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Cisplatin Resistance in Malignant Pleural Mesothelioma

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1. Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumour. Patients have a very short median survival after diagnosis. Inherent but importantly, also induced, tumour survival mechanisms of malignant pleural mesothelioma is a major contributor to the short survival time (Brida et al., 2007). No curative treatment is available. Mesothelioma has been subject to therapeutic trials, hitherto with little clinical gain. Most targeted therapies have focused on impairing signaling pathways on which cancers depend for survival. However, the efficacy of these approaches is impaired by signaling redundancy, adaptation and activating/silencing mutations.

Apoptosis is a physiological form of cell death that removes mutated and damaged cells. Deregulation of apoptosis in tumour cells allows them to bypass apoptosis during clonal expansion. Cancer-generating mutations disrupt apoptosis, leading to tumor initiation, progression or metastasis. The mutations that suppress apoptosis also reduce the sensitivity to cancer treatment (Johnstone et al., 2002). There are potential benefits to targeting tumors at the level of their anti-apoptotic defenses. Direct inhibitors of anti-apoptotic defenses could bypass the need to inhibit multiple pathways by moving to a distal level at which multiple signals are integrated. Also, stimulators of apoptosis through specific signaling pathways that emerge during cancer development or are acquired during cancer chemotherapy could be targeted. Such an approach may be effective in the different mesothelioma subtypes, against a variety of apoptosis-inducing therapies, and without substantial injury to normal tissues where apoptosis signaling is not disturbed.

Cisplatin, most often in combination with other drugs is still the treatment of choice for mesothelioma. Cisplatin chemotherapy activates several signaling pathways and can cause cell cycle arrest, DNA repair and cell survival, and execution of cell death (Wang et al., 2004). Cisplatin kills cancer cells by inducing apoptosis (Chu, 1994) and specifically activates the mitochondrial signaling pathway of apoptosis, which is initiated by activation of BH3-only proteins i.e. pro-apoptotic members of the Bcl-2 family of proteins.

Targeting the anti-apoptotic Bcl-2 family of proteins, thus restoring apoptotic pathways, may overcome drug resistance to cancer chemotherapy. Down-regulation of Bcl-x_L or its

cooperative Bcl-2 family member Mcl-1 expression or activity increases apoptosis *per se* as well as cisplatin sensitivity in MPM. The importance of these anti-apoptotic proteins in the protection against chemotherapy-induced apoptosis makes them obvious targets to (re-) chemosensitize malignant mesothelioma tumour cells (Varin et al., 2010).

Glycosylation defects seem universal in carcinogenesis. They may alter cell signalling, growth, adherence and motility. Essentially all experimental and human cancers have altered glycosphingolipid composition and metabolism (Hakomori & Zhong, 1997), and several tumour-associated antigens are indeed glycosphingolipids. The globotriaosylceramide membrane receptor (Gb3) is over-expressed in many human tumours and tumour cell lines including MPM with inherent or acquired MDR. Gb3 is co-expressed and interplays with the membrane efflux transporter P-gp encoded by the *MDR1* gene associated with multidrug resistance.

The bacterial toxin verotoxin-1 (VT-1) exerts its cytotoxicity by targeting Gb3. Recent work has shown that apoptosis and inherent or acquired multidrug resistance in Gb3-expressing MPM tumour cells could be affected by VT-1 holotoxin, a sub-toxic concentration of the holotoxin concomitant with chemotherapy or its Gb3-binding B-subunit coupled to cytotoxic or immuno-modulatory drug, as well as chemical manipulation of Gb3 expression (Johansson et al., 2010). The interplay between Gb3 and P-gp thus gives a possible physiological approach to augment the chemotherapeutic effect in multidrug resistant malignant mesothelioma.

We hereby summarize the possibility to target also Bcl-2 and Gb3 signalling as two novel treatment possibilities to overcome or re-sensitize resistant malignant pleural mesothelioma tumour cells to cisplatin.

2. Targeted chemotherapy of malignant pleural mesothelioma

Chemotherapy will continue to be important in the therapy of MPM. An increased understanding of the major molecular pathways of intrinsic and acquired resistance of MPM is needed to develop diagnostic, therapeutic, and prevention methods. Ideally, future MPM therapy should be guided by molecular characteristics of the tumour rather than clinical stage and patient features. Insights into the biological basis of MPM may lead to personalized treatment involving also early detection of poor prognostic indicators that will reduce the heterogeneity of the clinical response (Zucali et al., 2011).

None of the hitherto explored targeted treatments can currently be recommended as standard treatment in MPM. A much used combination of cisplatin and pemetrexed has yielded enhanced response rates. Most patients with MPM progress during or shortly after first-line treatment. Due to the limited efficacy of chemotherapy, new treatment options are clearly warranted and several targeted agents have thus been explored (Jakobsen & Sørensen, 2011).

Basic cancer research has given the insight that apoptosis and the genes that control it have a profound effect on the malignant phenotype. Most conventional treatments depend on an ability to induce apoptosis as a final common pathway (Zucali et al., 2011). The unresponsiveness of mesothelioma to most conventional agents may in part be

explained by a resistance to the induction of apoptosis. The mutations that suppress apoptosis also reduce the sensitivity to cancer treatment (Johnstone et al., 2002). Most targeted therapies have focused on impairing signaling pathways on which cancers depend for survival.

There are potential benefits to targeting tumors at the level of their anti-apoptotic defenses. Direct inhibitors of anti-apoptotic defenses could bypass the need to inhibit multiple pathways by moving to a distal level at which multiple signals are integrated. Also, stimulators of apoptosis through specific signaling pathways that emerge during cancer development or are acquired during cancer therapy could be targeted. Such an approach may be effective in the different mesothelioma subtypes, against a variety of apoptosis-inducing therapies, and without substantial injury to normal tissues.

3. Cisplatin signal transduction during therapy and cisplatin resistance

3.1 Cisplatin therapy

Cisplatin kills cancer cells by inducing apoptosis (Chu, 1994). The pathways leading to cisplatin-induced apoptosis are subject of considerable current interest, hence yet not fully understood, but is known to involve the activation of the MAP kinases SAPK/JNK (stress-activated protein kinases/c-Jun-N-terminal kinases) and p38 kinases, leading to the induction of caspase activity and apoptosis (Mansouri et al., 2003; Brozovic & Osmak, 2007). The mitochondrial signaling pathway to apoptosis has recently attracted attention and an increasing number of articles on the effect of cisplatin action and cisplatin resistance involving the expression of Bcl-2 family proteins have been published.

3.2 Inherent and induced cisplatin resistance

Tolerance might occur to platinum, through decreased expression or loss of apoptotic signaling pathways (either the mitochondrial or death-receptor pathways) as mediated through various proteins such as p53, anti-apoptotic and pro-apoptotic members of the BCL-2 family, and JNK (Adducci et al., 2002).

The challenge is thus to determine the mechanism of why some tumours, and MPM in particular, are intrinsically resistant to cisplatin and how enhanced cisplatin resistance eventually is acquired during the course of therapy. The acquired resistance may not necessary be an enhancement of the tumours intrinsic resistance mechanisms. All alternations of signaling pathway that may lead to enhancement of apoptosis must thus be exploited.

Reduced cellular accumulation of platinum either by impaired uptake or increased efflux is often found in cells selected for cisplatin resistance, both *in vivo* and *in vitro*, and is generally considered as one of the most consistent characteristics of platinum resistant cells (Gately & Howell, 1993). Many papers have reported that the expression of the ATP-binding cassette (ABC) transport proteins renders tumor cells resistant to chemotherapeutic drugs that are substrates of these transporter proteins. The ABC transporter protein family includes multidrug resistance proteins (ABCB1), multidrug resistance-associated proteins (ABCC), and breast cancer resistance protein (ABCG2).

3.3 Cisplatin chemotherapy and apoptosis

The failure of drug-induced apoptosis is an underlying cause of drug resistance. Some studies have identified a number of key mediators of apoptosis that are altered in chemoresistant cancer cells. Resistance to cisplatin might occur through decreased expression or loss of pro-apoptotic factors or increased expression of anti-apoptotic proteins (Brozovic & Osmak, 2007).

Apoptosis is initiated either by intrinsic death stimuli such as oncogene activation and DNA damage, or as response to extrinsic death stimuli such as binding to their cell surface receptors of the Fas or TNF α ligands as part of an immune response. Both apoptosis initiation signalling pathways meet at the mitochondrial outer membrane (MOM) where the Bcl-2 protein family members regulate apoptosis. Mitochondrial outer membrane permeabilization (MOMP), intermembrane space (IMS) protein release, activation of pro-apoptotic Bcl-2 and caspase family proteins have been identified as key events in the initiation, progression and execution of apoptosis. But, there is still much that we do not understand about how cell death signaling is fine-regulated, particularly how the signaling within a single cell is coordinated for its ultimate orchestration at the population level.

Apoptotic cysteine-aspartate proteases (caspases) are essential for the progression and execution of apoptosis, and detection of caspase fragmentation or activity is often used as markers of apoptosis. We compared a cisplatin-resistant pleural malignant mesothelioma cell line (P31res1.2) with its more cisplatin-sensitive parental cell line (P31) regarding the consequences of in vitro acquired cisplatin-resistance on basal and cisplatin-induced (equitoxic and equiapoptotic cisplatin concentrations) caspase-3, -8 and -9 fragmentation and proteolytic activity. Acquisition of cisplatin-resistance resulted in basal fragmentation of caspase-8 and -9 without a concomitant increase in proteolytic activity, and there was an increased basal caspase-3/7 activity. In P31s, we found that cisplatin exposure resulted in caspase-9-mediated caspase-3/7 activation, but in the induced cisplatin-resistant sub-line, P31res1.2, cisplatin-induced caspase-3/7 activation occurred before caspase-8 or -9 activation. We concluded that in vitro acquisition of cisplatin-resistance rendered P31res1.2 cells resistant to caspase-8 and caspase-9 fragments and that cisplatin-induced, initiator-caspase independent caspase-3/7 activation was necessary to overcome this resistance (Janson et al., 2010).

4. The intrinsic apoptotic pathway in cancer: The Bcl-2 family

The Bcl-2 family consists of three groups of proteins, the anti-apoptotic proteins Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1, the pro-apoptotic proteins, Bax, Bak and Bok, and the pro-apoptotic BH3-only proteins members such as Bad, Bim, Bid and Noxa (Shamas-Din et al., 2011).

The pro-survival members act to preserve the mitochondrial outer membrane permeabilization through the sequestration of pro-apoptotic proteins that induce cell death by triggering the release of cytochrome c and the subsequent caspases and nucleases activation. The BH3-only proteins act as cellular guards that sense the apoptotic signal and modulate the function of the other pro-apoptotic Bcl-2 family members. BH3-only proteins

inhibit the anti-apoptotic proteins and activate the pro-apoptotic proteins to cause MOM permeabilisation (MOMP) leading to release of cytochrome *c* and SMAC into the cytoplasm where they activate caspases leading to apoptosis and elimination of the cell. MOMP is regarded as the 'point-of-no-return' for a cell (Varin et al., 2010).

The intricate balance between antagonistic anti- and pro-apoptotic Bcl-2 family proteins may thus have a deciding huge role on the susceptibility of cells to apoptosis. Studies of the mechanisms of activation or inhibition of the MOMP proteins Bax and Bak and the activator BH3-only proteins with the aim of modifying these processes is an important therapeutic goal in order to restore the three possible blocks that cancer cells can exploit: loss of BH3-only proteins (or inhibition of their activation), a reduction or elimination of multi-region pro-apoptotic proteins, and increased expression of an apoptosis inhibitor such as Bcl-2 or Mcl-1 (Deng et al., 2007).

The most common block noted in human cancers is the over-expression of anti-apoptotic proteins. Tumours are often dependent on the presence of one or more anti-apoptotic protein for survival. Being able to abolish Bcl-2 anti-apoptotic signalling would therefore represent a form of synthetic lethality that would kill predominantly cancer cells rather than normal cells that do not automatically engage apoptotic machinery. In tumours with the loss or inhibition of BH3-only proteins, BH3 mimetics replace the need to induce expression or activate BH3-only proteins to initiate death. On the other hand, in cancers over-expressing anti-apoptotic Bcl-2 family proteins, BH3 mimetics can compete with endogenous activator BH3-only proteins for binding to anti-apoptotic proteins (Leber et al., 2010).

Over-expression of *BCL-2* and *BCL-XL* genes contribute to apoptotic inhibition and the development of the multidrug resistance of human cancers. Directly inhibiting the interaction between the anti-apoptotic proteins and BH3 proteins would lead to apoptosis of multi-drug resistant tumour cells (Shamas-Din et al., 2011).

The BH3 mimetic ABT-737 binds to Bcl- χ_L and Bcl-2 but not to Mcl-1 (Oltsersdorf et al., 2005). ABT-737 displaces Bad and Bim from the binding pocket of Bcl-2 stimulating apoptosis. Cancer cells with higher levels of Bcl-2 and Bcl- χ_L , but lower levels of Mcl-1 are therefore sensitive to ABT-737 (Del Gaizo Moore et al., 2007). Mcl-1 expression (maintaining anti-apoptotic function) is the determinant of resistance to ABT-737 (Deng et al., 2007).

Because resistance can be reversed by reducing Mcl-1 levels, combination with an inhibitor of Mcl-1 will permit a selective kill of a wide variety of tumours. Obatoclax is a derivate that in addition binds also to Mcl-1, and may overcome tumour cell resistance to ABT-737. BH3-mimetics that selectively antagonize the anti-apoptotic proteins may prove to be successful in cancer therapy (Shamas-Din et al., 2011).

5. Signal transduction to apoptosis in malignant pleural mesothelioma. Role of Bcl-2 family proteins

Apoptosis is an uncommon event in mesothelioma and low mean cleaved caspase-3 index has been demonstrated (Jin et al., 2010). Over-expression of anti-apoptotic proteins have been implicated in MPM resistance to therapy (O'Kane et al., 2006).

5.1 Apoptosis related to p53 inactivation

The tumour suppressor p53 is referred to as the guardian of the genome. It is mutated in a wide variety of human cancers. A key function is to induce growth arrest or apoptosis following stress. The apoptotic pathways regulated by p53 involve the Bcl-2 family of pro- and anti-apoptotic regulators. The p53 tumour suppressor transcriptionally regulates the expression of pro-apoptotic Bcl-2 members Bax, Noxa and Puma, which stimulate mitochondria-mediated apoptosis. Inactivating the apoptotic pathways regulated by p53 is crucial for emerging tumour cells and is most often accomplished by inactivation of p53 itself. Some tumours however retain wild type p53 expression but apoptotic suppression is instead performed by deregulating Bcl-2 family members such as Bcl-2 and Bcl-x_L (Yip & Reed, 2008).

Genetic inactivation of p53 by alteration of *TP53* is rare in MPM. Targets of the tumour suppressing microRNA miR-34b/c include down-regulation of Bcl-2. miR-34b/c is frequently down-regulated by aberrant methylation in MPM, resulting in the loss of tumor-suppressive p53 function. Epigenetic silencing of miR-34b/c might explain why p53 functions are impaired in MPM despite the presence of intact p53 in the majority of MPM. Normalization of miRNA expression could be a potential method of therapeutic intervention. As miR-34b/c acts as a tumor suppressor, its expression should be restored in targeted tumor cells by the delivery (Kubo et al., 2011).

5.2 MAP kinases activate cisplatin-induced apoptosis in malignant pleural mesothelioma

Mitogen-activated protein kinases (MAPKs) are activated in cisplatin-induced apoptosis in most investigated cell systems and induced cisplatin resistance is also associated with reduced activation of MAPKs. The mitogen activated protein kinase (MAPK) family of proteins (extracellular signal-regulated kinase (Erk) which are activated by growth hormone receptors via a Ras/Raf pathway and the c-Jun N-terminal kinase (Jnk) and p38 kinase are via Mkk4/Mkk7 and Mkk3/Mkk6 pathways, respectively, sequentially activated and regulate a number of cellular functions, including survival, growth, differentiation and apoptosis. The Erk pathway together with the PI3K/Akt pathway, are important mediators of cell growth, survival and differentiation i.e. pro-survival. The Jnk and p38 pathways are particularly associated with diverse apoptotic paradigms, mediated by transcription dependent and independent mechanisms (Brozovic & Osmak, 2007).

In pleural mesothelioma, p38 MAPKs have been suggested to play a major role in carcinogenesis and aggressiveness of tumours. The Erk1/2 and p38 MAPK signalling pathways can stimulate the expression of heat-shock-proteins (Hsp), Hsp27, Hsp40, and Hsp70 which can rescue tumour cells from cell death. Hsp70 increases the resistance of MPM cells to chemotherapeutic drugs. Intracellular Hsp has been implicated in the protection of tumour cells from apoptosis of other tumour types, while secreted Hsp stimulate the immune system to attack tumour cells (Roth et al., 2009).

The constitutively active p38 MAPK by MPM cells was essential for the expression of Hsp40 and Hsp70, while Erk1/2 MAPK seemed to play less important role. The p38 MAPK was constitutively activated and needed the interaction with Erk1/2 MAPK to increase Hsp expression. This constitutively active p38 MAPK together with Erk1/2 MAPK are central for

MPM cell proliferation. In contrast to other tumour types a central role of Hsp70 in heat stress survival is indicated for MPM. It might be more effective to suppress both Hsp40 and Hsp70 to sensitize MPM cells to heat. Therefore the inhibition of Hsp40/Hsp70 or Erk1/2 MAPK might present a new option to increase the success of hyperthermia in mesothelioma (Zhong et al., 2011).

5.3 Bcl-2 family member protein expression in MPM

Whereas expression of anti-apoptotic *BCL2* is rare, elevated levels of Bcl-x_L mRNA and protein have been detected in all mesothelioma cell lines and tumour samples examined (Hopkins-Donaldson et al., 2003). Pro-apoptotic Bax is also expressed in mesothelioma cell lines, including those that are highly resistant to pro-apoptotic stimuli, suggesting that over-expression of Bcl-x_L may be necessary to counteract the pro-apoptotic effect of Bax (Narasimhan et al., 1998).

Much less is known about pro-apoptotic Bcl-2 family proteins in human MPM. Pro-apoptotic BH3-only proteins (e.g. Bim EL, L and S; Puma α and β ; Bid; Bad; Bmf, Bik) bind to the pro-survival proteins and cause the release of pro-apoptotic Bax-like proteins (e.g. Bax, Bak) that mediate mitochondrial membrane permeabilisation (Willis et al., 2007). Loss of expression of Bad, Bid and Bim is described in human MPM samples (O'Kane et al., 2006).

During a 6-h exposure to a LD₅₀ concentration of cisplatin we found a transient increase in BH3-only proteins in the cisplatin-sensitive MPM parental cell line P31 whereas in the induced cisplatin resistant sub-line P31res1.2 most of the proteins were un-responsive or decreased. This indicates that BH3 mimetics could sensitize MPM cells to cisplatin exposure. The P31res1.2 cells had essentially unchanged Bcl-x_L expression, but interestingly increased Bcl-2 expression and increased phosphorylation of Bcl-2 at ser70 which is necessary for Bcl-2 pro-survival function. We however found also a decrease of potent pro-apoptotic proteins and an increase of the expression of the weak pro-apoptotic proteins that were increased in the P31res1.2 cells. This could contribute to the resistance, as both Bcl-2 and Bcl-x_L must be inhibited in the P31res1.2 cells, and activation of one or two weak pro-apoptotic proteins is insufficient for an optimal inhibition of these pro-survival proteins (Janson et al., 2011, unpublished results).

Targeting the anti-apoptotic Bcl-2 family of proteins, thus restoring apoptotic pathways, may overcome drug resistance to cancer chemotherapy (Letai et al., 2008). Studies have shown that the down-regulation of Bcl-x_L expression (using antisense strategies) (Smythe et al., 2002) or activity (Cao et al., 2007) increases both apoptosis *per se* and drug sensitivity in MPM. Furthermore, it was shown that a reduction of Bcl-x_L in human mesothelioma cell lines in combination with cisplatin causes reduction of tumor growth *in vivo* as well as increases survival in mouse models (Littlejohn et al., 2008). These studies underline the importance of this protein in the protection against chemotherapy-induced apoptosis and emphasize it as a pertinent target to chemosensitize malignant mesothelioma cells (Varin et al., 2010).

Bcl-x_L regulation is not entirely understood. In some cell lines phosphorylation of Bcl-x_L at serine 62 by stress response Jun kinase has been demonstrated to oppose the anti-apoptotic function of Bcl-x_L permitting cells to die by apoptosis by inhibiting its ability to bind to Bax.

Also caspase-3/CPP32-like proteases have been observed to cleave Bcl-x_L protein resulting in accelerated apoptotic cell death (Katz et al., 2009).

2-methoxy antimycin A3 induces apoptosis by neutralizing protective effects of Bcl-2/Bcl-x_L. Most tumour cells have acquired multiple defects in cell cycle and other checkpoints, and are, therefore, highly dependent on the anti-apoptosis function of Bcl-2/Bcl-x_L for survival. Any approach to alter this delicate anti- and pro- apoptosis balance will lead to apoptosis. Because the threshold of apoptosis is much lower in tumour cells than normal, Bcl-2/Bcl-x_L inhibitor could selectively induce apoptosis in tumour and spare normal cells at certain doses. Bcl-2/Bcl-x_L inhibitors could become promising single cancer therapeutic agents and also render tumour cells more sensitive to conventional chemotherapeutic agents

Potential drug resistance mechanisms against Bcl-2/Bcl-x_L inhibitors pose particular problems. Due to the redundancy function of anti-apoptosis Bcl-2 family genes, it should be no surprise to find that over-expressed Bcl-2 family genes such as Mcl-1, Bcl-w or A1 will compensate the loss of anti-apoptosis function due to Bcl-2/ Bcl-x_L inhibition. Generating Bcl-2/ Bcl-x_L resistant cell lines will be important for future studies (Cao et al., 2007).

Interestingly, mesothelioma cells cultured as spheroids 3D acquired resistance to bortezomib by failing to up-regulate Noxa, a pro-apoptotic sensitizer BH3-only protein that acts by displacing Bim, a pro-apoptotic Bax/Bak-activator protein, thereby acquiring both apoptotic resistance and sensitivity to Bcl-2 blockade. Immunocytochemistry of 48 mesotheliomas, demonstrated accordingly that 69% expressed elevated Bim. Therefore, mesothelioma, a highly resistant tumour, may have an intrinsic sensitivity to Bcl-2 blockade that can be exploited therapeutically. Tumors identified as 'primed for death' may respond to inhibition of the anti-apoptotic defenses with small molecules such as ABT-737, an inhibitor of Bcl-2/Bcl-x_L. Bim was essential for the response to ABT-737, and the level of Bim correlated with sensitivity to ABT-737 indicating that Bim may be a predictive biomarker for the response of mesothelioma to ABT-737 together with bortezomib. Bim over-expression was frequent (90%) in the more chemosensitive epithelioid MPM subtype and uncommon (20%) in the more chemoresistant sarcomatoid subtype (Barbone et al., 2011).

As indicated above, Mcl-1 might cooperate with Bcl-x_L for protection against cell death. Using RNA interference, Bcl-x_L depletion sensitized two highly chemoresistant mesothelioma cell lines to cisplatin. Inhibition of Mcl-1 by cisplatin may contribute to the induction of cell death observed after Bcl-x_L down-regulation. Additionally, Mcl-1 has also been found to be over-expressed in most malignant mesothelioma cell lines and tumor tissues. Down-regulation of Mcl-1 was also observed in response to cisplatin in two MPM cell lines. These observations thus suggest that resistance to apoptosis in MPM could be rather related to Bcl-x_L and/or Mcl-1 than to Bcl-2. After concomitant siRNA down-regulation of Bcl-x_L and Mcl-1, the proportion of viable mesothelioma cells was dramatically reduced. None or little cell death was induced after transfection with single siRNA. Combination of both siRNAs with a low cisplatin concentration led to a nearly complete annihilation of tumor cells whereas normal mesothelial cells were marginally affected. The development of BH3-mimetic small-molecule inhibitors together with siRNA for gene silencing in cancer, may yield effective targeted strategy within short (Varin et al., 2010).

It has been shown that the BimEL/Mcl-1 and BimEL/ Bcl-x_L complexes can be rapidly dissociated following activation of Erk1/2 by survival factors thus allowing Bax and Bak binding to Mcl-1 and Bcl-x_L and thus inhibiting apoptosis (Ewing et al., 2007).

6. Glycosphingolipids in cancer development and chemotherapy resistance

6.1 Glycosphingolipids and globotriaosylceramide (Gb3)

Glycosphingolipids (GSLs) are components of all vertebrate cells and play a fundamental role during development and cell differentiation (Erdmann et al., 2006). GSLs are involved in cellular growth, signal transduction and cell-cell interaction (Lahiri & Futerman, 2007). GSL profiling indicate that neutral globo series GSLs, including the neutral glycosphingolipid cell verotoxin-1 (VT-1) surface receptor globotriaosylceramide (Gb3), have important roles in mediating *MDR1* transactivation and expression (Liu et al., 2010).

The expression and metabolism of cell surface glycolipids is changed during oncogenic transformation and altered glycosylation patterns affect tumour invasion and metastasis (Hakomori & Zhang, 1997). Gb₃ is expressed in several human malignancies such as breast cancer (Johansson et al., 2009). Gb₃ expression in colorectal cancer correlates with invasiveness and metastatic potential. Elevated levels of Gb₃ have also been seen in drug-resistant cancers and cell lines and a functional interplay between membrane Gb₃ and *MDR1* has been suggested (Mattocks et al., 2006; De Rosa et al., 2008). Deletion of Gb₃ synthase needed for Gb₃ synthesis renders mice completely resistant to verotoxins (Okuda et al., 2006). GSLs are the only functional VT-1 receptors (Lingwood et al., 2010). These findings suggest that the Gb₃-binding specificity of VT-1 could be used to target tumours for the toxin in the receptive cancer cells.

GSLs in cells are clustered and assembled with specific membrane proteins and signal transducers to form GSL-enriched microdomains or lipid rafts. Rafts are rich in GSLs, cholesterol, lipid-modified- and transmembrane proteins. The length of the fatty acyl chain of Gb₃ influences its receptor function, intracellular sorting and retro-translocation of VT-1 to the cytosol (Lingwood, 1996). Binding of VT-1 B-subunit with clustered raft-localized Gb₃ receptors is a requirement for the retrograde transport (Falguieres et al., 2008) and for a cytotoxic effect in the ER (Smith et al., 2006). For cells with Gb₃ present in the non-raft plasma membrane fraction, the toxin receptor complex is internalized and trafficked to lysosomes where the toxin is degraded leading to VT-1 resistant cells. Furthermore, VT-1 B subunit binding to Gb₃ induces lipid reorganization of the cell membrane leading to enhancement of VT-1 uptake into the cell (Römer et al., 2007). The membrane organization of the glycosphingolipid receptor is the main discriminator for pathology *in vivo* (Lingwood et al., 2010).

6.2 Multidrug resistance to cancer chemotherapy

Tumour over-expression of the membrane efflux transporter P-glycoprotein (P-gp) is a common alteration in drug resistance (Gottesman, 2002). P-gp, encoded by the *MDR1* gene (Ueda et al., 1986), was the first ABC protein demonstrated to confer resistance to cancer chemotherapeutics (Gottesman et al., 1996). Other transporter proteins such as multidrug resistance protein (MRP1) and breast cancer resistance protein (BCRP) have also been described. P-gp plays roles in the absorption, distribution and excretion of compounds in

normal tissues. Over-expression of *MDR1* in tumours results in active efflux of several types of anticancer agents. P-gp is expressed by many types of primary solid tumours, as well as haematological malignancies (Sandor et al., 1998).

Exposure to chemotherapy can up-regulate tumour P-gp expression, which occurs in acquired drug resistance and severely limits the success of chemotherapy. *MDR1* inhibitors have been clinically tested in order to block drug efflux. Specific modulators or inhibitors such as GG918 and LY335979 have overcome the toxic adverse effects noted in first generation modulators but still have minor effect when co-administrated with chemotherapeutics in trials in part due to *MDR1* polymorphisms (Liu et al., 2010).

6.3 Globotriasosylceramide (Gb₃) and *MDR1* expression

Little is known about the molecular mechanism underlying *MDR1* over-expression and how it interacts with other genes to impart drug-resistance. Over-expression of glucosylceramide synthase (GCS), the first enzyme of GSL synthesis, can result in multidrug resistance. Many cells expressing *MDR1* show elevated levels of glucosylceramide (GlcCer) (Morjani et al., 2001) and inhibitors of GCS kill MDR cells (Nicholson et al., 1999). *MDR1* can translocate glucosylceramide into the Golgi apparatus for neutral GSL synthesis, including Gb₃. P-gp has been proposed as a Golgi glucosylceramide flippase that enhances neutral GSL synthesis as transfection of *MDR1* increases, and inhibition of P-gp decreases neutral GSL biosynthesis in cells (De Rosa et al., 2004). GCS up-regulates *MDR1* expression and modulates drug resistance of cancer.

Partial *MDR1* and Gb₃ cell surface co-localization has been observed and inhibition of GSL biosynthesis depletes cell surface *MDR1*. *MDR1* may therefore interact with Gb₃. A significant fraction of surface *MDR1* is not co-localized with Gb₃, and could therefore be VT-1-insensitive. *MDR1* can be expressed in cells lacking Gb₃. However, drug-resistant metastatic ovarian tumour cells have a particularly high Gb₃ content and Gb₃ is highly expressed in metastatic colon carcinoma (Arab et al., 1997; Kovbasnjuk et al., 2005).

The water-soluble Gb₃ mimic adamantylGb₃, but not other GSL analogues, reversed *MDR1*-MDCK cell drug resistance (DeRosa et al., 2004). Verotoxin-mediated Gb₃ endocytosis also up-regulated total *MDR1* and inhibited drug efflux (Pastan et al., 1988).

The Gb₃ content, which is regulated by the expression of Gb₃ synthase, determines the sensitivity of HeLa cells toward VT-1. We recently demonstrated extensive variability in breast cancer cell lines for apoptosis induction by VT-1. Sensitivity was correlated with Gb₃ expression, and use of the drug PPMP, which down-regulates glucosylceramide production, inhibited VT-1-mediated apoptosis (Johansson et al., 2009).

7. Verotoxin-1

7.1 Verotoxin-1 structure and induction of apoptosis

VT-1 consists of one A and five B subunits. After B subunit binding to Gb₃ (Wadell et al., 1990), it is endocytosed and follows the retrograde pathway to the endoplasmic reticulum where the A-subunit is translocated to the cytosol and inhibits protein synthesis (Raa et al., 2009).

Importantly, VT-1 also induces apoptosis through sequential activation of caspases, leading to nuclear changes, such as chromatin condensation and DNA fragmentation. VT-1-induced

apoptosis in monocytic THP1 cells requires retrograde transport through the Golgi apparatus to the ER and the activation of caspase-3, the executioner caspase (Kojio et al., 2000). Similar apoptotic signalling pathways are triggered by Shiga toxins in different cell lines.

VT-1 induces a prominent ribotoxic stress signalling response leading to disrupted ribosomal RNA (rRNA) functions, protein synthesis inhibition and altered mitogen-activated protein kinase (MAPK) pathway signalling (Johannes & Römer, 2010). The anti-apoptotic function of Bcl-2 requires Jnk-mediated phosphorylation of Bcl-2. Alternative Bcl-2 phosphorylation reactions, including p38 MAPK-directed phosphorylation of Bcl-2, inhibit Bcl-2 function. Bcl-2 was differentially phosphorylated by VT-1 treatment of monocyte- vs. macrophage-like THP-1 cells. Levels of anti-apoptotic phospho-Bcl-2 molecules were transiently increased in macrophage-like cells, while levels declined in monocyte-like cells. Thus, the ribotoxic stress response induced by VT-1 may regulate the activation of the Bcl-2 family of proteins that, in turn, control apoptosis (Tesh, 2011).

7.2 Effect of cisplatin and verotoxin-1 on malignant pleural mesothelioma cells

We found that Mkk3/6 and Jnk was phosphorylated after cisplatin treatment in the cisplatin-sensitive MPM P31 cells, but not in the corresponding P31res1.2 sub-line with acquired-cisplatin resistance. VT-1 induced phosphorylation of Mkk3/6, which was enhanced when VT-1 was combined with cisplatin (Johansson et al., 2010). Mkk3/6 is known to activate P38 (Derijard et al., 1995; Han et al., 1996). P38 as well as Jnk has been shown to promote apoptosis in response to cellular stress (Kim et al., 2006). Treatment of cells with chemical inhibitors or siRNA targeting P38 was recently shown to specifically inhibit VT-1 transport to the Golgi apparatus complex and reduce VT-1 toxicity (Walchli et al., 2008), and VT-1 prolonged Jnk and P38 MAPK activation of macrophage-like cells (Lee et al., 2007). We have previously demonstrated Jnk phosphorylation in response to VT-1 treatment also in glioma and breast cancer cell lines (Johansson et al., 2006; 2009).

Apoptosis induced by VT-1 was associated with enhanced expression of the pro-apoptotic protein Bax (Jones et al., 2000) and over-expression of Bcl-2 protects cells against VT-1-induced cell death (Suzuki et al., 2000). Shiga toxins also inhibit the expression of the anti-apoptotic Bcl-2 family member Mcl-1 (Erwert et al., 2003). Interestingly, acquisition of cisplatin resistance in MPM cells increased cisplatin activation also of weak proapoptotic proteins of the Bcl-2 family of proteins but apparently not enough to counteract the increased expression of also of anti-apoptosis proteins (Janson et al., 2011, unpublished results).

8. Globotriasosylceramide, verotoxin-1 and cisplatin in targeting of resistant mesotheliomas

The possibility that VT-1 through the A-subunit could cause protein synthesis inhibition and induce apoptosis in normal cells constitutes a concern for the use of the holotoxin as an anticancer agent. The non-toxic VT-1 B subunit is stable at extreme pH, resist proteases, cross tissue barriers, distribute in the organism and generally resist extra- and intracellular inactivation (Johannes & Decaudin, 2005). The receptor selectivity of the B subunit has therefore been used to couple it to cytotoxic compounds such as the topoisomerase I inhibitor SN38 (El Alaoui et al., 2007) or induce an immune response (Vingert et al., 2006) with preferential effects on cancer cells.

Of primary cultures of gastrointestinal tumours, 80% were found to bind the VT-1 B subunit and could be detected on tumour cells after 5 days. The stable association of VT-1 B subunit with cells might be a useful property for diagnostic or therapeutic delivery strategies. This subunit has little immunologic properties (Bast et al., 1997) and is well tolerated in a mouse model (Smith et al., 2006).

An apparent treatment possibility to reverse MDR is to inhibit GSL biosynthesis by inhibiting GCS or Gb3 synthase enzyme expression and/or activity, or use Gb3 mimics like adamantylGb₃ (Arab et al., 1997).

The treatment obstacle of acquired-cisplatin resistance in MPM and other cancers makes it necessary to find new strategies to overcome resistance. We showed that cisplatin can up-regulate Gb3 expression in MPM and NSCLC cells and thus sensitize the cells to VT-1-induced cytotoxicity. The increased proportion of Gb3-expressing cells after cisplatin treatment (Fig. 1) suggests that cisplatin induces Gb3 expression in cancer cells, that

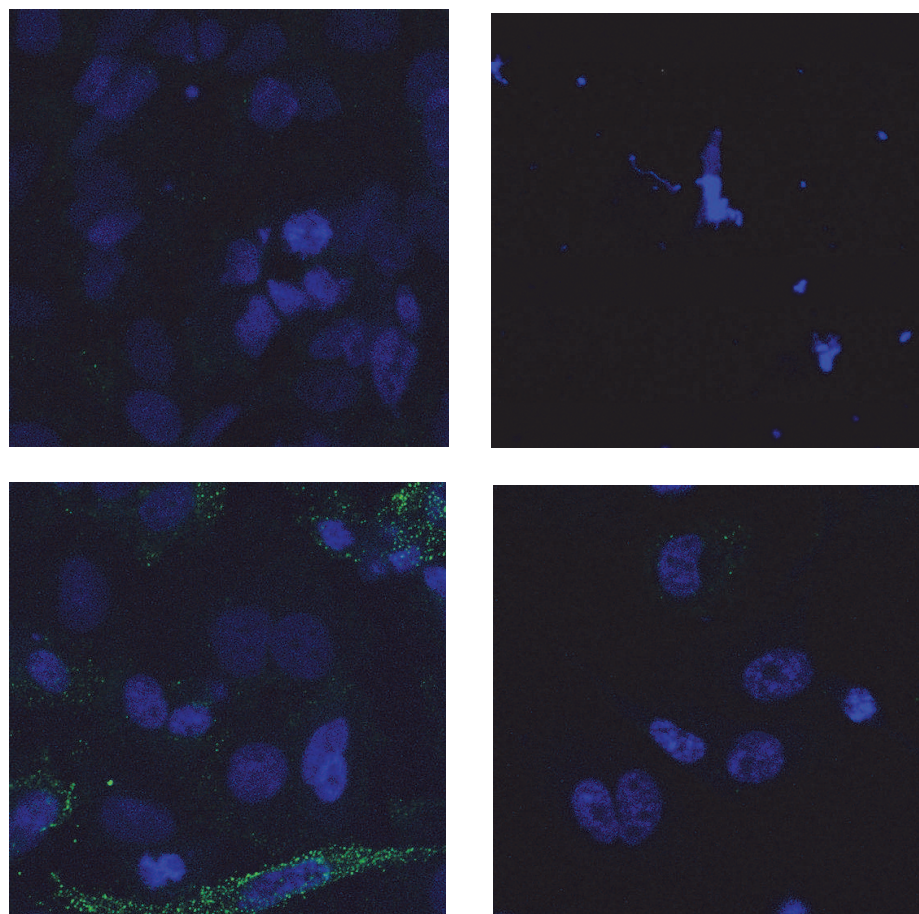


Fig. 1. The human mesothelioma cell line (P31 – upper panels) and its cisplatin-resistant sub-line (P31res1.2 – lower panels) were grown to confluence on glass cover-slips without (left panels) or with 15 µmol/L PPMP (right panels) for 72 h, then fixed with paraformaldehyde and stained for 1 h with rat primary IgM antibodies against globotriasylceramide (Gb3). Secondary goat anti-rat IgM antibodies labeled with Alexa Fluor® 488 Dye were added for 1 h. Nuclei were visualized with DAPI staining DNA and visualizing cell nuclei. Images were captured on a Zeiss upright confocal microscope and analyzed using Zeiss ZEN 2010 software.

cisplatin preferentially eradicates cell with low Gb3 expression and that Gb3 expression is linked to acquired cisplatin-resistance (Johansson et al., 2010). We could also correlate increased expression of Gb3 in cisplatin-resistant MPM (P31res1.2) cells to increased expression of MDR1/PgP. This is important since MDR1/PgP generally is not over-expressed in MPM but expression could possibly occur following chemotherapy. This needs to be investigated further. PPMP reduced Gb3 expression in the resistant sub-line cells (Fig. 1) and particularly of the Gb3-expressing fraction that was induced when the mother cell line was made cisplatin-resistant. A strong super-additive effect of combined cisplatin and a sub-toxic concentration of VT-1 in cisplatin-resistant MPM cells were observed, indicating a new potential clinical treatment approach (Johansson et al., 2010).

The MAPK pathway is involved in proapoptotic signalling of VT-1 in stressed cell systems and the pathway is also involved in cisplatin-induced apoptosis and induced cisplatin resistance (Salhia et al., 2002; Johansson et al., 2010). Targeting the MAPK signalling pathway could, therefore, be an additional way to reduce cisplatin-induced tumour cells resistance.

The partial cell surface co-localization of Gb3/MDR1, the modulation of MDR1 cell surface expression by GSL and chemotherapy and the possibility to inhibit MDR1 expression by VT-1/VT-1 B-sub-unit, all indicate a functional link between Gb3 and MDR1. Targeting the physiological regulation of MDR1 could be an efficient way not only to prevent the development of drug resistance during cancer chemotherapy but also to reverse inherent and acquired drug resistance of MPM.

9. Conclusion

Improving our knowledge of the molecular alterations and signaling pathways specific to MPM should help in the identification of biomarkers useful novel treatment approaches. Such treatments might include targeted agents in combination with effective chemotherapeutic regimes. Personalized treatments based on the biological characteristics that follow that of the tumour will offer better future outcomes for MPM patients.

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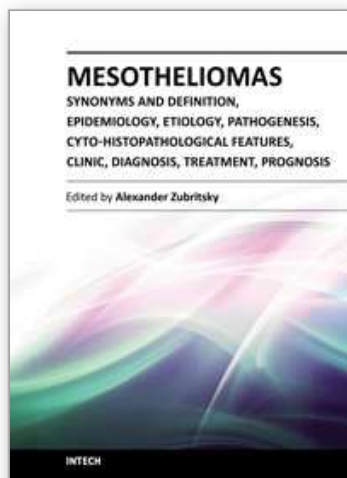
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Mesotheliomas - Synonyms and Definition, Epidemiology, Etiology, Pathogenesis, Cyto-Histopathological Features, Clinic, Diagnosis, Treatment, Prognosis

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Mesotheliomas are mysterious mesothelial tumors in that they are relatively rare, difficult to diagnose, with a large number of synonyms, and the etiology and pathogenesis of the disease are still not fully disclosed. This problem attracts the attention of various specialists in the field of medicine and biology every year. In recent years there has been a significant increase of mesothelioma morbidity in most of the countries, due to the further industrialization of society. In this regard, this book has been published with the participation of an international group of experts with rich experience from around the world . The book consists of 14 chapters containing the most advanced achievements of all aspects of the various types of mesotheliomas, both in humans and domestic animals, at a high methodological level. This book is intended for biologists and all health care workers, mostly oncologists of different profiles, as well as students of medical educational institutions engaged or even just interested in the problems of mesotheliomas.

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