CHAPTER 1

General introduction and scope of the thesis
Chapter 1

Introduction

Despite promising advances that have been made in detection, diagnosis and treatment of cancer in the last decades, it is still the second major cause of death in western countries. Physicians rely on surgery, chemotherapy and/or radiotherapy as the primary treatment strategies. Given that a large number of patients presents with disseminated disease at diagnosis, chemotherapy frequently forms an integral part of treatment. However, the efficacy of chemotherapy is often limited due to the occurrence of multidrug resistance. In this process cancer cells have become insensitive to the administered drug, and in addition to other, structurally or functionally unrelated, drugs. Therefore, novel strategies are required that reverse multidrug resistance. An extensively studied multidrug resistance mechanism involves drug efflux proteins, which pump cytotoxic drugs from the cytoplasm out of the cells. These proteins belong to the family of ABC transporter proteins and reside in the plasma membrane, where they are enriched in so-called 'lipid rafts'. These rafts are characterised by a high content of cholesterol, sphingolipids and specific proteins, which gives them different physical properties compared to the surrounding membrane. It may well be that these unique physical properties are essential to the functional activity of specific proteins, including ABC transporters. Thus, modulation of rafts, for example by changing the sphingolipid composition, may hold the key to hamper ABC transporter activity, thereby reversing multidrug resistance. Interestingly, increased levels of the sphingolipid glucosylceramide have been found in cells over-expressing ABC transporters, suggesting a relation between the two. Moreover, an increased synthesis of glucosylceramide implies that ceramide levels are decreased. Ceramide has been recognised as a second messenger, among others in apoptosis signalling. Thus, inhibition of glucosylceramide synthesis may lead to elevated levels of apoptosis-stimulating ceramide and decreased levels of multidrug resistance-associated glucosylceramide, making it an obvious target for chemosensitisation of cancer cells.

The research presented in this thesis was performed in neuroblastoma cells. Neuroblastoma is the most common extracranial solid tumour in childhood cancer. Treatment of the disease, in which chemotherapy plays a central role, is often limited due to the occurrence of multidrug resistance. This is one of the reasons that prognosis is generally poor for children with disseminated disease. Changes in sphingolipid composition have been observed in neuroblastoma cells compared to neural crest cells from which they originate, which may imply that sphingolipids play a role in the development of the disease.
Furthermore, neuroblastoma cells are characterised by high levels of the complex sphingolipid GD2, which is shed from these cells in considerable amounts, and affects the development of immune and haemopoietic cells in the environment.

Taken together, there appears to be a strong basis for the development of sphingolipid-based treatment strategies in cancer therapy in general and in neuroblastoma in particular.

This **General introduction** provides information on the main topics of this thesis; i.e. **neuroblastoma**, **multidrug resistance**, **ABC transporters** and **sphingolipids**. In addition, some information is provided on the topics cell cycle, cancer and lipid rafts. These issues are also relevant to the work presented in this thesis.

**Cell cycle**

Cellular division is an indispensable process during growth and in maintenance of an organism. In the adult human body millions of cellular divisions take place every second. Cellular division is a highly controlled process and part of the natural cell cycle. The cell cycle of a eukaryotic cell can roughly be divided into four successive phases:

- The first phase is the replication of nuclear DNA, which takes place in the so-called S phase.
- Prior to the actual cellular division, cells pass through the G\textsubscript{2} phase.
- The M phase is the phase in which the actual cellular division takes place. Within the M phase several sub phases can be recognised describing the progression state of nuclear division (mitosis). This phase ends with the physical separation of the two newly-formed daughter cells. The latter process is called cytokinesis.
- The time between two successive cellular divisions is designated as the G\textsubscript{1} phase. During both the G\textsubscript{1} and G\textsubscript{2} phase cells have time to grow. When the conditions are right, the cells can start a new round of cellular division (Alberts et al., 1994).

The cellular machinery continuously monitors all parameters essential to initiate or continue a successful cellular division. For a cell to progress to a next phase in the cell cycle, it has to pass checkpoints. The three major checkpoints are the G\textsubscript{1}, G\textsubscript{2}/M and metaphase (spindle assembly) checkpoints. Until a cell meets all requirements needed for a successful progression through the next phase of the cell cycle, cell cycle progression is arrested. Progression through the G\textsubscript{1} checkpoint requires parameters like intact DNA, favourable
environmental factors and cellular size, only then chromosomal duplication is initiated. Similarly, progression through the G2/M and metaphase checkpoints require parameters like fully duplicated chromosomes, favourable environmental factors, cellular size and alignment of the chromosomes on the spindle, respectively (Cleveland et al., 2003, Taylor et al., 2004). Many proteins are involved in cell cycle regulation (Nigg, 2001, Barr et al., 2004) but a central role is played by a combination of specialised proteins called the cyclin-dependent protein kinases (CDK) and cyclins (Alberts et al., 1994). The cyclins bind to the CDKs and thereby control their ability to phosphorylate target proteins on serines and threonines, which are then activated or deactivated. The activity of cyclins, on their turn, is controlled by proteins such as Cdc25 phosphatases (Coleman and Dunphy, 1994), p27 or the retinoblastoma (Rb) gene product (Murray, 2004). Two key CDKs in cellular division are CDK1, which controls the M phase (Nigg, 2001), and CDK2, which plays a central role in DNA and centrosome duplication (Meraldi et al., 1999).

Besides the duplication and subsequent separation of the chromosomes, cellular division also has major consequences for the microtubule network and organelles such as the endoplasmatic reticulum (ER) and Golgi apparatus. The microtubule network undergoes a drastic rearrangement, which is essential to the actual separation of the duplicated chromosomes (Alberts et al., 1994; Kline-Smith, 2003). Disturbance of the microtubules results in a cell cycle arrest or even cell death (Jordan, 2002). A number of cytotoxic drugs sort their effect by disturbing microtubules. Examples of such microtubule-perturbing drugs are paclitaxel (Abal et al., 2003; Burns et al., 2003) and vincristine (Jordan et al., 1991) (stabilising microtubules) and nocodazole (Thyberg and Moskalewski, 1999) (destabilising microtubules). The fate of the Golgi apparatus during mitosis is still subject of debate (Barr, 2004; Colanzi et al., 2003). Some investigators argue that the Golgi is fragmented into small cytoplasmic vesicles or tubulo-reticular elements (Shima et al., 1997; Jesch et al., 2001; Hammond and Glick, 2000; Prescott et al., 2001; Colanzi et al., 2000), while others favour the hypothesis that redistribution of Golgi proteins and membrane into the ER occurs (Thyberg and Moskalewski, 1992; Zaal et al., 1999). Important proteins for the coordinated assembly and disassembly of the Golgi apparatus during mitosis are among others: GM130 and p115 (Lowe et al., 2000), Arf1 (Altan-Bonnet et al., 2003) and Sar1 (Altan-Bonnet et al., 2004).

Cytotoxic drugs used in cancer therapy affect cells by induction of DNA damage, interference with RNA and DNA repair mechanisms or disruption of microtubules. These effects are most apparent during mitosis and often result in mitotic arrest due to the activation of a mitotic checkpoint. The microtubule-stabilising drug paclitaxel causes an arrest at the
metaphase/anaphase transition due to suppressed microtubule dynamics. This may result in a permanent activation of the mitotic spindle assembly checkpoint and eventually the induction of programmed cell death (apoptosis) (Burns et al., 2003). Alternatively, the mitotic arrest is transient and the cell progresses, despite suppressed microtubule dynamics, to a G₁-like state (Chen et al., 2003; Allman et al., 2003). This phenomenon is called mitotic slippage and usually involves an aberrant exit from mitosis with abnormal chromosome separation without cytokinesis. In the absence of a proper G₁ checkpoint, multinucleated cells are formed that can re-enter S phase. In contrast, cells with a proper G₁ checkpoint are arrested and subsequently apoptosis is triggered in a p53- or Rb-dependent way (Minn et al., 1996; Lanni and Jacks, 1998; Sablina et al., 1999; Tsuiki et al., 2001).

**Cancer**

The average life span of cells in an adult human body is about one month. This implies that a continuous formation of new cells needs to take place in order to maintain a steady state. In normal cells the process of cell growth and division is rigorously controlled. However, this regulation is lacking in cancers cells, resulting in uncontrolled cell growth and cell division. Uncontrolled cell proliferation is one of the fundamental characteristics of cancer cells. Furthermore, malignant cancer cells are distinguished from normal or benign cells by their poor differentiation state, invasive capacities and their capability to form metastases. Cancer cells also display a number of more or less specific morphological characteristics, such as enlarged nuclei, increased levels of nucleic acids, and increased number of nucleoli. Most cancer cells originate from poorly differentiated stem cells rather than from highly differentiated cells. Thus, the less committed state of cancer cells is generally not the result of dedifferentiation (Fiala, 1968; Reya et al., 2001). The theory that cancer cells originate from stem cells was first proposed by Fiala in 1968, and is known as the ‘cancer stem cell hypothesis’ (Fiala, 1968). This theory may also explain the recurrence potential of cancer cells after chemotherapy. Not only is drug resistance an innate characteristic of tumour stem cells, stem cells are also less prone to chemotherapy because they do not divide. Therefore, it is likely that stem cells survive to continue proliferating (Donnenberg and Donnenberg, 2005).

The abnormal proliferation of cancer cells is preceded by a number of hereditary and/or somatic mutations of specific genes. These genes belong to any of the following three
classes: the proto-oncogenes, the tumour suppressor genes, and genes involved in DNA repair mechanisms (Cooper, 1992). Proto-oncogenes are normal cell genes that play important roles in processes involved in cell growth and differentiation. Due to genetic alterations, these proto-oncogenes can turn into oncogenes. Although related to proto-oncogenes, oncogenes are only present in cancer cells, in which they cause abnormal oncogene gene expression or expression of structurally abnormal gene products. Oncogenes are activated as a result of the following genetic alterations: point mutations, rearrangements, or amplification of proto-oncogenes. A well-known example of gene amplification is the \textit{N-myc} gene in neuroblastoma, which is associated with rapidly progressing, highly malignant tumours. Other well-known examples of oncogenes are the \textit{src} and \textit{ras} gene families.

Genes belonging to the second class of genes that is affected in cancer cells are the tumour suppressor genes. These genes have important functions in normal cells concerning the inhibition of cell growth and tumour development. In cancer cells these genes are often lost or inactivated, thus resulting in uncontrolled proliferation. Well-known examples of tumour suppressor genes are \textit{Rb} and \textit{p53}.

DNA repair genes are involved in the correction of the numerous DNA damaging events that occur in a single cell every day. Without these genes, DNA damage would accumulate, which could result in cancer cell formation.

The nature of genetic changes is often determined in addition to tumour type, localisation and stage of the primary tumour, and the presence of metastases in order to make a prognosis and to map out the treatment strategy. In general, treatment of cancer is approached with surgery, chemotherapy and/or radiotherapy. In adults, surgery suffices in case of a localised tumour that has not invaded the adjacent normal tissues, or spread to distant parts of the body. Unfortunately, however, most tumours (about 70%) have already metastasised at the time of diagnosis. In these cases surgery is still an integral part of treatment, for example in removal of the primary mass or isolated metastatic tumours, and is supplemented with chemotherapy and/or radiotherapy. In children, chemotherapy often forms an integral part of treatment in addition to surgery due to the high chance of micrometastases. Whereas chemotherapy is applied in case of disseminated disease, radiotherapy, like surgery, is primarily used for treatment of localised tumours. The principle of radiotherapy is induction of irreversible and lethal DNA damage. This DNA damage specifically affects highly-proliferating cells, such as cancer cells, resulting in induction of apoptosis during mitosis. The efficacy of both radiotherapy and chemotherapy is limited by the toxic side effects. Besides cancer cells, these treatment modalities also affect normal cells with a high proliferation rate,
including: blood-forming cells in the bone marrow, epithelial mucosal cells, epidermal cells, cells of the hair follicles, and cells of the reproductive system. When these cells are affected, side effects occur, such as anaemia, infections, nausea, vomiting, diarrhoea, skin damage, hair loss and sterility (Cooper, 1992). In order to increase the efficiency of chemotherapy (i.e. increase cancer cell death, reduce side effects), often a combination of chemotherapeutics with different targets is used. A few examples of chemotherapeutics that are widely used in the clinic are the topoisomerase II inhibitors doxorubicin and etoposide (Estlin and Veal, 2003), the DNA damaging agents cisplatin and cyclophosphamide (Estlin and Veal, 2003) and the microtubule perturbing agents paclitaxel and vincristine (Jordan, 2002). However, as will be discussed later on, cancer cells can become resistant to chemotherapeutics. Therefore, the search for new or improved therapies continues (Novotny and Szekeres, 2003, 2005; Malecki et al., 2005).

Neuroblastoma

Neuroblastoma is the most common extracranial solid tumour in infancy and childhood. It accounts for approximately 5-7% of all childhood cancers. Neuroblastoma arises from neural crest cells, and can develop anywhere along the sympathetic chain, including the adrenal medulla (Haase et al., 1999; Maris, 2005). The majority of tumours develops at the adrenal gland and sympathetic ganglia of the abdomen, neck, thorax, and/or pelvis. Dissemination of neuroblastoma most commonly occurs to the regional lymph nodes, liver, bones, and bone marrow (Brodeur, 2003). The symptoms of neuroblastoma are dependent on the (primary) tumour location, but also include more systemic symptoms like weight loss, pallor and lethargy, especially when disseminated disease has occurred.

Determination of the clinical stage of neuroblastoma is of major importance to treatment strategy and prognosis. Therefore, a distinction has been made between four stages of neuroblastoma, which are described in the International Neuroblastoma Staging System (INSS) (Table I) (Brodeur et al., 1988; Brodeur et al., 1993). Patients diagnosed with stage 1 of the disease show a localised growth pattern of the tumour and have a good prognosis. In contrast, patients diagnosed with stage 4 tumours display metastatic growth and have a poor prognosis.
### Table I. International Neuroblastoma Staging System

<table>
<thead>
<tr>
<th>Stage</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Localised tumour with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumour microscopically (nodes attached to and removed with the primary tumour may be positive).</td>
</tr>
<tr>
<td>2A</td>
<td>Localised tumour with incomplete gross excision, representative ipsilateral non-adherent lymph nodes negative for tumour microscopically.</td>
</tr>
<tr>
<td>2B</td>
<td>Localised tumour with or without complete gross excision, with ipsilateral non-adherent lymph nodes positive for tumour. Enlarged contralateral lymph nodes must be negative microscopically.</td>
</tr>
<tr>
<td>3</td>
<td>Unresectible unilateral tumour infiltration across the midline, with or without regional lymph node involvement, or localised unilateral tumour with contralateral regional lymph node involvement; or midline tumour with bilateral extension by infiltration (unresectable) or by lymph node involvement.</td>
</tr>
<tr>
<td>4</td>
<td>Any primary tumour with dissemination to distant lymph nodes, bone, bone marrow, liver, skin and/or other organs (except as defined for stage 4S).</td>
</tr>
<tr>
<td>4S</td>
<td>Localised primary tumour (as defined for stage 1, 2A or 2B), with dissemination limited to skin, liver and/or bone marrow (limited to infant &lt;1 year of age).</td>
</tr>
</tbody>
</table>

(Haase et al., 1999; Brodeur et al., 1993)

The traditional ‘age and anatomic staging’ (Table I) has been supplemented with a staging system based on risk factors (Table II), which include primary tumour site (Maris and Matthay, 1999), serum markers (Berthold et al., 1991; Hann et al., 1985; Schulz et al., 1984; Valentino et al., 1990), genetic factors, histopathology (Shimada et al., 1999) and molecular indicators of progression (Castleberry et al., 1997; Haase et al., 1999). Many genetic features of neuroblastoma, such as the ploidy status, oncogene amplification, or allelic loss correlate with clinical outcome. Genetic features that correlate with aggressive tumours and poor prognosis are loss of heterozygosity or translocations in chromosome 1p, gain of 17q and MYCN oncogene amplification (Brodeur et al., 1981; Fong et al., 1992; Maris et al., 1995; Caron et al., 1996; Bown et al., 1999; Schwab et al., 1983; Brodeur et al. 1984; Seeger et al., 1985). Near-triploidy and high expression of the neurotrophin receptor TrkA, on the other hand, are favourable markers (Look et al., 1984; Nakagawara et al., 1993). MYCN amplification could also be related to drug resistance in neuroblastoma. A number of studies showed that over-expression of the drug resistance proteins MRP1 (multidrug resistance-related protein 1) and MRP4 are correlated with MYCN amplification (Norris et al., 1997;
Manohar et al., 2004; Norris et al., 2005). Therefore, genetic features have become essential to tumour characterisation and treatment stratification.

**Table II. Neuroblastoma risk groups based on clinical and biological features**

<table>
<thead>
<tr>
<th>Stage*</th>
<th>Low risk</th>
<th>Intermediate risk</th>
<th>High risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>All</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2A, 2B</td>
<td>Age &lt;1 year, or age 1-21 years and MYCN non-AMP, or age 1-21 years and MYCN AMP + FH</td>
<td>None</td>
<td>Age 1-21 years and MYCN AMP + FH</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>Age &lt;1 year and MYCN non-AMP, or age 1-21 years and MYCN non-AMP + FH</td>
<td>Age 0-21 years and MYCN AMP, or age 1-21 years and MYCN non-AMP + UH</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>Age &lt;1 year and MYCN non-AMP</td>
<td>Age &lt;1 year and MYCN AMP, or age 1-21 years</td>
</tr>
<tr>
<td>4S</td>
<td>MYCN non-AMP; FH; DI&gt;1</td>
<td>MYCN non-AMP; DI=1</td>
<td>UH; MYCN AMP</td>
</tr>
</tbody>
</table>

(Brodeur, 2003); * International Neuroblastoma Staging System; AMP, amplified; DI, DNA index; FH, favourable histology; non-AMP, not amplified; UH, unfavourable histology.

Progressive stage neuroblastoma is further stratified dependent on the clinical and biological prognostic factors and requires intensive treatment, which includes combinations of surgery, chemotherapy, autologous bone marrow transplantation and/or radiotherapy. The chemotherapeutics currently used are cyclophosphamide, ifosfamide, cisplatin, carboplatin, vincristine, doxorubicin, melphalan, etoposide and topotecan. In patients diagnosed with stage 1 or 4S of the disease surgical removal of the primary tumour mass often suffices. In some rare cases, spontaneous regression or maturation of the tumour into a benign gangliioneuroma may even occur. Unfortunately, despite intensive multimodality therapy, especially older children (>1 year) have a poor prognosis, which is often due to multidrug resistance (MDR; described in more detail in the next paragraph). In order to improve the outcome of treatment of patients with advanced stage neuroblastoma, and to reduce toxic side effects of treatment, new or improved therapies need to be developed. Currently, several new therapies are being developed, some of which have entered the clinical trial phase. One of these therapies is anti-GD2 antibody therapy. GD2 is a complex glycosphingolipid (ganglioside), which will be discussed in more detail in the paragraph ‘Sphingolipids’. In contrast to other cells of the
human body, neuroblastoma cells express high levels of ganglioside GD2 in their membranes (Schulz et al., 1984). The anti-GD2 antibodies that have been developed bind specifically to neuroblastoma cells and are subsequently internalised. Binding of these antibodies triggers cell- and complement-dependent tumour cell lysis (Mujoo et al., 1987), thereby suppressing tumour growth. Clinical trials using murine anti-GD2 monoclonal antibodies were not very successful due to serious side effects caused by human anti-mouse antibodies (Handgretinger et al., 1992). Preliminary results from clinical trials using modified (chimeric) anti-GD2 antibodies appear to be more promising, especially when antibodies are used in combination with cytokines to induce antibody-dependent cell-mediated cytotoxicity (Umaña et al., 1999; Ozkaynak et al., 2000; Bestagno et al., 2003). Other new therapies focus on: - induction of differentiation with retinoic-acid derivatives, such as 13-\textit{cis} retinoic acid (Matthay et al., 1999), or - induction of apoptosis, for example with the synthetic retinoid \textit{N}-(4-hydroxyphenyl)retinamide (fenretinide) (Lovat et al., 2000; Reynolds, 2000).

Until these new therapies make it to the clinic, neuroblastomas, and most other cancers, will have to be treated with the current generation of chemotherapeutics. This also implies that MDR will remain a major obstacle in the successful treatment of this disease. The next paragraph discusses the various MDR mechanisms.

**Multidrug resistance**

In everyday life cells are continuously exposed to all kinds of stress. Over millions of years cells have evolved to prevent or repair damage inflicted by stress like UV light, radiation, cytotoxic agents and shearing stresses. These protective mechanisms form a major obstacle in the treatment of cancer and other diseases. For example, as a result of chemotherapy tumour cells can become resistant to the employed cytotoxic drug, as well as to a broad range of other, functionally and structurally, unrelated drugs. This phenomenon is known as multidrug resistance (MDR) and was first defined by Ling and Thompson in 1974 (Ling and Thompson, 1974; Ling et al., 1983). The cellular mechanisms contributing to MDR can be divided into three categories based on the sorted effect, and work independently or as a combination (Biedler, 1994; Simon and Schindler, 1994):

- Mechanisms that lower intracellular drug levels. This can be achieved by changing the composition and hydrophobicity of the plasma membrane, thereby preventing that drugs can diffuse freely through the membrane (Carlsen et al., 1976; Bradley et al., 1988), or by
increasing drug efflux (Gros et al., 1986; Gottesman and Pastan, 1993; Borst et al., 2000; Gottesman et al., 2002).

- Mechanisms that reduce the efficacy of cytotoxic drugs (Simon and Schindler, 1994). Examples of these strategies are trapping of drugs in intracellular organelles, thereby preventing them from reaching their targets (Simon et al., 1994), and increasing detoxification pathways that convert drugs to harmless products (McFadyen et al., 2004).

- Mechanisms that compensate for all drug-inflicted damage, which includes increased DNA-repair activity (Spanswick et al., 2002) and inhibition of apoptotic pathways (Pardo et al., 2003).

One mechanism in particular has been studied extensively, i.e. the ATP-driven transport of cytotoxic drugs out of the cell. The transport of cytotoxic drugs out of tumour cells is mainly facilitated by two members of the ATP-binding cassette (ABC) transporter protein superfamily, P-glycoprotein (Pgp) and MRP1. Transporter proteins have been found in all living organisms, ranging from bacteria to complex multicellular organisms (Borst et al., 2000; Dean et al., 2001). In eukaryotes, ABC transporter proteins serve to shuttle hydrophobic molecules over membranes, either within the cell as part of a metabolic process, or to the outside of the cell for transport to other organs or secretion from the body. Of the 48 ABC genes in the human genome the \textit{ABCB1} (Pgp) and \textit{ABCC1} (MRP1) genes encode for the proteins that are often over-expressed in tumour cells, causing MDR. Pgp is a membrane protein with 12 membrane spanning domains, which are divided over two homologues membrane domains. These membrane domains both contain a cytoplasmic ATP-binding domain (nucleotide binding domain, NBD) inside a large cytoplasmic loop. Pgp has a broad substrate specificity of neutral and basic organic compounds. Among the chemotherapeutics that are recognised and transported by Pgp are colchicine, doxorubicin, etoposide, adriamycin, vinblastin, paclitaxel and vincristine (Scotto, 2003).

However, how these substrates are transported to the extracellular space is still not completely clear. At least three models have been proposed that could describe how Pgp shuttles molecules across the membrane.

- One model describes that Pgp works as a selective, ATP-dependent channel or membrane pore.
- Another model, which is also known as the ‘hydrophobic vacuum cleaner’ model describes that Pgp removes drugs from the inner leaflet of the plasma membrane and transports them to the extracellular space (Gottesman and Pastan, 1993).
Chapter 1

- In the third model Pgp was proposed to work like a flippase, moving drugs from the inner to the outer leaflet of the plasma membrane. Once in the outer leaflet, drugs eventually diffuse into the extracellular space (Bolhuis et al., 1996; Borst et al., 2000; Chang and Roth, 2001; Chang, 2003, Gottesman and Pastan, 1993).

MRP1-mediated transport differs from Pgp-mediated transport in the sense that MRP1 transports glutathione conjugates (Jedlitschky et al., 1994; Muller et al., 1994; Borst et al., 2000). This has implications for the substrate specificity of MRP1, which partly overlaps with that of Pgp. However, unlike Pgp, MRP1 is able to transport lipophilic anions, while Pgp substrates are neutral or mildly positive lipophilic compounds (Cole et al., 1994; Kruh and Belinsky, 2003). MRP1 and Pgp are also structurally different, although both have a similar two-membrane-domain core. MRP1 (and several other MRP family members) has an additional membrane domain consisting of five membrane spanning domains (Schinkel and Jonker, 2003).

In vitro models have shown that tumour cells can acquire MDR when they are grown under selective pressure of a cytotoxic drug (Roninson et al., 1984; Batist et al., 1986). Moreover, it has been shown that tumour cells possess MDR as an innate characteristic (Shoemaker et al., 1983). It is hypothesised that the more or less differentiated cancer cells can only acquire MDR, while MDR is an innate characteristic of undifferentiated cancer (stem) cells (Donnenberg and Donnenberg, 2005).

Pgp and MRP1 reside in the plasma membrane, where they are surrounded by a large variety of lipids. The lipid composition to a large extent determines the physical properties of the membrane and can thus affect functional activity or localisation of proteins, including those of Pgp and MRP1. In this respect one class of lipids, the sphingolipids, is particularly interesting. In vitro models have shown that the composition of sphingolipids changes when cancer cells become multidrug resistant (MDR) (Lavie et al., 1996; Kok et al., 2000; Ferte, 2000). The next paragraph describes the functions and relevance of sphingolipids, among others in relation to cancer (Negendank, 1992; Leach, 1996) and MDR.
Sphingolipids

Sphingolipid metabolism

Sphingolipids are one of the three major classes of lipids that can be found in animal cell membranes. They were named by J.L.W. Thudichum in 1884 after the sphinx of Greek mythology because of their enigmatic nature and unknown function. So far over 300 sphingolipid species have been identified. Sphingolipids share a basic structure that can be divided into three parts: a sphingoid backbone, which is usually a sphingosine, a fatty acid, and a headgroup (Fig. 1) (Futerman and Hannun, 2004). The large number of different sphingolipids is made possible by variations in the sphingoid backbone, differences in fatty acid chain length, degree of saturation and degree of hydroxylation of the fatty acid, and variation of the headgroup.

Sphingolipid synthesis takes place in the ER and the Golgi apparatus. The first step in the de novo sphingolipid synthesis is also the rate-limiting step and involves the coupling of serine and palmitoyl-CoA by serine palmitoyl transferase (SPT) to form 3-keto-sphinganine (Fig. 2). 3-keto-sphinganine is then converted via sphinganine and dihydroceramide into the central and most simple sphingolipid ceramide (Cer) (Fig. 1) (Ong and Brady, 1973; Michel et al., 1997; Geeraert et al., 1997; Kok et al., 1997; Merrill, 2002; Smith and Merrill, 2002). These first steps of sphingolipid synthesis take place in the ER, while the Golgi apparatus is the platform of synthesis of more complex sphingolipids by glycosylation or addition of a phosphorylcholine headgroup (Futerman and Pagano, 1991; Mandon et al., 1992). Whereas glucosylceramide (GlcCer) is formed at the

**Figure 1.** Chemical structure of Cer and SM. Sphingolipids consist of a sphingoid base, a fatty acid and a headgroup.
cytosolic side of the Golgi, all other glycolipids, i.e. lactosylceramide (LacCer) and gangliosides, are formed at the luminal side of the Golgi. Another abundant metabolite of Cer is sphingomyelin (SM) (Fig. 1), which is formed by the coupling of a phosphorylcholine group to Cer by sphingomyelin synthase (Futerman et al., 1990; Jeckel et al., 1990; Huitema et al., 2004). Besides the formation of new Cer via the de novo pathway, the SM pool can also serve as a source of Cer, as will be described in more detail later. Upon synthesis, most

Figure 2. Sphingolipid metabolism.
sphingolipids are then transported to cellular membranes of other organelles via vesicular or non-vesicular transport, to perform structural and functional roles. The transport of lipids is an ordered process, as is illustrated by the specific trafficking of sphingolipids to either the apical or basolateral membrane in polarised cells (Ait Slimane and Hoekstra, 2002; Maier et al., 2001).

Ceramide and apoptosis

Sphingolipids have been implicated in cellular processes like proliferation, differentiation, apoptosis and cellular senescence (Hannun and Luberto, 2000; Huwiler et al., 2000). The best characterised bioactive sphingolipids are Cer and one of its metabolites sphingosine-1-phosphate (S-1-P) (Futerman and Hannun, 2004; Spiegel and Milstien, 2003). A number of studies have shown that enhanced Cer levels induce differentiation and apoptosis, while S-1-P is associated with cell growth and inhibition of Cer-induced apoptosis. Cer levels can be induced in response to extracellular stimuli such as cytokines, cytotoxic agents and environmental stresses, which cause the breakdown of SM into Cer and phosphorylcholine by sphingomyelinases (SMases) (Okazaki et al., 1989; Andrieu-Abadie and Levade, 2002). To date, several different SMases have been identified based on their pH optimum, cation requirement and subcellular localisation (Levade and Jaffrezou, 1999; Goni and Alonso, 2002). The neutral plasma membrane-bound Mg$^{2+}$-dependent sphingomyelinase (often referred to as N-SMase) and the acid sphingomyelinas (A-SMase) have both been implicated in SM hydrolysis preceding ceramide-induced apoptosis signalling (Marchesini and Hannun, 2004). Besides these breakdown routes, induction of the de novo synthesis of sphingolipids by means of cytotoxic drugs also results in elevated Cer levels. Examples are the paclitaxel-mediated induction of SPT activity and the daunorubicin-mediated induction of sphinganine N-acetyltransferase activity (Charles et al., 2001; Wang et al., 2002; Bose et al., 1995). Whether elevated Cer levels eventually result in apoptosis signalling depends on factors such as the subcellular localisation of the newly formed Cer pool, the Cer acyl chain length and the availability of Cer targets. The subcellular localisation of Cer formation depends mainly on the nature of the (extracellular) stimulus. While Cer is formed in the plasma membrane or lysosomes/endosomes upon stimuli that induce the activity of N-SMase or A-SMase, induction of the de novo Cer synthesis results in elevated ER Cer levels. In addition, the acyl chain length of the newly formed Cer molecules could also be crucial to the apoptotic properties of Cer. A study in B cells demonstrated that particularly endogenous long chain ceramides (C16:0) are involved in induction of apoptosis signalling (Kroesen et al.,
Finally, the effectiveness of Cer signalling depends on the availability of its molecular targets. Several targets of Cer have been identified that are likely candidates in Cer-induced apoptosis signalling. Among these are protein kinase \( \zeta \) (PKC\( \zeta \)), cathepsin D, Cer-activated protein kinase (CAPK) and the Cer-activated protein phosphatases (CAPP) PP1 and PP2A (Pettus et al., 2002; Ruvolo, 2003). However, the exact mechanisms of interaction between Cer and these regulatory proteins need further research.

A number of studies suggest that mitochondria play a central role in Cer-induced apoptosis (Gudz et al., 1997; Garcia-Ruiz et al., 1997; Quillet-Mary et al., 1997; Arora et al., 1997; Kroesen et al., 2001; Birbes et al., 2002). In one of these studies Birbes et al. (2001) made use of a bacterial SMase (bSMase) fused to a green fluorescent protein (bSMase-GFP) construct to show that apoptosis was only induced when bSMase was specifically targeted to mitochondria, and not when it was targeted to either the ER, Golgi, nucleus or plasma membrane. The mechanism of action of Cer involves an increased permeability of the mitochondrial outer membrane for, among other small proteins, cytochrome c (see BOX 1).

**BOX 1  Caspase-dependent apoptosis pathways**

Dependent on the circumstances, cells die either via necrosis (uncontrolled cell death) or via apoptosis (programmed cell death). The latter involves the activation of a series of signaling pathways, which are governed by proteins that propagate, control or inhibit the apoptosis signal (Debatin and Krammer, 2004; Norbury and Zhivotovsky, 2004; Baliga and Kumar, 2003). Two apoptosis pathways will be briefly discussed here (Fig. 3A). The first pathway involves ligand-induced activation of death receptors belonging to the tumour necrosis factor (TNF) receptor gene superfamily (Smith et al., 1994; Wajant et al., 2003).

One death receptor example is Fas, which is activated upon binding of its ligand, Fas ligand (FasL). Death receptors contain a cytoplasmic sequence called the death domain (Tartaglia et al., 1993; Nagata, 1997), which triggers the intracellular apoptotic machinery. Typically, caspase-8 is activated and eventually the effector caspase, caspase-3. The latter activates proteins involved in processes such as DNA fragmentation, and thereby apoptosis.

The other pathway involves the disruption of mitochondria. Various apoptotic stimuli induce mitochondrial membrane permeability transition, which causes the release of cytochrome c molecules from molecules from mitochondria (Siskind et al., 2002). After release into the cytoplasm, cytochrome c complexes with Apaf-1 and caspase-9, thereby activating caspase-9 (Li et al., 1997; Green and Reed, 1998). Caspase-9 activates effector caspases (e.g. caspase-3), which in turn activate downstream apoptosis signals (Green, 2005).
**Figure 3.** Schematic representation of apoptosis pathways. The top panel (A) shows the receptor-mediated (1) and the mitochondrial (2) apoptosis pathways. The lower panel (B) shows the signalling pathway of GD3-induced apoptosis. First, SM is converted into Cer by TNF receptor-activated aSMases in the plasma membrane. The Cer is transported to the Golgi apparatus, were it is metabolised into GD3 (2). These GD3 molecules are then targeted to mitochondria (via the plasma membrane) (3), where they cause the release of cytochrome c and thus induction of the apoptosis pathway.
Although the involvement of Cer in apoptosis signalling is generally accepted based on the impressive amount of accumulative evidence, some of this evidence, especially the part concerning the use of short chain ceramides, remains controversial. Because short chain lipids are easy to use, water soluble and are readily taken up by cells, they have often been used as mimics of natural long chain lipids. However, the physical properties of short chain (C2- or C6-)Cer analogues are very different from those of the endogenous long chain ceramides (Venkataraman and Futerman, 2000; Sot et al., 2005). Therefore, results obtained with short chain ceramides cannot be extrapolated to explain the behaviour of natural long chain ceramides. On the other hand, Ogretmen et al. showed that adding exogenous short chain C6-Cer to cells results in the generation of long chain ceramides. This process involved the recycling of the C6-Cer sphingosine backbone after deacylation (Ogretmen et al., 2002).

Sphingolipids and MDR

Metabolism of Cer into other sphingolipids is a potential mechanism to avoid Cer-induced apoptosis signalling and to ensure cell survival. Not surprisingly, the relative amount of SM and Cer, rather than absolute Cer levels, was proposed to be important in balancing apoptosis, differentiation or proliferation (Okazaki et al., 1989; Hannun, 1996; Flores et al., 2000). However, in MDR cells, Cer is preferably metabolised towards GlcCer. A high amount of GlcCer was first observed in the MDR breast cancer cell line MCF-7-AdrR (Lavie et al., 1996), which was also characterised by the over-expression of the ABC transporter protein Pgp. Later, elevated GlcCer levels were also found in other MDR cancer cell lines, which either over-expressed Pgp or MRP1 (Kok et al., 2000; Veldman et al., 2002; Gouaze et al., 2004). Because of the consistent findings of GlcCer accumulation in MDR cancer cells, it was proposed as a diagnostic marker for MDR cancer cells (Lucci et al., 1998). An increased activity of glucosylceramide synthase (GCS) in MDR cancer cells was proposed as the underlying mechanism of GlcCer accumulation. This was supported by studies in which MDR was reversed by inhibiting, both genetically and pharmacologically, the GCS activity (Liu et al., 2000; Liu et al., 2004). Furthermore, over-expression of GCS in MCF-7-AdrR cells further increased MDR. On the basis of these results it was suggested that GCS activity by itself could act as an independent MDR mechanism (Liu et al., 2001). However, observations in the mouse melanoma cell line GM95, which lacks a functional GCS, do not support this theory. Stable transfection of these GM95 cells with functional GCS cDNA failed to make these cells more resistant to cytotoxic drugs compared to the parental cells (Veldman et al., 2003).
**Gangliosides**

Gangliosides are, like the more simple sphingolipids Cer and GlcCer, involved in important cellular processes, including signalling, apoptosis, proliferation, differentiation and tissue development. The initial step of ganglioside biosynthesis involves the sialosylation of lactosylceramide by sialyl(NeuAc)-transferase I (Fig. 2). In this process GM3 is formed, which can be sialosylated once more by sialyl(NeuAc)-transferase II forming GD3. To both GM3 and GD3 a residue of N-acetylgalactosamine, galactose and sialic acid can be introduced in that particular order to form GM2, GM1, GD1a and GD2, GD1b, GT1b respectively (van Echten and Sandhoff, 1993) (Fig. 2). Gangliosides of the branch starting with GM3 are called “a”-series gangliosides and those of the branch starting with GD3 “b”-series gangliosides. Gangliosides are synthesised in the Golgi, from where they are transported to the outer leaflet of the plasma membrane. Because of their hydrophilic and bulky headgroup, gangliosides are more easily shed into the extracellular space than other sphingolipids (Dyatlovitskaya et al., 1983; Li and Ladisch, 1991; Olshefski and Ladisch, 1998). Neural tissue expresses high ganglioside levels. Also some specific tumours express and shed high levels of gangliosides, for example neuroblastoma and melanoma. Gangliosides originating from tumours have been found to circulate in patients’ plasma. A well known example is GD2, which is often found in plasma of neuroblastoma patients (Schulz et al., 1984; Ladisch et al., 1987). GD2, shed from neuroblastoma cells, has been shown to inhibit murine cellular immune responses *in vivo* (Li et al., 1995). The inhibitory properties of gangliosides negatively affect the immunological anti-tumour response, thereby enhancing tumour formation (McKallip et al., 1999). Gangliosides derived from neuroblastoma cells are also known to inhibit murine and human haemopoiesis *in vitro* (Floutsis et al., 1989; Shurin et al., 2001; Sietsma et al., 1998). The exact mechanisms are not clear yet, but it is known that gangliosides can bind to target cells and become incorporated in the plasma membrane (Chigorno et al., 1996; Radsak et al., 1982; Schwarzmann et al., 1983; Chigorno et al., 1985; Orlando et al., 1979; Riboni et al., 1993). Once in the plasma membrane, very low ganglioside concentrations could be sufficient to alter protein activity (Leon et al., 1981), or even induce signalling pathways. Because GD2 expression and shedding is typical to neuroblastoma it can be used as a molecular marker in neuroblastoma detection and as a target antigen for immunotherapy (Li et al., 1996, Bestagno et al., 2003).

Like most other sphingolipids, gangliosides are primarily found in the outer leaflet of the plasma membrane, where they primarily reside in rafts. Rafts are membrane domains rich...
in sphingolipids and cholesterol and a number of proteins involved in signal transduction, such as receptor tyrosine kinases, Src family kinases, small GTP-binding proteins, G protein-coupled receptors and protein kinase C (Hakomori et al., 1998; Brown and London, 1998; Masserini et al., 1999). These signalling proteins are affected by changes in the lipid environment and these changes could well involve gangliosides because they were shown to modulate the functional properties of membrane proteins (Hakomori and Igarashi, 1995; Partington and Daly, 1979; Davis and Daly, 1980; Leon et al., 1981; Kreutter et al., 1987; Chan, 1987; Yates et al., 1989; Bassi et al., 1991). The presence of gangliosides in rafts is also exploited by bacteria. *Vibrio cholerae* secretes a toxin (cholera toxin) that binds specifically to the ganglioside GM1. This results in uptake of the toxin and eventually elicits a Cl⁻ secretory response, which is the primary event responsible for the massive secretory diarrhoea seen in cholera patients (Lencer et al., 1999).

The ganglioside GD3, like Cer, has the ability to directly interact with mitochondria, thereby inducing apoptosis (see **BOX1** and **Fig. 3B**). Although the underlying mechanism is not completely understood, it was clearly shown that GD3 induced the release of cytochrome c from mitochondria (Scorrano et al., 1999; García-Ruiz et al., 2000). The rapid synthesis of GD3 appears to be the source of the apoptogenic GD3 molecules, since knockdown of GD3 synthase (sialyl(NeuAc)-transferase II) using antisense RNA prevented Fas-induced apoptosis in human lymphoma cells (De Maria et al., 1997). The same study also showed that GD3 levels were elevated in human lymphoma cells treated with cell-permeating ceramides. Furthermore, Colell et al. (2002) showed that TNF-induced apoptosis in human colon epithelial cells was dependent on active aSMase and GD3 synthase (sialyl(NeuAc)-transferase II), suggesting that Cer could be involved in induction of GD3 generation. Because GD3 is synthesised in the Golgi, it has to be targeted to the mitochondria. Several pathways have been suggested, which are reviewed by Bektas and Spiegel (2004) and Morales et al. (2004).

In one study, GD3 has also been related to MDR. Plo et al. (2002) showed that gangliosides, specifically GM3 and GD3, regulate Pgp activity through modulation of Pgp phosphorylation in acute myeloid leukemia cells.

*Sphingolipid metabolism modulation*

Natural and synthetic inhibitors of enzymes involved in biosynthesis of sphingolipids have been indispensable to sphingolipid research. Some of these inhibitors will be briefly discussed here.
The first inhibitor that will be discussed is an inhibitor of the first step in de novo sphingolipid biosynthesis: ISP-1 (myriocin). ISP-1 was originally isolated from fungi and recognised as a potent immunosuppressant. Later it was found to be a structural analogue of sphingosine and an efficient inhibitor of SPT (Miyake et al., 1994). Therefore, ISP-1 can be used to abrogate sphingolipid biosynthesis and deplete cells from all sphingolipids.

The next four inhibitors that will be discussed here were designed to inhibit GCS: D,L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (t-PDMP) and D,L-erythro-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (e-PDMP) (Inokuchi and Radin, 1987), D,L-threo-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (t-PPPP) (Lee et al., 1999) and N-butyldeoxynojirimycin (NB-dNJ) (Platt et al., 1994). t-PDMP, an isomer of the less effective e-PDMP, was the first successful GCS inhibitor. However, more efficient inhibitors have been designed, such as the PDMP analogue t-PPPP and the iminosugar NB-dNJ, which were less toxic and had fewer intracellular side-effects. t-PDMP used in relatively high concentrations caused induction of cell cycle arrest in mouse fibroblast cells via inhibition of CDK1 and CDK2 (Rani et al., 1995), inhibition of lysosomal phospholipase A2 (Shayman et al., 2004), inhibition of intracellular membrane and protein transport (Rosenwald et al., 1992; Kok et al., 1998; Nakamura et al., 2001), induction of calcium responses (Kok et al., 1998; Filipeanu et al., 2000), inhibition of nucleoside transport (Griner and Bollag, 2000) and strengthening of microtubule-nucleating activity of the centrosome (Takatsuki et al., 2002).

**Lipid rafts**

The plasma membrane of animal cells is formed by an asymmetric bilayer of lipids in which a wide range of (trans)membrane proteins reside. The large variety in lipids, their differential physical properties and the presence of proteins that (actively) interact with certain lipids causes a heterogeneous distribution of lipids over the membrane.

- So-called lipid translocases translocate specific lipids either to the inner or outer leaflet of the plasma membrane. A well-known example is the phospholipid phosphatidylserine (PS), which is restricted to the inner leaflet of the plasma membrane under normal conditions and becomes partially redistributed to the outer leaflet during the apoptotic process (Bretscher, 1973; Fadok et al., 1992; Balasubramanian and Schroit, 2003). In contrast to most glycerophospholipids, sphingolipids are mainly found in the outer leaflet of the plasma membrane (Muller et al., 1994; Pike, 2004).
Lipids are also heterogeneously distributed over the plane of the membrane. This heterogeneity is induced as a result of certain lipid-lipid interactions that are more favourable than others and the recruitment of certain lipids by proteins. As a consequence of these interactions, clusters of lipids and proteins are formed that are referred to as membrane microdomains or lipid rafts (see BOX 2). Caveolae, which form flask-shaped invaginations in the plasma membrane, are a subset of these lipid rafts and are characterised by the presence of the coat protein caveolin and, like other lipid rafts, are enriched in cholesterol and (glyco)sphingolipids (Rothberg et al., 1992; Anderson, 1998; Stan, 2005; Brown and Rose, 1992; Rietveld and Simons, 1998; Brown and London, 2000). The lipid chains of sphingolipids are characterised by a higher degree of saturation than those of, for example, glycerophospholipids. This high degree of saturation is favourable to the close packing of lipids and the stability of rafts. The presence of cholesterol contributes further to the stability of rafts. As a result of the close packing, lipid acyl chains are in an extended conformation, thereby increasing the thickness of the membrane (Fig. 4). Furthermore, the close packing of

---

**Figure 4.** Schematic representation of a lipid raft in the plasma membrane. Lipid rafts are membrane domains enriched in sphingolipids, cholesterol and specific proteins. Rafts are believed to display differential physical properties compared to the surrounding membrane, which is the result of a cholesterol-induced tight packing of sphingolipids. This tight packing of sphingolipids is possible because of the high saturation of their acyl chains. Raft lipids are in an extended conformation due to the tight packing. Consequently, lipid raft domains are somewhat thicker than the surrounding membrane.
Lipids also have implications on the fluidity of raft membranes, making them less fluid than the surrounding membrane. The latter is characterised by a higher content of unsaturated glycerophospholipids, which does not allow a close packing (Ahmed et al., 1997; London and Brown, 2000). Lipid rafts are relatively insoluble in detergents due to their physico-chemical characteristics, and, because of their enrichment in lipids, they have a low buoyant density. With the use of detergents like Triton X-100, membrane fractions characterised by this low buoyant density can be isolated from the rest of the membrane. Originally, lipid rafts were defined by their low density and insolubility in cold 1% Triton X-100 (Brown and Rose, 1992; London and Brown, 2000). Hence, they were named detergent-insoluble glycosphingolipid-enriched membrane domains (DIGs) or detergent-resistant membrane domains (DRMs) (see BOX 2). In addition to Triton X-100, other detergents have been used to isolate lipid rafts from cells. Because of the difference in stringency of the detergents, the composition of the isolated DRMs varies markedly. DRMs, isolated using the stringent detergents Triton X-100 or CHAPS, are highly enriched in sphingolipids and cholesterol, in contrast to DRMs isolated with the less stringent detergents Lubrol or Brij, which were only moderately enriched in sphingolipids and cholesterol (Schuck et al., 2003). Given these observations concerns were raised regarding the possible introduction of artefacts through the use of detergents (Munro, 2003). However, results from detergent-free raft isolation procedures (Macdonald and Pike, 2005) and recent visualisation of lipid clustering in model membrane systems argue in favour of the existence of (biological) rafts (Kahya et al., 2004). Except for caveolae, the morphology of rafts has still to be resolved. So far, several models of raft structure have been proposed that could explain differential compositions of rafts obtained with different detergents.

- The first model, the so-called ‘layered raft’ describes rafts as concentric layers of lipids, consisting of a well-ordered cholesterol and (glyco)sphingolipid-enriched core that gradually becomes less ordered and eventually merges into the bulk plasma membrane.

- The second model is that of ‘homogeneous rafts with selective extraction of lipids’. This model describes rafts as distinctive well-ordered homogeneous domains, from which different detergents selectively extract lipids and/or proteins.

- The third model describes rafts as distinctive but heterogeneous domains, which differ in lipid and protein composition and are differentially sensitive to detergents (Pike, 2004).

Which model is closest to reality remains to be determined, but the coexistence or hybrids of these three raft models in biological membranes is likely. Raft morphology could also depend on the conditions under which they are formed. It has been proposed that rafts are formed in...
the trans-Golgi network and are part of transport vesicles involved in apical and basolateral transport of proteins in polarised epithelial cells (Simons and Ikonen, 1997; Simons and van Meer, 1988). However, other rafts might be formed within the plasma membrane upon recruitment of lipids, for example as a result of conformational changes in signalling molecules (Li et al., 2003; ffrench-Constant and Colognato, 2004).

<table>
<thead>
<tr>
<th>BOX 2</th>
<th>Rafts and DRMs, two of a kind?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In this thesis a distinction is made between membrane microdomains that might actually exist in cells, and membrane fractions isolated from cells using detergent or non-detergent techniques. Although both are sometimes referred to as ‘rafts’, no evidence exists that membrane fractions isolated from cells resemble cell membrane microdomains. Therefore, membrane fractions isolated with detergent techniques are referred to as ‘detergent-resistant membrane domains’ (DRMs) (Fig. 5A) and the term ‘raft’ or ‘lipid raft’ is used for (theoretical) cell membrane domains (Fig. 5B). In addition, the term ‘raft’ is also used in interpretative discussion, for example when results of isolation procedures and microscopy are projected onto the possible shape and composition of cellular membrane domains.</td>
</tr>
</tbody>
</table>

**Figure 5.** Rafts and DRMs. Panel A shows a schematic representation of a cell. Domains within the membrane of cells are referred to as ‘rafts’. Panel B shows a sucrose gradient, which is used in the isolation of membrane fractions with detergent techniques. The membrane fractions that are obtained with these techniques are referred to as ‘DRMs’.

In accordance with the specific physical properties and lipid composition of rafts (ordered phase, thickness, sphingolipids, cholesterol), the protein composition of rafts differs from that of the rest of the plasma membrane. Besides caveolin (Rothberg et al., 1992), GPI
General introduction and scope of the thesis

(glycosyl phosphatidylinositol)-linked proteins (Brown and Rose, 1992), Src family kinases (Brown and London, 2000) and numerous other proteins involved in cell signalling (Foster et al., 2003; von Haller et al., 2001) are found in rafts. Indeed, several studies have shown that rafts are directly involved in signalling processes (Simons and Toomre, 2000; Pike, 2003). Rafts have also been implicated in cellular processes such as endocytosis (Nabi and Le, 2003; Parton and Richards, 2003) and trafficking of cholesterol (Ikonen and Parton, 2000). A relation between rafts and MDR was also proposed because the ABC transporter protein Pgp was found in caveolin-1-containing Triton X-100 insoluble membrane domains in Pgp over-expressing cells (Lavie et al., 1998). In contrast, a recent study showed that Pgp and MRP1 are predominantly located in Lubrol-based DRMs, which are independent of caveolae (Hinrichs et al., 2004). Other evidence that supports the implication of rafts in MDR comes from cholesterol depletion studies. These studies showed that 1/ Pgp- and MRP2-mediated drug transport was significantly affected (Luker et al., 2000; Yunomae et al., 2003) 2/ Pgp shifted out of DRM fractions (Luker et al., 2000). Besides cholesterol, Pgp and MRP1 activity were shown to be affected by the fluidity of the membrane and the proximity of certain lipids. Romsicki and Sharom (1999) showed that Pgp had a higher affinity for its substrates when the surrounding membrane was in gel phase (typical to rafts) rather than in liquid-crystalline phase. Phosphatidylethanolamine (PE) and PS as well as other glycerophospholipids were shown to affect ATPase activity of both Pgp and MRPI (Chang et al., 1997; Doige et al., 1993; Liu and Sharom, 1998; Mao et al., 2000; Romsicki and Sharom, 1998; Sharom et al., 1995). Furthermore, a study in acute myeloid leukaemia cells showed that the gangliosides GM3 and GD3 could regulate Pgp activity through modulation of Pgp phosphorylation (Plo et al., 2002).

In summary, the literature discussed in this ‘General introduction’ shows that a relationship might exist between ABC transporters and rafts, rafts and sphingolipids, and sphingolipids and ABC transporters. These relationships play a central role in our approach to chemosensitise neuroblastoma cells, as will be explained in the next paragraph.
Chapter 1

Scope of the thesis

This thesis describes how modulation of sphingolipid metabolism can be explored as a tool to sensitise neuroblastoma cells to cytotoxic drugs. We focussed on two strategies to sensitise both human and murine neuroblastoma cell lines in a sphingolipid-dependent way. The first strategy is based on studies that relate sphingolipids, ABC transporter protein activity and specific membrane domains, so-called rafts. The second strategy is based on evidence that the sphingolipid ceramide is involved in apoptosis signalling, induced by chemotherapy.

In chapter 2, three human neuroblastoma cell lines, SK-N-AS, SK-N-DZ and SK-N-FI were characterised for their sphingolipid content in relation to their ABC transporter protein expression and activity. Based on the outcome of the characterisation, the effects of ganglioside depletion on ABC transporter protein function and localisation was investigated in the cell lines SK-N-AS and SK-N-FI (chapter 3). Through depletion of all sphingolipids in the Neuro-2a murine neuroblastoma cell line, the role of these lipids in ABC transporter function and raft localisation was studied (chapter 4). The effects of cholesterol depletion on raft integrity and raft localisation of ABC transporter proteins were also assessed.

In chapter 5 cellular and molecular mechanisms underlying the PDMP-induced chemosensitisation of Neuro-2a murine neuroblastoma cells to paclitaxel were studied. This included potential involvement of ceramide-induced apoptosis as well as effects on cell cycle regulation.

The findings presented in this thesis are summarised in chapter 6. In this chapter, our findings are also discussed in relation to the state of the art in the field, and future perspectives are provided.