Targeting Gb3 and apoptosis-related proteins to overcome cisplatin resistance

Andreas Tyler
To Sarah, Morris and Fred
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Abstract

Background  Cisplatin is used for treatment of malignant pleural mesothelioma (MPM) and non-small cell lung cancer (NSCLC) but treatment with cisplatin often leads to acquired resistance to cisplatin, resulting in poor patient survival. Globotriaosylceramide (Gb3) and multidrug resistance protein 1 (MDR1) have been associated with cisplatin resistance. Gb3 serves as a receptor for verotoxin-1 (VT-1), which induces apoptosis, and has been shown to have a functional dependency to MDR1 and heat shock protein 70 (HSP70). The Bcl-2 family of proteins and inhibitors of apoptosis (IAPs) are key regulators of apoptosis. BH3-mimetics mimic pro-apoptotic BH3-only proteins, while Smac mimetics mimic the IAP-binding protein Smac/Diablo. These drugs have shown great promise in reversing cisplatin resistance. Exosomes are small bio-nanoparticles secreted and taken up by both cancer cells and normal cells. They have the ability to transfer properties between cells and have been shown to confer resistance to cisplatin.

Methods  In this thesis, NSCLC cell line H1299 and MPM cell line P31 were studied using western blot, flow cytometry, proteome profilers, confocal microscopy and gene expression arrays to investigate changes in protein and gene expression after acquisition of cisplatin resistance (P31res and H1299res) or after incubation with exosomes or drugs that target these. The cytotoxic and apoptotic effects were studied using fluorometric cytotoxicity assay (FMCA) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

Results  This thesis confirms that Gb3 is a potential target for cisplatin resistance reversal. Incubation with glycosphingolipid production inhibitor DL-threo-1-phenyl-2-palmitoylamo-no-3-morpholino-1-propanol (PPMP) and VT-1 led to reduced Gb3 cell surface expression and increased cytotoxic effect of cisplatin in all cell lines. Gb3 and MDR1 was not co-localized in any studied cell line, but Gb3 and HSP70 were co-localized on the cell surface and PPMP and VT-1 led to a decrease of both Gb3 and HSP70. Both BH3-mimetic obatoclax and Smac mimetic AT-406 had an additive effect on cisplatin-induced cytotoxicity and apoptosis in P31 and a synergistic effect in P31res. Results indicate that exosomes from cisplatin-resistant cell lines can transfer HSP70 to the surface of cells.

Conclusion  Cell surface Gb3 and HSP70, the Bcl-2/IAP-family proteins and exosomal transfer of cisplatin resistance characteristics are potential targets in combatting cisplatin resistance that show therapeutic promise and warrant further research.
# Abbreviations

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<tr>
<td>17-AAG</td>
<td>17-N-Allylamino-17-demethoxygeldanamycin</td>
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
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<tr>
<td>BH3</td>
<td>Bcl-2 homology domain 3</td>
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<tr>
<td>FDA</td>
<td>Fluorescein diacetate</td>
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<td>FMCA</td>
<td>Fluorometric microculture cytotoxicity assay</td>
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<td>Gb3</td>
<td>Globotriaosylceramide</td>
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<td>GCS</td>
<td>Glucosylceramide synthase</td>
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<td>GSL</td>
<td>Glycosphingolipid</td>
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<td>HSP</td>
<td>Heat shock protein</td>
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<td>HSP70</td>
<td>Heat shock protein 70</td>
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<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis</td>
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<tr>
<td>IBM</td>
<td>IAP-binding motif</td>
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<td>Mcl-1</td>
<td>Induced myeloid leukemia cell differentiation protein</td>
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<td>MDR1</td>
<td>Multidrug resistance protein 1</td>
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<tr>
<td>miR</td>
<td>MicroRNA</td>
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<td>MPM</td>
<td>Malignant pleural mesothelioma</td>
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<td>MRP1</td>
<td>Multidrug resistance-associated protein 1</td>
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<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PBST</td>
<td>PBS Tween</td>
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<td>PPMP</td>
<td>DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol</td>
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<tr>
<td>Smac</td>
<td>Second mitochondria-derived activator of caspases</td>
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<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
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<tr>
<td>VT-1</td>
<td>Verotoxin-1</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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Populärvetenskaplig sammanfattning

Vid cancerbehandling vill man helst att cancerceller ska dö genom s.k. apoptos, en slags kontrollerad celldöd, som inte påverkar övriga, friska celler i kroppen. Cisplatin är ett cellgift som används vid cancerbehandling. Det orsakar apoptos hos cancerceller, men ett kliniskt problem är att cancercellerna efter ett tag blir resistent, d.v.s. motståndskraftiga och försvarar sig mot cisplatin och därmed överlever behandlingen. För att åter göra cancercellerna känsliga för cisplatin så att de dör av cisplatinbehandlingen, kan man kombinera cisplatin med andra typer av cellgifter. I denna avhandling har jag undersökt vad som händer med olika proteiner när odlade cancerceller blir resistent mot cisplatin och när cisplatinbehandling kombinerats med andra cellgifter eller molekyler som motverkar eller drar nytta av dessa förändringar.

BH3-mimetika är molekyler som ökar apoptos genom att påverka produktionen av proteiner som tillhör Bcl-2-familjen. AT-406 är ett ämne som också ökar apoptos genom att påverka s.k. IAP-proteiner. Denna avhandling visar att BH3-mimetikat obatoclax samt AT-406 ökar celldöd och apoptos av cisplatin, särskilt hos cancerceller som är resistent mot cisplatin.

Verotoxin-1 (VT-1) är ett bakteriellt gift som också ökar den celldödande effekten av cisplatin. VT-1 tar sig in i cellen genom att binda till Gb3-receptorn på utsidan av cellen. Gb3 finns hos cancerceller som är resistent mot cisplatin. Vi har funnit att Gb3 ofta är lokalisert nära proteinet HSP70, ett protein som skyddar andra proteiner inuti cellen, men när det uttrycks på utsidan av cellen kan både leda till att kroppens immunförsvar angriper cellen och att tumören blir mer resistant mot cellgifter och då sprider sig snabbare. När vi använde olika cellgifter för att minska Gb3-uttrycket på ytan av resista cancerceller så minskade också HSP70 och när cellgiften 17-AAG ökade HSP70 så ökade också Gb3. 17-AAG ökade den celldödande effekten av VT-1, sannolikt för att det hos resista cancerceller fanns mer Gb3 som VT-1 kunde binda till.

Många celler, både vanliga celler och tumörceller, utsöndrar små partiklar kallade exosomer. Dessa exosomer kan tas upp av andra celler, som då kan få nya egenskaper beroende på innehållet i dessa exosomer. Man tror t.ex. att resistens mot cellgifter kan överföras från cell till cell via exosomer. Vi fann att de tumörceller vi studerat utsöndrade exosomer samt att HSP70 i dessa exosomer hamnade på cellytan hos de celler som tog upp exosomerna.

Avhandlingen visar att det är möjligt att motverka cisplatinresisten hos tumörceller genom att rikta in sig på Gb3 och Bcl-2-familjen, samt att exosomer kan orsaka förändringar i tumörceller som kan leda till cisplatinresistens. Det vi kommit fram till i avhandlingen kan förhoppningsvis leda till nya behandlingsmetoder i framtiden.
1. Introduction

1.1 Malignant pleural mesothelioma (MPM) and non-small cell lung cancer (NSCLC)

Malignant pleural mesothelioma (MPM) is a rare but severe form of cancer linked to the exposure to asbestos and erionite (Carbone et al., 2012; Delgermaa et al., 2011). Median survival after diagnosis is low, around 14 months (Baas, 2007; Saint-Pierre et al., 2015). Treatments include radiotherapy, pneumonectomy and chemotherapeutic combination therapies, including platinum-based drugs such as cisplatin or pemetrexed (Rintoul et al., 2015; Stahel et al., 2015). The short survival is contributed by various drug resistance mechanisms, both inherent and acquired (Bridda et al., 2007). Lung cancer was the most frequently diagnosed cancer and the leading cause of cancer-related death worldwide in 2012 (Torre et al., 2015). Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancers (Torre et al., 2015) and has a short median survival, only 10-12 months (Rothschild, 2015). Treatments include resection surgery, radiotherapy and chemotherapeutic combination therapies including paclitaxel, erlotinib, gefinitib or platinum-based drugs such as cisplatin (Cho et al., 2015; Rusthoven et al., 2015; Yang et al., 2015).

1.2 Cisplatin

Chemotherapy with cisplatin, usually in combination with other chemotherapeutic agents, is used for treatment of both MPM and NSCLC (Schmid-Bindert et al., 2013; Stahel et al., 2015), resulting in partial tumor responses or disease stabilization and only a slight increase in patient median survival time. Cisplatin exerts its toxicity by adding crosslink purine bases on the DNA which interferes with DNA repairs, activates apoptosis and hinders rapidly dividing cells from duplicating their DNA for mitosis (O’Grady et al., 2014; Siddik, 2003; Zwelling & Kohn, 1979). The clinical usefulness of cisplatin treatment is however limited by the high incidence of acquired drug-resistance (Andrews & Howell, 1990; Kasibhatla & Tseng, 2003).

1.2.1 Mechanisms of cisplatin resistance

Strategies to overcome cisplatin resistance is crucial. Overcoming resistance by targeting multiple targets or pathways related to the diverse mechanisms of resistance is a promising approach (O’Grady et al., 2014). Resistance to cisplatin is mediated by multiple separate mechanisms (Rose et al., 2014). Reduction of intracellular platinum accumulation is one of the major resistance mechanisms (Ling, 1997; Zhang & Ling, 2000). This can be achieved by reduced uptake of cisplatin (Galluzzi et al., 2012) and by
increased efflux through trans-membrane pumps, such as multidrug resistance protein 1 (MDR1), an ABC protein demonstrated to confer resistance to cancer chemotherapeutics (Aksentijevich et al, 1996; Gottesman et al, 1996; Juliano & Ling, 1976). Other transporter proteins involved in cisplatin resistance include breast cancer resistance protein (BCRP) and multidrug resistance-associated protein 1 (MRP1), an ABC protein which exerts cisplatin resistance by extruding glutathione-cisplatin conjugates from the cells (Ishikawa, 1992). Evading apoptosis by deregulation of apoptosis-related proteins is another important drug resistance mechanism and several drug-resistant tumors display upregulated anti-apoptotic proteins or downregulated pro-apoptotic proteins (Fernald & Kurokawa, 2013).

1.3 Multidrug resistance protein 1 (MDR1)
Overexpression of MDR1 has been shown to enhance DNA damage repair and reduce induction of apoptosis (Liu et al, 2013; Ohmichi et al, 2005) but the importance of ATP-cassette protein efflux in cisplatin resistance (Zhou, 2008) has been questioned, since specific MDR1 transport inhibitors were unable to restore cisplatin accumulation and sensitivity (Galluzzi et al, 2012; Jin et al, 2005; Zhou, 2008). The expression of MDR1 on the nuclear membrane has been associated with extrusion of drugs from the nucleus of multidrug-resistant cells (Maraldi et al, 1999; Solazzo et al, 2006).

1.4 Apoptosis
Programmed cell death plays a major role in development and tissue homeostasis and protects the organism by eliminating abnormal and potentially harmful cells. Apoptosis is the most studied form of programmed cell death (Fuchs & Steller, 2011). Evading apoptosis is one of the hallmarks of cancer (Hanahan & Weinberg, 2000) and most anti-cancer treatments depend on an ability to induce apoptosis (Zucali et al, 2011). The key to a successful cancer chemotherapy is to promote apoptosis in tumour cells without affecting normal tissue negatively (Zucali et al, 2011).

1.4.1 Apoptotic pathways
There are two major apoptotic pathways: the intrinsic pathway (the mitochondrial mediated pathway) and the extrinsic pathway (the death receptor mediated pathway) (Hassen et al, 2012). The extrinsic pathway can initiate the intrinsic pathway but the two can also function separately. An important step in the execution of apoptosis, both intrinsic and extrinsic, is the activation of caspases, a family of cysteine proteases that are expressed as inactive precursors and indispensable for apoptotic cell death (Chandra et al, 2004; Hengartner, 2000). Caspases are classified as initiator (caspase-8
and -9) and effector or executioner caspases (caspase-3, -6 and -7), based on their position in apoptotic signaling cascades (Hu et al, 2013).

In addition to the intrinsic and extrinsic pathways there are other less well known pathways, such as the caspase-12 or caspase-2-initiated apoptosis, which are activated by endoplasmic reticulum stress (Szegedi et al, 2006). Apoptosis can also be caspase-independent, where apoptosis inducing factor (AIF) plays a crucial role by translocating to the nucleus, causing DNA fragmentation independent of caspases (Susin et al, 1999). Caspase-3 can also be activated in a caspase-8/caspase-9-independent manner (Chen et al, 2005).

The extrinsic pathway is mediated by death ligands that bind to death receptors on the surface of target cells. Apoptosis triggered by death ligands is thought to represent a form of innate immunity. Traditional chemotherapeutic drugs target the intrinsic pathway of cell death, activating apoptosis from within cells through induction of DNA damage or other cellular stresses (Ashkenazi, 2015). This thesis will focus on the intrinsic pathway. The caspase-8/caspase-9-independent pathway is also given some interest since cisplatin-resistant MPM cell line P31res, which is featured in this thesis, seems to enter cisplatin-induced apoptosis without activation of caspase-8 or caspase-9 (Janson et al, 2010).

Previous studies published by our group indicates that caspase-7 was upregulated and substituted caspase-3 in cisplatin-resistant NSCLC H1299res cells (Janson et al, 2010). The P31res cells, MPM cells with acquired cisplatin resistance, were resistant to initiator caspase fragmentation and demonstrated an increased basal caspase-3/7 activity and initiator-caspase-independent cisplatin-induced activation of caspase-3/7. Cells of the parental cell line of P31res, P31, which are more sensitive to cisplatin, appeared to have caspase-9-mediated cisplatin-induced caspase-3 activity (Janson et al, 2010).
1.4.2 The intrinsic pathway of apoptosis and the Bcl-2 family

The Bcl-2 (B-cell lymphoma 2) family of proteins regulate the intrinsic signalling pathway of apoptosis and consists of three groups of proteins, the anti-apoptotic proteins (Bcl-2, Bcl-x, Bcl-w, Mcl-1 and A1), the pro-apoptotic proteins (Bax, Bak and Bok) and the pro-apoptotic Bcl-2 homology domain 3 (BH3)-only proteins members (e.g. Bad, Bim, Bid, Noxa etc.) (Shamas-Din et al, 2011) (Fig. 1). The anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-x and Mcl-1) preserve the mitochondrial outer membrane permeabilization through inhibition of pro-apoptotic proteins. 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, which causes mitochondrial outer membrane permeabilisation leading to release of cytochrome c and second mitochondria-derived activator of caspases (Smac) into the cytoplasm where they activate caspase-9, leading to activation of apoptosis through caspase-3 (Wesarg et al, 2007). Apoptosis is an uncommon event in mesothelioma and cleaved caspase-3 index has been demonstrated as low (Jin et al, 2010).

![Diagram of the intrinsic pathway of apoptosis](image)

**Figure 1.** Brief summary of the intrinsic pathway of apoptosis, the caspase-independent pathway and some of the apoptosis-related proteins featured in the thesis.
Pro-apoptotic BH3-only proteins bind to the anti-apoptotic proteins and cause the release of pro-apoptotic Bax-like proteins (e.g. Bax, Bak) that mediate mitochondrial membrane permeabilisation (Willis et al, 2005; Willis et al, 2007). While all anti-apoptotic proteins bind to Bax, only Mcl-1 and Bcl-xL can bind to Bak. Bim, Puma, and truncated Bid (tBid) are potent pro-apoptotic proteins that can inhibit all known anti-apoptotic Bcl-2 proteins (Willis et al, 2005). The weak pro-apoptotic BH3-only proteins selectively inhibit (Wesarg et al, 2007) a subset of anti-apoptotic Bcl-2 proteins - e.g. Bcl-xL is inhibited by Bad, Bmf, Bik and Hrk whereas Bcl-2 is inhibited by Bad and Bmf (Willis et al, 2005). Loss of expression of Bad, Bax, Bak, Bid and Bim has been noted in human MPM samples (O’Kane et al, 2006).

1.4.3 The role of the Bcl-2 family in cisplatin resistance
Over-expression of anti-apoptotic Bcl-2 and Bcl-xL genes contribute to apoptotic inhibition and the development of multidrug resistance of human cancers (Shamas-Din et al, 2011). In human mesothelioma, Bcl-xL is often over-expressed, whereas elevated levels of Bcl-2 are less common (Hopkins-Donaldson et al, 2003; O’Kane et al, 2006). Mcl-1 has also been found to be over-expressed in most malignant mesothelioma cell lines and tumor tissues (Varin et al, 2010).

1.4.4 BH3-mimetics
BH3-mimetics are BH3-domain containing peptides that mimic the effects of BH3-only proteins, which potentially could lead to induction of apoptosis (Zhang et al, 2007a). In tumours with loss or inhibition of BH3-only proteins, BH3-mimetics replace the need of BH3-only proteins to initiate apoptosis. 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). BH3-mimetics that selectively antagonize the anti-apoptotic proteins may prove to be successful in cancer therapy (Shamas-Din et al, 2011).

ABT-737 is a potent and specific BH3-mimetic that induces apoptosis by antagonizing the antiapoptotic Bcl-2, Bcl-xL, Mcl-1, Bcl-w and Bcl-xL proteins (Fulda et al, 2010). Cells overexpressing the anti-apoptotic Bcl-2 family protein Mcl-1 have been found to be resistant to ABT-737 and down-regulation of Mcl-1 increases the effect of ABT-737 (van Delft et al, 2006).

Obatoclax (GX15-070) is a small compound that antagonizes Bcl-2, Bcl-xL, Bcl-w and Mcl-1. Unlike ABT-737, obatoclax also disrupts the interaction between Bak and Mcl-1 which means it can overcome Mcl-1-dependent resistance to ABT-737 (Fulda et al, 2010). Obatoclax has been suggested to be useful for targeting the apoptosis pathway in platinum resistant cancers (Crawford et al, 2011) and has so far shown promising results in enhancing
cisplatin-induced cell death (Li et al, 2008; Pan et al, 2010). There is evidence that suggests that obatoclax also may induce cell death independent of the Bcl-2 family of proteins (Bajwa et al, 2012; Elkholi et al, 2011).

1.4.5 Inhibitors of apoptosis (IAPs)

The inhibitors of apoptosis (IAPs) are negative regulators of apoptotic signaling and keep it under control to prevent accidental cell death. IAP proteins are overexpressed in many malignant tumors and correlates with poor prognosis. The eight IAP proteins found in humans are; X-linked IAP (XIAP), cIAP1, cIAP2, neuronal apoptosis inhibitory protein (NAIP), melanoma IAP, apollon, survivin, and IAP-like protein 2 (Obexer & Ausserlechner, 2014). XIAP is the best characterized and most potent of the IAPs and is the only inhibitor that directly binds to both initiator and effector caspases (Obexer & Ausserlechner, 2014). To induce caspase-dependent apoptosis it is often necessary to counterbalance the function of IAPs. This can be achieved by proteins containing an IAP-binding motif (IBM), such as Smac/Diablo and HtrA2 (Bai et al, 2014). There are a number of different Smac mimetics, one such is AT-406. As a single agent it has been found to effectively induce cell death in ovarian cancer. In the same study it also sensitized cancer cells to platinum-based drugs (Brunckhorst et al, 2012). Combined use of XIAP siRNA (small interfering RNA) and cisplatin has been shown to increase cytotoxic activity (Zhang et al, 2007b).

The expression levels of cIAP-1, cIAP-2, survivin and XIAP were also increased in the P31res cells according to a previous study (Janson et al, 2010). The possible role of XIAP inhibition of caspase-9 activity was therefore investigated by determining the expression and fragmentation of XIAP. HtrA2 protease activity was suggested to be involved in XIAP inactivation (Janson et al, 2010).

1.5 Glycosphingolipids and ceramide

Glycosphingolipids (GSL) consist of hydrophobic ceramide and hydrophilic sugar chains and are located on the surface plasma membranes where they form lipid rafts together with different proteins and signaling molecules. Ceramide and GSLs affect important cell functions such as apoptosis, proliferation, endocytosis, transport and migration, thereby regulating cancer progression and treatment efficiency (Patwardhan & Liu, 2011). Ceramide increases in response to chemotherapy, leading to cell proliferation arrest and apoptosis. However, ceramide can be eliminated by glucosylceramide synthase (GCS) conversion of ceramide to glucosylceramide and GSLs. Tumor cells often up-regulate GCS to deplete ceramide pools induced by chemotherapy through conversion to a more benign GSL format such as Gb3 (Gouaze-Andersson & Cabot, 2006). Persistent GCS activity leads to reduction of ceramide, stimulation of cell
proliferation and blocked apoptosis, thereby increasing tumor progression (Liu et al, 2008). Excessive GCS expression has been identified as a drug resistance mechanism in many cancer cell lines. Silencing GCS expression or inhibition of GCS activity sensitized resistant cells to more than 20 anticancer agents, including cisplatin (Liu et al, 2013). Introduction of the GCS gene into drug-sensitive cells conferred cellular resistance to several drugs, in most of the tested cell lines, indicating that drug resistance to some extent is cell-type or cancer-dependent (Liu et al, 2013). This mechanism of drug resistance has so far never been investigated in MPM or lung cancer. The GCS inhibitor DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) inhibits a number of GSLs, including Gb3 and increases cisplatin-induced cell death (Alam et al, 2015). Cyclosporin A, an MDR1 inhibitor, has also been shown to reduce Gb3 levels (Mattocks et al, 2006).

1.5.1 Globotriaosylceramide (Gb3)  
Gb3 is synthesized from lactosylceramide (Fig. 2), the common synthetic precursor to the majority of GSL, through α1,4-galactosyltransferase (Gb3 synthase) (Keusch et al, 2000; Yu et al, 2009). Lactosylceramide itself is synthesized by the stepwise addition of carbohydrate molecules, e.g. galactose, N-acetylgalactosamine, and sialic acid to glucosylceramide by galactosyl transferase 2 (Yu et al, 2009).

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**Figure 2.** The step-by-step conversion of ceramide to Gb3.
Gb3 is expressed in several human malignancies such as lymphoma (LaCasse et al, 1999), breast cancer (Johansson et al, 2009) and testicular carcinoma (Kang et al, 1995). Gb3 is generally not found in non-malignant tissue, except in the vasculature of some tissues. Gb3 expression in colorectal cancer correlates with invasiveness and metastatic potential (Kovbasnjuk et al, 2005) and overexpression of Gb3 have been seen in drug-resistant cancers and cell lines (De Rosa et al, 2008; Hanashima et al, 2008).

The bacterial toxin verotoxin-1 (VT-1) induces apoptosis by binding to its functional receptor, Gb3, thereby activating caspases (Kojio et al, 2000). The binding of VT-1 to Gb3 results in internalization and retrograde transport of the VT-1/Gb3 complex via endosomes and the golgi network to the endoplasmatic reticulum (Engedal et al, 2011; Falguieres et al, 2001). The proteolytically cleaved A1 subunit of VT-1 is translocated to the cytosol to inactivate protein synthesis by depurination of the 28S RNA of the 60S ribosomal subunit (Saxena et al, 1989). Cellular glucosylceramide is required to maintain Gb3 in VT-1-detectable plasma membrane lipid rafts (Smith et al, 2006) and is also required for ER retrograde transport of VT-1. Cells without lipid raft expression of Gb3 are insensitive to VT-1. In our previous study, a sub-toxic concentration of VT-1 potentiated cisplatin-induced apoptosis and cytotoxicity in cisplatin-resistant MPM P31res cells and NSCLC H1299res cells due to the upregulated expression of cell surface Gb3 (Johansson et al, 2010).

1.5.2 Interplay of Gb3 and MDR1

MDR1 has been suggested to flip glucosylceramide across the membrane from the cytosol to the Golgi as a mechanism to provide glucosylceramide as a substrate for the various luminal glucosyl transferases involved in neutral GSL biosynthesis. MDR1 is therefore considered a prerequisite for neutral GSL biosynthesis (De Rosa et al, 2004; Lala et al, 2000). A functional dependency between Gb3 and MDR1 in plasma membrane lipid rafts has been suggested (De Rosa et al, 2008; Lavie & Liscovitch, 2000; Mattocks et al, 2006) possibly by upregulation of MDR1 expression through GCS (Liu et al, 2010). Cell surface MDR1 has been found to partially co-localize with Gb3 in MDR1-transfected canine kidney cells (Lala et al, 2000) and MDR1 and GCS are coincidently overexpressed in several drug-resistant cell lines (Gouaze et al, 2004; Liu et al, 2008).

Tumour chemotherapy induces cell stress and generates ceramide, driving cells to proliferation arrest and apoptosis. This transactivates GCS expression via the specificity protein 1 (Sp1) transcription factor leading to an increase of GSLs (e.g. Gb3). This in turn activates cSrc kinases, thereby increasing nuclear β-catenin and transactivating MDR1 expression. In MDR1-transfected cells, cell surface MDR1 is co-localized with Gb3 and a soluble analog of Gb3 (adamantylGb3) proved to inhibit MDR1 and increase
drug sensitivity (De Rosa et al., 2008). Ceramide as well as the GSLs may therefore up-regulate GCS and MDR1 expression in response to anticancer drugs, and confer acquired tumour cell resistance by preventing ceramide-induced apoptosis and possibly MDR1-mediated drug efflux (Liu et al., 2010; Liu et al., 2008). Inhibitors of GCS may therefore prove useful in preventing chemotherapy resistance (De Rosa et al., 2008; De Rosa et al., 2004).

1.6 Heat shock proteins
Heat shock proteins (HSPs) are stress proteins, functioning as chaperones, assisting the proper folding of misfolded proteins and aiding to prevent their aggregation. HSP70 expression is generally only seen in cells under cellular stress (Nylandsted et al., 2004). Overexpression of HSPs in tumour cells is often associated with poor prognosis (Goloudina et al., 2012). A high expression of HSP70 is also associated with resistance to cisplatin in ovarian cancer and inhibition of HSP70 can resensitize cells to cisplatin (Yang et al., 2012). The ability to inhibit lysosomal membrane permeabilization is one of the mechanisms behind the antiapoptotic abilities of HSP70. Depletion of HSP70 has been seen to lead to massive caspase-independent apoptosis (Nylandsted et al., 2004). Inhibitors such as VER-155008 and MKT-077 can be used to target HSP70, but specific HSP70 inhibitors have not yet been found (Goloudina et al., 2012).

HSP70 in the cytosol is known to offer protection from apoptosis (Garrido et al., 2006), but membrane-bound HSP70 has other properties. On the cell surface, HSP70 acts as recognition sites for activated natural killer (NK) cells (Multhoff et al., 1999) and cell surface HSP70 can induce immunological and inflammatory responses (Henderson & Pockley, 2010). It is also believed that cell surface HSP70 might facilitate metastases, support adherence of tumor cells to endothelial cells and organs, or confer resistance to an unfavorable milieu during metastasis (Multhoff et al., 2011).

Studies have shown that HSP70 is present in glycosphingolipid and cholesterol-rich microdomains called lipid rafts. Cells expressing HSP70 in the cell membrane have also been shown to express higher levels of Gb3, and HSP70 and Gb3 are co-localized on the cell surface. Gb3 is believed to be a prerequisite for integration of HSP70 in the plasma membrane as depletion of Gb3 from tumors reduces the levels of membrane-bound HSP70 (Gehrmann et al., 2008).

17-N-Allylamino-17-demethoxygeldanamycin (17-AAG) is a HSP90 inhibitor that binds to the N-terminal ATPase domain of HSP90. Inhibition of HSP90 in cells may sometimes be compensated by induction of HSP70 expression (Kuballa et al., 2015). Studies have also shown that 17-AAG can induce ceramide production through activation of CD95 (Toyomura et al., 2012; Walker et al., 2010), a member of the tumor necrosis factor
superfamily (Peter & Krammer, 2003), which is also upregulated by cisplatin (Bagnoli et al, 2007).

1.7 Exosomes
Exosomes are small bio-nanoparticles, measuring from 30 to 100 nm in diameter. They are secreted by most biological cells and by many kinds of tumor cells. They are also present in most body fluids (Andre et al, 2002; Thery et al, 2002; Wolfers et al, 2001). Exosomes transport various biological molecules, ranging from membrane receptors and proteins to mRNA and microRNA (miR) (Camussi et al, 2010). The multivesicular endosome is formed from endosomes by invagination of the endosomal membrane. In most cells, multivesicular endosomes fuse with or mature into lysosomes but in certain cells, they fuse with the plasma membrane, extruding their vesicular cargo into the extracellular space, thereby releasing exosomes (Stahl & Barbieri, 2002).

The contents of the exosomes vary between different physiological and pathological conditions and cell types. The composition of exosomes can be distinct from the cells they originated from due to selective sorting of the cargo into exosomes (Zhang et al, 2015). The protein composition is similar in all exosomes and can be categorized into three major groups: genuine raft proteins, cytoskeleton-like proteins and heat shock proteins (Valadi et al, 2007).

Exosomes are able travel to surrounding cells or distant tissues to introduce properties such as immune stimulation, immune suppression (Whiteside, 2013), induction of proliferation and resistance to drugs (Corcoran et al, 2012; Katakowski et al, 2013; Skog et al, 2008). RNA can be present in exosomes and thereby be transferred from one cell to another (Valadi et al, 2007), which could contribute to the proliferation and metastasis of cancer and cancer development as well as drug resistance (Katakowski et al, 2013; Ohshima et al, 2010; Skog et al, 2008; Xin et al, 2012; Zhang et al, 2015).

MDR1 is found in exosomes and is correlated with increased resistance to docetaxel (Kato et al, 2015). Ceramide and GSLs can be present in exosomes as well. Inhibition of GCS synthase with PPMP has been shown to affect the composition of released exosomes (Llorente et al, 2013; Phuyal et al, 2014; Trajkovic et al, 2008). HSP70 has also been found in exosomes and a study found that the amount of HSP70 exosomes secreted by breast, ovarian and lung cancer cells increased after incubation with cisplatin (Gobbo(Lv et al, 2012) et al, 2016).

miRs are a class of short (~ 20 nucleotides) non-coding RNAs which can regulate the expression of nearly one third of all human genes (He et al, 2015; Shen et al, 2012). Some miRs are highly enriched in specific cell or tissue types and their potential as biomarkers has gained increasing interest.


miRs that have been associated with cisplatin resistance include miR-21, miR-155 (Challagundla et al., 2015). miR-98, miR-133b, miR-138, miR-181a, miR-200c (Crawford et al., 2009; Galluzzi et al., 2010; Xiang et al., 2013; Zhang et al., 2013), miR-181b (Zhu et al., 2010), miR-181d (Dai et al., 2011), miR-372/373 (Duale et al., 2007) and annexin A3 (Yin et al., 2012). During exposure of platinum-based drugs, adenocarcinoma A549 cells secreted exosomes which decreased the sensitivity of other A549 cells to platinum-based drugs, this may be mediated by miRs and mRNAs exchange by exosomes via cell-to-cell communication (Xiao et al., 2014). The miR-21-3p has been found to increase resistance to cisplatin in breast cancer cells, while miR-21-5p had the opposite effect and increased cisplatin sensitivity in some cell lines (Pink et al., 2015).
2. Objectives

The overall objective of this thesis was to analyze the role of Gb3, the Bcl-2 family of proteins and other apoptosis-related proteins in acquired cisplatin resistance of MPM and NSCLC cell lines in order to identify therapeutic targets to overcome cisplatin resistance.

2.1 Specific objectives

• To investigate the possible relationship between Gb3 and multidrug resistance protein expression and if inhibitors could re-sensitize cisplatin-resistant tumour cells to cisplatin cytotoxicity (Study I).
• To investigate the possible relationship between Gb3 and heat shock protein expression and if inhibitors could re-sensitize tumour cells to cisplatin cytotoxicity (Study II).
• To investigate the possible effects of BH3-mimetics ABT-737 and obatoclax on the expression of the Bcl-2 family of proteins and assess whether these or IAP-inhibitor AT-406 could potentiate the cytotoxic and apoptotic effects of cisplatin (Study III).
• To characterize exosomes secreted by cultured MPM and NSCLC cells and determine if exosomes from cisplatin-resistant tumour cell sublines could confer resistance to the more cisplatin-sensitive parental cell lines (Study IV).
3. Materials and methods

3.1 Cell lines

The P31 cell line used originates from a male patient with MPM in the early 1980s (Marklund et al., 1982). H1299 (American Type Culture Collection, CRL-5803, Manassas, VA, USA) is a NSCLC cell line derived from the lymph node and is widely used in research.

To analyze the mechanisms of acquired cisplatin resistance, cisplatin-resistant sublines of P31 and H1299 named P31res and H1299res were established (Janson et al., 2008). The cisplatin-resistant cell sublines were created by culturing the parental cells in medium with gradually increasing concentrations of cisplatin. The LD50 concentration of cisplatin after 72 h incubation was approximately 4 times higher in the sublines with acquired cisplatin resistance compared to the parental cell lines. 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) and supplemented with 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, respectively.

<table>
<thead>
<tr>
<th>Drug</th>
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<th>Study</th>
<th>Application**</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Sigma</td>
<td>II</td>
<td>CM, FC, FMCA</td>
<td>72h</td>
</tr>
<tr>
<td>ABT-737</td>
<td>Selleck</td>
<td>III</td>
<td>FMCA, PP, TUNEL, WB</td>
<td>6h</td>
</tr>
<tr>
<td>AT-406</td>
<td>Selleck</td>
<td>III</td>
<td>FMCA</td>
<td>72h</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>BMS</td>
<td>I</td>
<td>FMCA</td>
<td>72h</td>
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<tr>
<td></td>
<td></td>
<td>III</td>
<td>FMCA, PP, TUNEL, WB</td>
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<td></td>
<td></td>
<td>IV</td>
<td>GA</td>
<td>6h</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>Sigma</td>
<td>I</td>
<td>CM, FC, FMCA</td>
<td>72h</td>
</tr>
<tr>
<td>HA14-1</td>
<td>Sigma</td>
<td>II</td>
<td>FMCA, FC</td>
<td>72h</td>
</tr>
<tr>
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<td>Sigma</td>
<td>II</td>
<td>FMCA, FC</td>
<td>72h</td>
</tr>
<tr>
<td>Obatoclax</td>
<td>Selleck</td>
<td>III</td>
<td>FMCA, PP, TUNEL, WB</td>
<td>6h, 72h</td>
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<td>I, II</td>
<td>CM, FC, FMCA</td>
<td>72h</td>
</tr>
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<td>VER155008</td>
<td>Sigma</td>
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<td>FMCA, FC</td>
<td>72h</td>
</tr>
<tr>
<td>VT-1</td>
<td>Sigma</td>
<td>II</td>
<td>CM, FC, FMCA</td>
<td>72h</td>
</tr>
</tbody>
</table>

Table 1 Summary of drugs used in thesis.

* BMS – Bristol-Myers Squibb, New York, NY, USA; Selleck – Selleck Chemicals, Houston, TX, USA; Sigma – Sigma-Aldrich, St Louis, MO, USA
** CM – Confocal microscopy; FC – Flow cytometry, FMCA – Fluorometric microculture cytotoxicity assay, PP – Proteome profiler; WB – Western blot; GA – Gene expression array
3.2 Cell death analysis

3.2.1 Fluorometric microculture cytotoxicity assay (FMCA)
The fluorometric microculture cytotoxicity assay (FMCA) was used to quantify viable cells. Cells (2 - 10 x 10^3) were plated in the wells of 96-well microtiter plates with 100 µL of medium. Medium was removed by flicking the plate and wells were washed three times with 200 µL PBS (phosphate-buffered saline) buffer. To each well, 100 µL of PBS containing 10 mg/L fluorescein diacetate (FDA) (Amersham International, Amersham, UK) was added and the plates were 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. Viability was determined as fluorescence in proportion to the fluorescence of viable control cells (Lindhagen et al., 2008).

3.2.2 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay
The cells (50 x 10^3 cells and 150 x 10^3 cells for P31 and P31res respectively) were cultured in Ø = 10 cm petri dishes with 15 mL MEM at 37 °C, 5% CO2 for seven days. Cells were incubated with 2.5 or 10 mg/L cisplatin, 1 µmol/L ABT-737 and 0.4 µmol/L obatoclax, alone or in combination, for 6 h followed by drug-free medium for 72 h. Cells were harvested through trypsination, washed (free cells in the medium were also washed and added to the trypsinated cells) and fixed in 2% paraformaldehyde for 1 h. The cells were then permeabilised with 0.5 % Triton X-100 and 0.1% sodium citrate and labeled with tetramethylrhodamine (TMR) red (Roche, Basel, Switzerland). DNA fragmentation was analysed in a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

3.2.3 Analysis of synergistic drug combinations
To assess synergy effects of the 17-AAG+VT-1 combination, the Chou-Talalay method was used (Chou, 2010). 36 different 17-AAG and VT-1 concentration combinations were added to 96-well microtiter plates with P31, P31res, H1299 and H1299res cells. Cell viability was determined by FMCA and synergy effects were analyzed using computer software CompuSyn by Chou & Martin, 2005 (Combosyn Inc., Paramus, NJ, USA). The definition of an additive effect is when CI = 1. Synergy effect is when CI < 1 and antagonistic effect when CI > 1 (Chou, 2010).
### 3.3 Detection of proteins

**Table 2** Antibodies used in flow cytometry, western blot and confocal microscopy. mAb – Monoclonal antibody  
* Abcam – Abcam, San Francisco, CA, USA; Beckman – Beckman Coulter Inc., Brea, CA, USA; Chemicon – Chemicon International, Temecula, MA, USA; Dako, Glostrup, DK; Novex – Novex, Frederick, MD, USA; R&D – R&D Systems Inc., Minneapolis, MN, USA; T. Fisher – Thermo Fisher Scientific, Waltham, MA, USA  
** CM – Confocal microscopy, FC – Flow cytometry, FMCA – Fluorometric microculture cytotoxicity assay; WB – Western blot

<table>
<thead>
<tr>
<th>Protein</th>
<th>Type</th>
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<th>Dilution</th>
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</thead>
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<td>Beckman</td>
<td>FC, CM</td>
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</tr>
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<td>Mouse, IgG2a, mAb</td>
<td>AbCam</td>
<td>FC, CM, WB</td>
<td>1:50, 1:50, 1:200</td>
</tr>
<tr>
<td>HSP70</td>
<td>Rabbit, IgG, mAb to Alexa Fluor 647</td>
<td></td>
<td>WB</td>
<td>1:100</td>
</tr>
<tr>
<td>MDR1</td>
<td>Mouse, IgG2a, mAb</td>
<td>Chemicon</td>
<td>FC</td>
<td>1:50</td>
</tr>
<tr>
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<td>R&amp;D</td>
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<td>1:40</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Mouse, IgG1κ, mAb</td>
<td>Novex</td>
<td>WB</td>
<td>1:200</td>
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</table>

<table>
<thead>
<tr>
<th>Reactivity</th>
<th>Host</th>
<th>Label</th>
<th>Company*</th>
<th>Application**</th>
<th>Dilution</th>
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<tr>
<td>Rat IgM</td>
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<td>T. Fisher</td>
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<tr>
<td>Mouse IgG2a</td>
<td>Goat IgG</td>
<td>Alexa Fluor 647</td>
<td>T. Fisher</td>
<td>FC, CM, WB</td>
<td>1:100, 1:100, 1:10000</td>
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<tr>
<td>Mouse IgG1κ</td>
<td>Rabbit IgG</td>
<td>Horse-radish peroxidase</td>
<td>Dako</td>
<td>WB</td>
<td>1:5000</td>
</tr>
</tbody>
</table>
3.3.1 Flow cytometry

Cells were trypsinized, suspended in PBS and washed with PBS twice. Cells used for detection and quantification of total protein expression were fixed by incubating with 2% formaldehyde for 1 h in room temperature, followed by one wash with PBS with 2% BSA, 0.5% Triton X-100 and 0.1% sodium citrate at 4 °C for 10 min to permeabilize the membranes. Both fixed and unfixed cells were washed twice with PBS-BSA. Cells were then stained with desired primary antibodies (Table 1). To distinguish from non-specific binding, negative controls stained with corresponding isotype antibodies were used (Table 1). Cell pellets were re-suspended and incubated with secondary antibodies (Table 1) for 1 h at 4 °C. Cells were washed and centrifuged for 10 min and analysed with a FACScan flow cytometer (Becton Dickinson Immunotech Systems, San Jose, CA, USA). Data was processed using the BD CellQuest® software (Becton Dickinson). Debris and cell aggregates were gated out and the data was dot plotted.

3.3.2 Western blot

A bicinechonic acid (BCA) Protein assay (Pierce) was used to determine protein concentration. For quantification of HSP70 in exosomes, 5–10 μg protein were separated by 10% and 5–14% Mini-PROTEAN TGX gels (Bio-Rad, USA). The separated protein fractions in the gels were transferred to a polyvinylidene difluoride (PVDF) membrane (Midi format 0.2 μm, Bio-Rad, USA) using Trans-Blot Turbo (Transfer System, Bio-Rad, USA). The membrane was blocked with Odyssey Blocking Buffer (LI-COR Biosciences, Nebraska, USA) for 1 h at 21 °C, washed with PBS Tween (PBST) and then incubated with desired primary antibodies (Table 1) at 4 °C overnight. The membrane was washed with PBST before incubation with secondary goat anti-mouse antibody (Table 1) for 1 h at 21 °C. After washing with PBST the proteins were visualized using LI-COR ODYSSEY GLx. For quantification of Mcl-1, a previously described western blot method was used (Janson et al, 2008).

3.3.3 Confocal microscopy

Cells were grown in Ø 10 cm petri dishes until 80% confluence was reached. To achieve a similar confluence, the seeding densities for cells incubated with an LD50 concentration of a drug were doubled. Cytospin preparation followed manufacturer’s manual (Thermo Fisher Scientific, Waltham, MA, USA). A coverslip with ProLong Gold Anti-fade DAPI (Invitrogen) including nuclear staining was mounted on the slide to prevent photobleaching. Z-stacks were used to acquire images in ZEN 2010 imaging software on a LSM 710 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).
Quantification was made using IMARIS software (Bitplane, Zürich, Switzerland).

3.3.4 Proteome profiler array
Proteome profiler™ arrays for determination of apoptosis-related proteins and Phosphorylation of mitogen-activated protein kinases (MAPK) (R&D Systems, Minneapolis, MN, USA) were performed on P31, P31res, H1299 and H1299res cells incubated with or without cisplatin for 6 h. In P31 and P31res, the apoptosis profiler array was also performed on cells incubated with and without BH3-mimetics ABT-737 and obatoclax. Cells were plated on Ø 15 cm culture plates which were incubated with and without 2.5 or 10 mg/L cisplatin, 1 µmol/L ABT-737 and 0.4 µmol/L obatoclax, alone or in combination, for 6 h. The cells were washed with cold PBS and lysed with the lysis buffer included in the kit. To determine the total protein content of lysates, a BCA protein assay reagent kit was used according to the manufacturers’ instructions (Pierce Biotechnology). The array membranes were incubated overnight with 300 µg protein lysate per membrane and processed according to manufacturer’s instructions. The array spots were detected (ECL Advance Western Blotting Detection Kit, GE Healthcare), visualized and quantified with the Chemidoc XRS system and Quantity One 1-D Analysis software (Bio-Rad Laboratories) by determining pixel density and correcting for background.

3.4 Gene expression analysis
Total RNA was isolated using Gen Elute TM mammalian total RNA mini prep kit (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer’s instructions. RNA concentration was measured using ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA quality was evaluated using the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc, Palo Alto, CA).

Total RNA (250 ng) from each sample were used to generate amplified and biotinylated sense-strand cDNA from the entire expressed genome according to the GeneChip® WT PLUS Reagent Kit User Manual (P/N 703174 Rev 1 Affymetrix Inc., Santa Clara, CA). GeneChip HTA Arrays (GeneChip Human Transcriptome Array 2.0) were hybridized for 16 h in a 45°C incubator, with a rotation of 60 rpm. The arrays were then washed and stained according to manufacturer’s instructions using the Fluidics Station 450 (Affymetrix Inc.) and were finally scanned using the GeneChip Scanner 3000 7G (Affymetrix Inc.). The raw data was normalized in the free software Expression Console provided by Affymetrix (http://www.affymetrix.com) using the robust multi-array average (RMA)

3.5 Exosome isolation
Cells were cultivated for 72 h in complete medium. The medium was collected and centrifuged at 3000 g for 30 min. The supernatant was collected and passed through a 0.2 µM filter for removal of apoptotic bodies and an Amicon-15 100 kDa centrifugation filter (Merck Millipore) was used for reducing volume and serum protein of the samples. Samples (0.5 mL) were loaded on the qEV column (iZON Science, Christchurch, New Zealand). Fractions of 0.5 mL were collected according to manufacturer’s instructions. The protein content of all fractions was determined by the Bradford assay (Thermo Scientific) to ensure the purity of the fractions. Particle size and concentration were determined by Nanoparticle Tracking Analysis on the Nanosight 300 system (Malvern Instruments LTB, Worcestershire, UK), after being diluted to the instruments working range of 1-10 x 10⁸ particles/mL. Camera shutter was set to 33.31 ms and gain to 400. All fractions were measured during 60s and the final sample concentration was calculated by summing particle values of all vesicle-containing fractions.

3.6 Exosome analysis and quantification
Electron microscopy of isolated endovesicular vehicles was performed at Umeå University, Sweden. 3.5 µL of purified exosomes were applied to 300 mesh formvar and carbon coated Ni-grids to adhere for 2 min. Grids were washed in MQ-water to remove excess sample and negatively stained for 2 x 15 sec with 1.5% Uranyl acetate. Exosomes were examined with a JEM1230 transmission electron microscope (JEOL) operating at 80kV. Micrographs were acquired with a Gatan Orius 830 2kx2k CCD camera using Digital micrograph software.

Microvesicles were quantified by using Nanoparticle tracking analysis (NTA) (NanoSight N300, Malvern Instruments Ltd. Malvern, United Kingdom). The exosomes were incubated with PKH67 (Sigma-Aldrich) according to manufacturer’s instructions to analyze exosome uptake in H1299, H1299res, P31 and P31res 3-6 h after transfection with exosomes. To analyze uptake of exosomal HSP70, exosomes were stained with rabbit monoclonal anti-HSP70 conjugated to Alexa Fluor 647 (1:100) (Abcam, San Fransisco, CA, USA) for 24 h at 4 °C. The cells were transfected with these exosome for 3-6h.
4. Results and discussion

4.1 Interrelationship of cell surface Gb3, MDR1 and MRP1 (Study I)
Flow cytometry results and confocal microscopy alike confirmed previous findings (Johansson et al, 2010; Johansson et al, 2009) that cell surface Gb3 is increased in the cisplatin-resistant sublines of P31 and H1299 (Table 3). While several reports (Kok et al, 2000; Lavie & Liscovitch, 2000; Lucci et al, 1998; Morjani et al, 2001; Veldman et al, 2002) state a relationship between MDR1 and Gb3, we found no evidence of correlation or co-localization between the two, indicating that MDR1 might not be an important factor of cisplatin resistance in these tumor cell lines. However, confocal microscopy did display an increased MDR1-expression in the cisplatin-resistant sublines, which means the importance of MDR1 to cisplatin resistance can’t completely be ruled out. Flow cytometry results showed that cell surface Gb3 expression of resistant cells was annihilated by PPMP in all cell lines (H1299, H1299res, P31 and P31res). No decrease of MDR1 by PPMP or cyclosporin A was noted using flow cytometry, however, a decrease of MDR1 in H1299 was indicated by confocal microscopy.
Table 3. Brief summary of the effect of 17-AAG, VT-1, Gb3 synthase, PPMP and cyclosporin A on Gb3 and its connected proteins HSP70 and MDR1. Up/down arrow indicates up- or downregulation of protein, ‘-’ indicates no change. The effects of PPMP were tested on HSP70, Gb3 and MDR1 expression. The effects of 17-AAG, VT-1 and Gb3 synthase siRNA were only tested on HSP70 and Gb3, while cyclosporin A was tested on Gb3 and MDR1.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Drug</th>
<th>HSP70</th>
<th>Gb3</th>
<th>MDR1</th>
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<tbody>
<tr>
<td>H1299</td>
<td>17-AAG</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>VT-1</td>
<td>–</td>
<td>–</td>
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<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Cyclosporin A</td>
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<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1299res</td>
<td>17-AAG</td>
<td>↑</td>
<td>↑</td>
<td>–</td>
</tr>
<tr>
<td>VT-1</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>–</td>
</tr>
<tr>
<td>Gb3 synthase siRNA</td>
<td>↓</td>
<td>↓</td>
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4.2 Interrelationship of cell surface Gb3 and HSP70 (Study II)

Gb3 and HSP70 were consistently co-expressed and co-localized on the cell surface of the resistant sublines H1299res and P31res, as shown by flow cytometry and confocal microscopy, supporting the suggestion that Gb3 is a prerequisite for presentation of HSP70 on the cell surface (Gehrmann et al., 2008) (Table 3). Both Gb3- and HSP70-expression on the cell surface of the parental cell lines H1299 and P31 was low or non-existent. GCS inhibitor PPMP, Gb3 synthase siRNA, and VT-1 all reduced both Gb3 and HSP70 expression in the resistant cell lines, according to flow cytometry, indicating that targeting Gb3 affects cell surface HSP70. The HSP90-inhibitor 17-AAG, previously shown to potentially increase HSP70 expression and ceramide production (Kuballa et al., 2015; Toyomura et al., 2012; Walker et al., 2010), did not only induce HSP70 cell surface expression, but also Gb3-expression, further supporting that Gb3 and HSP70 are strongly intertwined. Confocal microscopy demonstrated a significant decrease of Gb3- and HSP70-expression after VT-1 incubation only in P31res, an effect that seemed to have no effect on co-localization. A decreased HSP70 expression may be either beneficial or unfavorable depending on the tumor environment (Henderson & Pockley, 2010; Multhoff et al., 2011). Decreasing cell surface HSP70 by targeting Gb3 could therefore prove therapeutically useful, since there are only a few and unspecific HSP70-inhibitors available clinically today.

4.3 Effects of cisplatin and obatoclax on apoptosis-related proteins (Study III)

The effects of cisplatin and obatoclax on the expression of Bcl-2 and IAP family of proteins in P31 and P31res were determined by a proteome profiler array (Figure 3). Cisplatin increased the expression of Bcl-x and decreased the expression of cleaved caspase-3 in P31 cells. In P31res cells cisplatin decreased Bad, Bax, Bcl-2 and cleaved caspase-3. Obatoclax decreased protein expression of Bad, Bax, pro-caspase-3 and cleaved caspase-3 in P31. In P31res cells, Bad, Bax and pro-caspase-3 were also decreased, as well as Bcl-2 and Bcl-x. When cisplatin and obatoclax were combined, the effects were similar to those of obatoclax alone in P31 cells. In P31res cells, the combination of cisplatin and obatoclax affected protein expression similarly to cisplatin alone, except for Bcl-x expression which remained very low. It is uncertain exactly how cisplatin and obatoclax execute their apoptotic effect with regards to the Bcl-2, since both pro-survival and pro-apoptotic members are decreased, but it seems plausible that the Bcl-2-family is deregulated by cisplatin as well as obatoclax.
Figure 3. Summary of changes of expression of Bad, Bax, Bcl-2, Bcl-x, pro-caspase 3, caspase 3, cytochrome c, cIAP-1, cIAP-2, XIAP, livin, survivin, Smac/Diablo and HTRA2 in A.) P31 cells and B.) P31res cells when incubated with cisplatin, obatoclax and cisplatin combined with obatoclax for 6 h. Up arrow indicates increase of expression, down arrow decrease of expression and straight line indicates no change. Light grey indicates incubation with cisplatin, mid-grey incubation with obatoclax and black incubation with both cisplatin and obatoclax.
Cisplatin exposure had a marginal effect on P31 and P31res cell expression of IAPs cIAP1, cIAP2, XIAP, livin and survivin, while obatoclax decreased the expression of studied IAPs in both P31 and P31res cells. This indicates that the apoptotic and cytotoxic effects of obatoclax might be due to inhibition of IAP family of proteins expression. Targeting both the IAP and Bcl-2 family of proteins could therefore have synergistic effects. Cisplatin in combination with obatoclax resulted in reduced IAP contents in a similar manner to obatoclax per se in P31 cells. However, in P31res cells the addition of cisplatin to obatoclax resulted in IAP levels similar to those of P31res cells exposed to cisplatin only—i.e. no major changes compared to control.

Smac/Diablo expression was unaffected by cisplatin in both P31 and P31res cells whereas Htra2/Omi was slightly increased in P31 cells but decreased in P31res cells. Even though the effects on these IBM-containing proteins were small, this might cast some light on why cisplatin is 4 times more potent in the parental cell line compared to the cisplatin-resistant.

4.4 Cytotoxic effects of cisplatin combination therapies (Study I and III)

FMCA was used to study the effects of a combination of cisplatin and drugs that target pathways involving cisplatin resistance. Targeting multiple pathways could lead to synergistic effects and aid in the development of new chemotherapeutic treatment regimes.

4.4.1 Combining cisplatin with PPMP and cyclosporin A (Study I)

The GCS activity inhibitor PPMP increased the cytotoxic effects of cisplatin in all cell lines in a synergistic manner. The effects of cisplatin combined with MDR1 pump inhibitor cyclosporin A were additive. While PPMP decreased cell surface MDR1 expression in H1299 and P31res cells, which could make the cells more sensitive to chemotherapeutics since MDR1-overexpression correlates with enhanced DNA damage repair and reduced apoptosis induction (Liu et al, 2013; Ohmichi et al, 2005), the more likely explanation for the cisplatin-potentiating effect of PPMP is the reduction of cell surface Gb3, which occurred in all cell lines. The reduction of cell surface HSP70 expression could also contribute to the increased sensitivity to cisplatin. The different cellular responses to PPMP and cyclosporin A is probably due to the fact that PPMP is a GCS inhibitor, which directly inhibits an important enzyme in the synthesis of Gb3, while cyclosporin A inhibits MDR1, which would only have an indirect effect on Gb3. Since no co-localization between Gb3 and MDR1 and no effects on cell surface Gb3 was noted after incubation with cyclosporin A, a role of MDR1 in the cisplatin resistance of studied cell lines seem unlikely.
4.4.2 Cytotoxic effects of combining cisplatin with BH3-mimetics ABT-737 and obatoclax (Study III)

The effects of the BH3-mimetic obatoclax and cisplatin were studied after both 6 h and 72 h of incubation. After 72 h of incubation, obatoclax potentiated the cytotoxic effect of cisplatin in both P31 and P31res cells, a synergistic effect was noted in P31res cells, while the effect in P31 cells was additive.

After only 6 h of incubation, obatoclax, proved to have an additive effect on cisplatin cytotoxicity in P31 cells but not in P31res cells at the tested concentrations. However, obatoclax potentiated the apoptotic effect of cisplatin in both P31 and P31res cells. After 6 h of incubation, the BH3-mimetic ABT-737 did not potentiate the cytotoxic or apoptotic effects of cisplatin at the tested. In fact, the effects were slightly antagonistic.

Bcl-2 and Bcl-xL contribute to drug resistance in many cancers (Shamas-Din et al., 2011) and especially Bcl-xL is often overexpressed in mesothelioma cells (Hopkins-Donaldson et al., 2003; O’Kane et al., 2006; Soini et al., 1999). The inhibitory effect of obatoclax on Bcl-2 and Bcl-xL-expression in P31res cells likely contributes to the cytotoxic and apoptotic effects of obatoclax. While the potentiating effect of obatoclax on cisplatin cytotoxicity after 72 h was evident in both P31 and P31res cells, the potentiating effect on apoptosis was more prominent in P31res cells, possibly due to its effect on Bcl-xL. The proapoptotic Bcl-2 family protein Bax was unchanged in P31 cells when exposed to obatoclax alone, but was reduced in P31res cells, which could explain why LD₅₀ of obatoclax was 10 times higher in P31res cells. While obatoclax is a BH3-mimetic which targets the Bcl-2 family of proteins, it’s inhibitory effect on IAP expression, which was noted in both P31 and P31res cells, may also contribute to the cytotoxic and apoptotic effects.

Combining obatoclax with ABT-737 could potentially have a synergistic effect, since cell lines that are resistant to ABT-737 often overexpress Mcl-1 (van Delft et al., 2006) and obatoclax, unlike ABT-737, targets Mcl-1. Combining obatoclax with ABT-737 had no effect in P31 cells, but in P31res cells cytotoxicity increased compared to obatoclax or ABT-737 alone. A triple combination comprised of cisplatin, obatoclax and ABT-737 had no further effect on cytotoxicity or apoptosis compared to the cisplatin+obatoclax combination in P31 cells, but in P31res cells, cytotoxicity was higher than cisplatin+obatoclax, cisplatin+ABT-737 or cisplatin alone. Western blot results demonstrated that Mcl-1 expression were similar in both parental P31 cells and cisplatin-resistant P31res cells and also unaffected by cisplatin, indicating that resistance to ABT-737 may not be due to high Mcl-1-expression. Another study showed that Mcl-1 expression was higher in P31res cells (Michels et al., 2014), though this was not supported by our observations. Mcl-1 activity can be altered by cleavage, ubiquitination and phosphorylation (Thomas et al., 2010), it’s therefore possible that Mcl-1
activity differs in the two cell lines, which could explain why the ABT-737 and obatoclax combination had an effect in the resistant P31res cell line.

4.4.3 Cytotoxic effects of combining cisplatin with IAP inhibitor AT-406 (Study III)
The Smac mimetic AT-406 had an additive effect on cisplatin toxicity in P31 cells and a synergistic effect in P31res cells. While cisplatin had no effect on IAP-protein expression, proteome profiler showed that HTRA2 expression is decreased by cisplatin in P31res cells but increased in the parental cell line. Downregulation of HTRA2 in response to cisplatin exposure has been proposed as a possible cisplatin resistance mechanism (Yang et al., 2005). This could explain why the response to cisplatin+AT-406 exposure differed between the two cell lines. The downregulation of HTRA2 may thus be targeted by inhibiting IAP-protein expression with AT-406. The effects of AT-406 and cisplatin were similar to those of cisplatin combined with obatoclax, but there was no additional effect when cisplatin was combined with both obatoclax and AT-406.

4.5 Cytotoxic effects of combining VT-1 with 17-AAG (Study II)
The effect of combination of 17-AAG and VT-1 on cytotoxicity was analyzed using the Chou-Talalay method (Chou, 2010) and found to be highly synergistic. 17-AAG induced an increase of cell surface Gb3 expression, thereby maximizing VT-1 binding potential, which may be an explanation for the synergistic effects. The synergistic effects were higher in the resistant cell lines, which favors this hypothesis.

4.6 Analysis of exosomes and effects of exosomal transfection (Study IV)
All studied cell lines were found to secrete exosomes, as determined by Nanosight analysis and electron microscopy. No Gb3 was found in the exosomes, but HSP70 was present in the exosomes from all the cell lines. Cisplatin has previously been shown to increase the amount of HSP70 exosomes (Gobbo et al., 2016), but there was no difference in the amount of HSP70 found in the cisplatin-resistant cell lines (H1299res and P31res) compared to their cisplatin-sensitive parental cell lines (H1299 and P31). Cisplatin may however have a short term effect on secretion of HSP70 through exosomal release and we therefore plan to study the effect of cisplatin incubation on HSP70 content in the exosomes.

When all cell lines (H1299, H1299res, P31 and P31res) were transfected with exosomes from their corresponding cisplatin-resistant cell line (H1299res and P31res), confocal microscopy revealed that the exosomes were taken up by the cells and that Hsp70 were transferred from the
exosomes to the surface of the cells. Cell surface HSP70 on the surface of tumor cells may promote either cell death or survival depending on the tumor environment (Henderson & Pockley, 2010; Multhoff et al, 1999; Multhoff et al, 2011).

To complete the study we intend to examine if transfecting H1299 and P31 cells with exosomes secreted by their cisplatin-resistant sublines H1299res and P31res causes the cells to become more cisplatin-resistant and if the transfection changes the expression of Gb3 and HSP70 on the cell surface.
5. Conclusions

Previous studies have already highlighted Gb3 as an important biomarker for drug resistance in cancer (Johansson et al, 2009; Kang et al, 1995; Kovbasnjuk et al, 2005; LaCasse et al, 1999). This thesis puts further stress on the importance of Gb3 and delves into its relationship with other important biomarkers for drug resistance such as MRP1, MDR1 and HSP70. It also focuses on possibilities to resensitize cisplatin-resistant cancer cells to cisplatin by targeting Gb3 and/or its related proteins.

In line with previous results (Johansson et al, 2010; Johansson et al, 2009) we found, using flow cytometry and confocal microscopy, that Gb3 is expressed on the cell surface of the cisplatin-resistant cell lines of P31, H1299 and H1975.

Previous findings have suggested that Gb3 is colocalized with MDR1 on the cell surface of tumor cells (Kok et al, 2000; Lavie & Liscovitch, 2000; Lucci et al, 1998; Morjani et al, 2001; Veldman et al, 2002) but we found no evidence of co-localization in H1299 or P31 or their cisplatin-resistant cell lines. However, confocal microscopy demonstrated an increased expression of MDR1 in both studied cisplatin-resistant cell lines compared to their parental cell lines and cyclosporin A also decreased Gb3 in H1299 and H1299res cells, still providing the possibility of a functional dependency between Gb3 and MDR1.

Gb3 and HSP70 were found to be co-localized and co-expressed on the cell surface of H1299, H1299res and P31res cells. Targeting Gb3 with PPMP, VT-1 or Gb3 synthase siRNA decreased both Gb3 and HSP70 expression, pointing to a functional dependency between the two. 17-AAG increased Gb3 and HSP70 and had a synergistic cytotoxic effect with VT-1, suggesting that targeting extracellular Gb3 and HSP70 could lead to therapeutical benefits.

Acquired cisplatin resistance led to changes in the expression of the Bcl-2-family of proteins expression following exposure to cisplatin. While the BH3-mimetic ABT-737 had no potentiating effect on cisplatin cytotoxicity, obatoclax synergized with cisplatin cytotoxicity in cisplatin-resistant P31res cells and therefore shows promise as a drug candidate for treating MPM. The decrease of Bcl-x and the IAP-binding HTRA2 protein by obatoclax in P31res cells likely contributed to the synergistic effects in P31res cells. Combining obatoclax with ABT-737 had no effect on cytotoxicity in P31 cells, but in P31res cells there was a potentiating effect, both in the presence and absence of cisplatin. IAP inhibitor AT-406 had a synergistic effect with cisplatin on cytotoxicity in P31res, similar to that of obatoclax.

We found that exosomes from the cisplatin-resistant cells H1299res and P31res were taken up by cells of all studied cell lines and that exosomal HSP70 were presented on the surface of the cells. Inhibition of exosome
release may potentiate the effect of cisplatin (Li et al, 2016) and studies of
the role of exosomes in the acquisition of cisplatin may result in improved
anti-cancer treatments.

Taken together, the mechanisms of cisplatin are many and varied. To
reverse cisplatin resistance, several of these mechanisms, such as apoptosis-
related protein deregulation, cell surface protein expression and exosomal
transfer of resistance characteristics, must be considered.
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