 2 μmol/L PPMP or 10 μmol/L cyclosporin A for 72 h. Medium was removed by flicking the plate and wells were washed once with 200 μL PBS buffer. To each well 100 μL of PBS containing 10 mg/L FDA was added, and the plates incubated for 45 min at 37°C, followed by fluorescence determination in a fluorometer (LS 55, Perkin Elmer, MA, USA) using 485 and 538 nm for excitation and emission, respectively. The combined cytotoxicity of two drugs incubated simultaneously was compared against a combination index calculated for an expected additive cytotoxic effect of the drugs per se.

3. Statistics

SPSS 22.0 was used for statistical analysis. Mann–Whitney U-test was performed. The level of significance to reject the null hypothesis was p<0.05. All results are presented as mean ± 2 S.E.M.

4. Results

4.1. Flow cytometry analysis of MDR1 and MRP1 expression

4.1.1. Flow cytometry on fixed and permeabilised cells

The distribution of individual cell total (mostly intracellular) expression of MDR1 and MRP1 was evaluated by FACs analysis on fixed and permeabilised cells. All parental as well as subline cells demonstrated a high expression of MDR1 protein, whereas MRP1 was expressed to a less extent by a high proportion of P31 parental (99%) and subline (79%) cells but less by H1299 parental (62%) and subline (72%) cells (Table 1). MRP1 levels were 2.7 to 5.3 times that of the isotype control and with no apparent mean fluorescence differences between parental and cisplatin-resistant sublines (Table 1). MDR1 protein was however expressed to a much higher extent in all cell lines with fluorescent means 44.0–79.3 times that of the isotype control, again with no differences between parental and resistant cells (Table 1).

Incubation of the cells with 2 μmol/L PPMP or 10 μmol/L cyclosporin A for 72 did not significantly affect MDR1 and MRP1 fluorescence of any of the tested cells/subline cells (data not shown).
4.1.2. Flow cytometry on non-permeabilised cells

Cell surface expression of Gb3 in relation to MDR1 and MRP1 protein expression was evaluated by FACS analysis on non-fixed and non-permeabilised cells. Only a small number of cells expressed cell surface MRP1 protein expression (results not shown) and further studies of this protein was discontinued. Gb3 was detected in 25% of the cisplatin-resistant p31res cells and 19% of the cisplatin-resistant H1299res cells (Fig. 1), MDR1 was detected in 1% and 2% of the cells in resistant sublines (Fig. 2), respectively. Flow cytometry scattergrams showed a correlated co-expression of MDR1 and Gb3 only in a small streak of cells in the upper right flow cytometry quadrant of the resistant cell sublines (Fig. 1) and at least partly due to unspecific binding as the isotype control demonstrated a similar expression pattern.

Incubation of resistant subline cells with 2 μmol/L PPMP for 72 h before FACS analysis reduced the subfraction of cells with increased Gb3 cell surface expression to 3–8% of all cells whereas MDR1 expression was largely unaffected (Fig. 1) Incubation with 10 μmol/L cyclosporin A did not affect Gb3 or MDR1 expression in these cells (Fig. 1).

4.1.3. Confocal microscopy analysis of Gb3, MDR1 and MRP1 expression

Laser-scanning confocal microscopy was then used for visualization of the distribution and possible co-localization as well as software quantification of Gb3, MDR1 and MRP1 cell surface overexpression as cells below the isotype antibody limits was gated away. No expression of MRP1 was noted. There was no sign of co-localization of Gb3 and MDR1.

Using the Imaris software to objectively quantify the mean fluorescence intensity of confocal microscopy images of cells with cell surface Gb3 and MDR1 expression with and without PPMP and cyclosporin A demonstrated that P31 cells on acquisition of cisplatin resistance on average expressed extensively more (p<0.001) Gb3 and MDR1 (p<0.01) (Fig. 3). The Gb3 expression of H1299 cells on acquisition of resistance was not affected (p=N.S.), whereas MDR1 was markedly increased (p<0.001).

Incubation with 10 μmol/L cyclosporin A decreased (p<0.01) MDR1 and Gb3 cell surface mean fluorescence in H1299 parental cells but did not affect the expression in the other cell lines (Fig. 3).

Incubation with 2 μmol/L PPMP almost eradicated cell surface Gb3 expression in all cell lines except possibly in P31 parental cells with already very low Gb3 expression, whereas MDR1 expression was decreased in H1299 (p<0.05) and P31res cells (p<0.05) (Fig. 3).

4.1.4. Effect of PPMP and cyclosporin A exposure on cisplatin cytotoxicity

Exposure of all NSCLC and MPM cells (not only the fraction with increased Gb3 expression) to LD_{50} concentrations of cisplatin for 72 h demonstrated cytotoxicity to the parental cells and cisplatin-resistant cell sublines (Fig. 4). The cytotoxicity of 2 μmol/L PPMP per se reduced cell viability to approx. 80% with the exception of P31 cells where it was approx. 60% and 10 μmol/L cyclosporin A per se reduced the viability to 30–60%. The effect of PPMP on cisplatin cytotoxicity using LD_{50} concentrations was more than additive on all parental and sublines cells but not H1299res subline cells where an approx. additive effect was noted. The effect of cyclosporin A was additive on all parental and subline
cells but not P31 parental cells where cytotoxicity was found to be more than additive (Fig. 4).

5. Discussion

Cell surface Gb3 expression was induced in MPM and NSCLC cell lines with acquired cisplatin resistance. The glycosphingolipid synthesis inhibitor PPMP radically decreased the induced cell surface Gb3 but not MDR1 protein expression and also augmented cisplatin cytotoxicity of the resistant MPM cell subline. The results indicate that expression of cell surface Gb3 is a biomarker of acquired cisplatin resistance in MPM and NSCLC. Perhaps cell surface Gb3 reflects the functional resistance to cisplatin better than cell surface expression of MDR1. Therapy with GCS inhibitors reducing ceramide glycosylation or with Gb3 receptor-binding verotoxin-1 may overcome or reduce acquired cisplatin drug resistance in NSCLC and MPM [27].

Lung cancer is the first and second leading cause of cancer-related death in men and women, respectively [30,31]. The most common type is non-small cell lung cancer (NSCLC), which accounts for over 75% of all cases [32]. Malignant pleural mesothelioma (MPM) is a high mortality malignancy with poor prognosis partially because of treatment resistance [33]. Treatment options often include platinum-based drugs such as cisplatin (cis-diamminedichloroplatinum (II), which is extensively used. However, the occurrence of inherent resistance and especially, treatment-induced acquired resistance to cisplatin is a major clinical problem that undermines the curative potential of the drug [34].

A well characterized form of drug resistance is due to overexpression of drug efflux pumps, especially MDR1 of the ABC (ATP-binding cassette) transporter super-family, capable of efflux of many different chemotherapeutic agents, leading to decreased drug accumulation [6,21]. The importance of ATP-cassette protein efflux in cisplatin resistance [35] has however been questioned.
as specific MDR1 transport inhibitors were unable to restore cisplatin accumulation and sensitivity [36].

Ceramide and glycosphingolipids (GSL) are important molecules as they affect cell functions such as apoptosis, proliferation, endocytosis, transport and migration, and thereby regulate tumor genesis, cancer progression, and cancer treatment efficiency [37]. Glucosylceramide synthase (GCS) is a key enzyme for ceramide glycosylation and GSL synthesis. Following cell stress, such as
chemotherapy, tumor cells persistently up-regulate GCS to deplete (drug-induced) ceramide pools, which would otherwise prove cytotoxic, via conversion to a more benign GSL format such as Gb3 [38]. Excessive GCS expression has been detected as a cause of drug resistance in a multitude of cancer cell lines [39].

Silencing GCS expression or inhibition of GCS activity sensitized these resistant cells to more than 20 anticancer agents of diverse types including cisplatin. Introduction of the GCS gene into drug-sensitive cells confers cellular resistance to several drugs, in most, but not to all tested cell lines, indicating that drug resistance to

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**Fig. 3** – Quantification of cell surface (A) Gb3 (globotriaosylceramide) and (B) MDR1 (multidrug resistance 1) expression in parental cell lines non-small cell lung cancer cell line H1299 and the malignant pleural mesothelioma cell line P31 as well as their cisplatin-resistant sublines H1299res and P31res. Quantitation of the fluorescence intensity (voxels) of the confocal microscope images using the Imaris software (Bitplane, Switzerland). The fluorescence threshold was set against the fluorescence of isotype antibodies so that only cells with elevated protein expression were quantified. Mean expression ± 2 S.E.M. from 9 images (taken from three different experiments, total number of cells was at least 300). Mann–Whitney U-test was used to compare expression between groups.

***p < 0.001, **p < 0.01, *p < 0.05 compared to control.

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**Fig. 4** – Cell viability assays of exposed cells to LD50 cisplatin, with or without PPMP and cyclosporin A alone or in combination. Cell viability (fluorescence of FMCA assays – control cells set to 100%) after exposure of malignant pleural mesothelioma cell line P31 and non-small cell lung cancer cell line H1299 NSCLC cell lines and their cell sublines with acquired cisplatin resistance (P31res, H1299res) incubated with LD50 cisplatin (1 mg/L for P31 and H1299 cells, 5 mg/L for P31res and H1299res cells without or with 2 μmol/L PPMP or 10 μmol/L cyclosporin A alone or in combination for 72 h. Arrows on/above histogram bars of drug combinations denote the expected result of an additive cytotoxic effect when calculating on the effect of the drugs alone. Mean viability ± 2 S.E.M. (n = 6). Mann–Whitney U-test was used to compare viability between groups.

***p < 0.001 (compared to control), ###p < 0.001 (compared to cisplatin and cyclosporin A/PPMP).
some extent is cell-type or cancer-dependent [39]. However, this mechanism has hitherto not been tested in MPM or lung cancer.

MDR1 has been proposed as a specific glucosylceramide transporter for the molecule across the Golgi to deliver it for the synthesis of GSLs [22]. Tumour chemotherapy increases cell stress and generates ceramide which drives cells to proliferation arrest and apoptosis but in addition also transactivates GCS expression via the Sp1 transcription factor leading to globo-series GSLs (e.g. globotriaosylceramide (Gb3)) increase which in turn activates cSrc kinases, and thereby increases nuclear β-catenin and transactivates MDR1 gene expression. In MDR1-MDCK cells, cell surface MDR1 co-localized with globotriaosylceramide (Gb3) and a soluble analog of Gb3 proved to be an MDR1 inhibitor [23]. Ceramide as well as the GSLs therefore up-regulate GCS and MDR1 expressions in response to anticancer drugs, and confer acquired tumour cell resistance by preventing ceramide-induced apoptosis and possibly MDR1-mediated drug efflux [14,26]. Inhibitors of GCS may therefore be useful in preventing chemotherapy resistance [22,23].

We have previously demonstrated that Gb3 expression was significantly increased on the cell surface of MPM and NSCLC cell sublines with acquired cisplatin resistance [26]. In the present study, cell surface Gb3 expression was increased in a large fraction of the resistant subline cells and MDR1 was co-expressed in only a minute fraction of cells noted by flow cytometry whereas MRP1 cell surface expression was not detected. PPMP, almost eradicated the induced cell surface Gb3-expression in resistant subline cells whereas cell surface MDR1 expression was reduced only in H1299 cells. It is noteworthy that silencing of Gb3 with PPMP augmented the sensitivity to cisplatin in cisplatin-resistant MPM subline cells possibly back to the cisplatin sensitivity of the parental cells as we previously demonstrated [27]. MDR1 expression was only decreased by cyclosorpin in H1299. This was also the only cell line where a decreased Gb3 expression was noted following cyclosorpin incubation, indicating that MDR1 might have a role in the extracellular expression of Gb3 in these cells.

The bacterial toxin verotoxin-1 exerts its cytotoxicity by targeting cell surface Gb3, which is the functional receptor of verotoxin-1 [40]. Combination of cisplatin and subtoxic concentrations of verotoxin-1 led to a super-additive increase of cytotoxicity and TUNEL staining, especially in the cisplatin-resistant cell sublines [27]. Verotoxin-1-Gb3 binding results in internalization, retrograde transport via endosomes, TGN, and Golgi to the ER [40,41]. Here the proteolytically cleaved A1 subunit is translocated to the cytosol to inactivate protein synthesis by depurination of the 28S RNA of the 60S ribosomal subunit [42]. Cellular glucosylceramide is required to maintain Gb3 in verotoxin-1-detectable plasma membrane lipid rafts [16], and is also required for ER retrograde transport of verotoxin-1. Cells without lipid raft expression of Gb3 are thus insensitive to VT-1 [16].

Cell surface Gb3 but less likely MDR1, but not MRP1 up-regulation seems to be required for drug resistance to cisplatin in MPM and NSCLC cells. This means that cell surface Gb3 could be an important resistance determinant and that the correlation to MDR1 is of less importance to acquired cisplatin resistance in MPM and NSCLC. This may however vary with the tumour type or cell line studied, as several reports suggest that cells which overexpress MDR1 have higher levels of specific glycosphingolipids compared to their parental sensitive cells [25,43–46]. Inhibition of GSL biosynthesis results in the loss of drug resistance and decreased expression of MDR1 [21,47]. In addition, newly synthesized Gb3 seems important for verotoxin toxicity which might indicate that Gb3 expressed as a consequence of acquired cisplatin resistance might be more important for conveying cytotoxicity than intrinsically expressed Gb3 [48].

MDR1-mediated glucosylceramide flipping within the Golgi is a primary mechanism by which glucosylceramide is provided as a substrate for the various luminal glucosyl transferases involved in neutral GSL biosynthesis [22]. In addition to the interaction of MDR1 with glucosylceramide, cell surface MDR1 was found to be partially co-localized with Gb3 in MDR1 transfected cells [21]. Inhibition of GSL biosynthesis resulted in the loss of drug resistance and of cell surface MDR1. In MDR1-MDCK cells, cell surface MDR1 co-localized with globotriaosyl ceramide (Gb3), and a soluble analog of Gb3 proved an MDR1 inhibitor [23]. MDR1 and GCS have been shown to be coincidently overexpressed in several drug-resistant cell lines [14,49]. Localization of MDR1/Pgp in intracellular membranes has also been reported [50–52]. Its expression on the nuclear membrane has been associated with extrusion of drugs from the nucleus of multidrugresistant cells [52,53]. There was no sign of colocalization in any cell line. This indicates that co-localization of Gb3 with MDR1 was without significance for cisplatin resistance development or for the re-sensitation of the resistant NSCLC or MPM cell sublines to cisplatin with PPMP.

6. Conclusions

Taken together, these results demonstrate that GCS as reflected by ensuing Gb3 expression might have a regulatory role in acquired cisplatin drug resistance in NSCLC and MPM cells. Inhibition of GCS or direct verotoxin-1 targeting of over-expressed cell surface Gb3 could be efficient approach to reverse acquired cisplatin resistance of non-small cell lung cancer and malignant pleural mesothelioma.

Competing interests

The authors declare that they have no competing interests.

Authors contributions

The work presented was carried out in collaboration between all authors. KG, AJ, AT and PBM defined the theme, designed methods and experiments. AT and PBM carried out the flow cytometry, confocal microscopy and cytotoxicity tests. TK and SKG aided AT and PBM to confocal microscopy and KG, AJ, PBM and TB interpreted the data. All authors read and approved the final manuscript.

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