CHAPTER 6

Summary, general discussion and future perspectives
Summary

The research presented in this thesis focused on the question whether modulation of sphingolipid metabolism can be used as a tool to enhance chemosensitivity of neuroblastoma cells. Neuroblastoma is the most common extracranial solid tumour in childhood and despite progress in treatment strategies, prognosis remains poor for the large majority of patients with advanced stage disease at diagnosis. Depending on age, INSS classification and tumour biology, treatment varies from surgery to multimodal treatment including surgery, chemotherapy and/or radiotherapy. A major obstacle in the ultimate success of chemotherapy is the occurrence of intrinsic or acquired multidrug resistance, which is usually due to the increased expression of the ABC transporter proteins P-glycoprotein (Pgp) or multidrug resistance-related protein 1 (MRP1). These membrane proteins are enriched in detergent-resistant membrane domains (DRMs), suggesting an interaction between them and (sphingo)lipids enriched in these DRMs. The relationship between ABC transporter proteins and sphingolipids was investigated in human as well as murine neuroblastoma cell lines and is discussed in chapters 2, 3 and 4.

Chapter 2 describes the characterisation of the sphingolipid composition and multidrug resistance status of three human neuroblastoma cell lines with a differential Pgp and MRP1 expression level. These cell lines are derived from patients and acquired their MDR protein expression levels in vivo. Therefore, they offer a good model to study the relationship between sphingolipid metabolism and ABC transporter protein activity, in addition to model systems where differential expression of Pgp or MRP1 is acquired through transfection or selection with a cytotoxic drug. The highest Pgp expression level and efflux activity were found in the SK-N-FI cell line and the highest MRP1 expression level and efflux activity in the SK-N-AS cell line. These two cell lines also displayed higher (glyco)sphingolipid levels than the SK-N-DZ cell line, with sphingomyelin (SM) being especially abundant in SK-N-AS. All three cell lines were relatively abundant in gangliosides, with SK-N-FI and SK-N-AS expressing predominantly a-series gangliosides and SK-N-DZ b-series gangliosides.

In chapter 3 we show that depletion of these gangliosides has only marginal effects on the ABC transporter protein activity and localisation in the human neuroblastoma cell lines SK-N-FI and SK-N-AS. With the use of the glucosylceramide synthase (GCS) inhibitors t-PPPP and NB-dNJ the ganglioside content was efficiently depleted from these cells, including from their DRMs. The depletion of ganglioside content did affect Pgp and MRP1 activity, but
these effects were marginal compared to the effects of established inhibitors of Pgp and MRP1, i.e. cyclosporin A and MK571, respectively. Moreover, the effect of ganglioside depletion on cell survival was small and did not correspond with the effect on efflux activity. These results were consistent with the observations that DRM localisation of MRP1 was not affected by ganglioside depletion and that MRP1 and gangliosides appear to be in a different subset of DRMs. The results are in contrast with those of Plo et al. (2002), which suggested that the gangliosides GM3 and GD3 stimulate Pgp activity in acute myeloid leukaemia cells. Our study shows that ganglioside-ABC transporter protein interactions appear to be cell type dependent and that gangliosides are not a promising therapeutic target to modulate multidrug resistance (MDR) in neuroblastoma.

The results of our studies reported in chapter 3 raised questions regarding the relevance of sphingolipids to the integrity of lipid rafts and the localisation of certain proteins in these rafts, given that depletion of gangliosides (and all other glycosphingolipids) had no effect on DRM localisation of MRP1. The relevance of sphingolipids and cholesterol, generally acknowledged as important components of DRMs, to raft integrity and raft localisation of ABC transporter proteins and the raft markers caveolin-1 and Src was investigated in Neuro-2a murine neuroblastoma cells. The study described in chapter 4, shows that highly efficient depletion of the pool of all sphingolipid classes neither affects raft integrity, nor the raft localisation of MRP1, Src or caveolin-1. This raises concerns regarding the importance of enrichment of sphingolipids to lipid raft integrity. In contrast, depletion of cholesterol using methyl-β-cyclodextrin did cause a shift of MRP1 to higher sucrose gradient density fractions, as well as a slight decrease of MRP1 efflux activity. However, in this case also DRMs could be isolated and were shown to contain classical raft markers.

Thus, cholesterol seems to be a possible modulatory lipid in MDR. Another way of chemosensitisation of cancer cells besides interfering with the cellular defence mechanisms, such as decreasing the activity of ABC transporters, may be supporting the induction of apoptosis by cytotoxic drugs. In this respect, an interesting effect of some cytotoxic drugs is the induction of ceramide (Cer) synthesis. Cer, which is the simplest sphingolipid, has been implicated in apoptosis signalling in a number of studies. Therefore, increasing intracellular Cer levels has been explored as a tool to sensitisise tumour cells to cytotoxic drugs. Using GCS inhibitors in addition to cytotoxic drugs further increases Cer levels, whereas glycosphingolipid levels are decreased. We previously showed that PDMP, a GCS inhibitor, sensitised Neuro-2a murine neuroblastoma cells to paclitaxel and vincristine in a synergistic manner. The research presented in chapter 5 of this thesis is a follow-up on that study and
deals with the involvement of Cer in PDMP-induced sensitisation of Neuro-2a cells to paclitaxel. It was concluded that Cer has no causal role in the PDMP-induced sensitisation to paclitaxel. Instead, PDMP itself induced aberrant cell cycle progression and hyperploidy in paclitaxel-arrested cells. Indeed, depletion of the total sphingolipid pool did not abrogate hyperploidy, ruling out the involvement of Cer. Instead, paclitaxel and PDMP synergistically decreased CDK1 and CDK2 activities. Thus, this study dissociates PDMP-mediated chemosensitisation of Neuro-2a from Cer accumulation and suggests that PDMP affects cell cycle related proteins by itself.

General discussion and future perspectives

Sphingolipids were long considered solely as structural components of cellular membranes, until it became clear that sphingolipids are implicated in a number of important cellular processes. This is perhaps best illustrated by sphingosine-1-phosphate (S-1-P), which is one of several sphingolipids that has been recognised as a second messenger, and, so far, is the only sphingolipid known to have its own receptors. Through activation of these receptors diverse processes are regulated, including cell migration, angiogenesis, vascular maturation and neurite retraction (Toman et al., 2001). S-1-P, as an intracellular second messenger, is also involved in promotion of cell growth and suppression of apoptosis (Spiegel and Milstien, 2000; Pyne and Pyne, 2000). Another extensively-studied bioactive sphingolipid is ceramide (Cer). In contrast to S-1-P, Cer has been related to inhibition of proliferation and induction of apoptosis. Cer is formed when either de novo sphingolipid biosynthesis is induced or sphingomyelin (SM) is metabolised by sphingomyelinases (SMases) during cell stress induced by cytotoxic drugs. Cer, in turn, activates one or more of its proposed targets, thereby initiating a downstream signalling cascade ultimately leading to apoptosis.

More complex sphingolipids, such as the glycosphingolipids, have also been associated with a variety of cellular processes. In multidrug resistant (MDR) cancer cells over-expressing the ABC transporter proteins Pgp or MRP1, glucosylceramide (GlcCer)/ganglioside levels are significantly increased (Lavie et al., 1996; Kok et al., 2000; Veldman et al., 2002; Gouaze et al., 2004). The apparent importance of glycosphingolipids or, alternatively, of glucosylceramide synthase (GCS) activity to the MDR status of cancer cells was supported by pharmacological and genetic evidence. Glycosphingolipids could be beneficial to the functional activity of ABC transporter proteins through direct modulation of their phosphorylation state (Plo et al., 2002) or through formation of a favourable lipid
environment for these proteins, for example in the form of lipid rafts (Lavie et al., 1998; Hinrichs et al., 2004). Their synthesis also facilitates the metabolic removal of Cer (Liu et al., 1999). Thus, increasing glycosphingolipid synthesis could contribute to drug resistance in two ways: 1/ by facilitation of ABC transporter function, and 2/ by metabolic removal of Cer and subsequent reduction of apoptotic tendency.

The evidence for Cer as a mediator in apoptosis signalling, and the correlation between glycosphingolipids/GCS activity, ABC transporter protein-mediated drug resistance and lipid rafts prompted the research presented in this thesis.

The major objective of this research was to find novel sphingolipid-based strategies to sensitise neuroblastoma cells to cytotoxic drugs. In addition, we studied the cell-biological and molecular mechanisms deferring the role of sphingolipids in ABC transporter function and drug resistance, including apoptotic contribution. The first part (chapters 2-4) concerned the depletion of neuroblastoma cells from specific sphingolipids in order to modulate ABC transporter activity. The second part (chapter 5) involved induction of cancer cell death by increasing intracellular Cer levels using pharmacological inhibitors of GCS.

Two of the three human neuroblastoma cell lines, of which the sphingolipid composition and MDR status was established in chapter 2, were efficiently depleted from their ganglioside content in order to determine the effect of these lipids on ABC transporter function. In chapter 3 we show that ganglioside depletion did not affect Pgp and MRP1 function in SK-N-FI and SK-N-AS cells respectively. These results were in contrast with a previous study in acute myeloid leukaemia cells, where the gangliosides GM3 and GD3 were shown to directly modulate Pgp activity through phosphorylation (Plo et al., 2002). Besides cell type specific effects, other explanations may exist for the absence of a ganglioside-dependent effect on ABC transporter function. Firstly, selective retention of glycosphingolipids in rafts would render ABC transporter function unaffected in glycosphingolipid-depleted cells. However, we found that glycosphingolipids were also efficiently depleted from rafts. Secondly, other sphingolipids, such as Cer or SM, could be equally important to support ABC transporter function. However, results presented in chapter 4 of this thesis show that ABC transporter function remained unaffected, even when Neuro-2a murine neuroblastoma cells were efficiently depleted from the pool of all sphingolipid classes. Altogether, our results show that sphingolipids are not directly involved in modulation of ABC transporter function in neuroblastoma cells. Recent findings showing that ABC transporters and sphingolipids are primarily located in a different subset of lipid rafts
support these results. ABC transporters were enriched primarily in Lubrol-based DRMs, but less so in Triton X-100-based DRMs, while sphingolipids were more enriched in the latter (Hinrichs et al., 2004; Hinrichs et al., 2005a). The sphingolipids in Lubrol-based DRMs were found to be substituted by the phospholipids PE and PS. It has been shown that ABC transporters are dependent on PE and PS for their ATPase activity (Doige et al., 1993; Sharom et al., 1995; Romsicki and Sharom, 1998; Liu and Sharom, 1998; Chang et al., 1997; Mao et al., 2000). Based on results presented in chapter 4 of this thesis, ABC transporters also appear to be dependent on either the presence of cholesterol or the presence of intact rafts for optimal function. In contrast to depletion of the sphingolipid pool, depletion of Neuro-2a neuroblastoma cells from cholesterol did decrease ABC transporter (i.e. MRP1) function. Interestingly, depletion of cholesterol also resulted in a shift of MRP1 out of Lubrol-based DRM fraction to higher density fractions in sucrose gradients. In contrast to MRP1, the typical raft markers Cav-1 and Src were not shifted out of Lubrol-based DRM fractions, indicating that these proteins are probably in different membrane domain subsets. However, a very interesting outcome of these experiments was that Lubrol-based DRM fractions could be isolated despite the absence of sphingolipids or cholesterol from these cells. Consequently, these results raise important questions such as: ‘How do rafts exist without cholesterol and/or sphingolipids?’ or ‘Do rafts really exist in living cells, or are they artefacts of the isolation procedure?’ In contrast to our data, which challenge the definition of lipid rafts, other studies support the existence of rafts. These studies show that:

- Membrane fractions isolated with either a detergent-free method or the controversial detergent methods have a similar composition (Macdonald and Pike, 2005), indicating that rafts are not detergent-induced artefacts.
- Membrane domain formation also takes place in model membrane systems, such as giant unilamellar vesicles (GUVs), in which they can even be visualised (Kahya et al., 2004). Nevertheless, results obtained with detergent methods to isolate rafts should always be interpreted with care.

The results presented in chapters 2-4 thus show that in contrast to cholesterol depletion, depletion of gangliosides/sphingolipids does not affect ABC transporter function or their DRM localisation. Moreover, depletion of gangliosides/sphingolipids does not contribute to chemosensitisation of neuroblastoma cells. On the other hand, accumulation of Cer correlated with chemosensitisation of Neuro-2a cells by t-PDMP (Sietsma et al., 2000). However, the results presented in chapter 5 of this thesis show that this correlation is not based on a mechanistic relation, because the observed decrease in cell viability turned out to
be independent of Cer accumulation or GCS inhibition. In fact, the paclitaxel/t-PDMP-induced decrease in cell viability was not due to apoptosis but the result of formation of hyperploid cells. We concluded that the induction of hyperploidy is a sphingolipid-independent effect of t-PDMP. It is very likely that this involves the cell cycle machinery, since paclitaxel/t-PDMP synergistically decreased the activity of the cyclin-dependent kinases CDK1 and CDK2. This was an indirect effect of paclitaxel/t-PDMP, since this drug combination did not affect CDK1/CDK2 activities directly in the assay. The direct target of paclitaxel/t-PDMP in cell cycle regulation is currently unknown. Our interpretation of these results is that paclitaxel/t-PDMP affects a cell cycle regulation pathway, which is upstream of induction of apoptosis. In cells with an intact G₁ checkpoint, the resulting aberrant cell cycle progression is terminated and apoptosis follows. In cells with a dysfunctional G₁ checkpoint, such as the Neuro-2a cells in our study, cell cycle proceeds in an aberrant fashion leading to hyperploidy. We do not know whether the G₁ checkpoint dysfunction is an inherent property of Neuro-2a cells or the result of the specific treatment. The formation of hyperploid cells shows that increased Cer levels do not necessarily result in induction of apoptosis. This could imply that in a number of studies increased Cer levels have been misinterpreted as the apoptosis trigger, while actually unknown effects of inhibitors were responsible. Future research is required to identify the paclitaxel/PDMP-specific target, and to determine whether apoptosis is triggered in cells with functional mitotic checkpoints. We also need to know more about the ultimate fate of hyperploid cells, i.e. if they eventually die after a number of aberrant cell cycles, or remain viable cells. In addition, we may have to look for alternatives for PDMP, since the clinical use of this drug is hampered by in vivo toxicity (in mice).

In summary, the research presented in this thesis shows that sphingolipids are not essential to Pgp or MRP1 function. Even more surprisingly, sphingolipids do not appear essential for membrane domain (DRM) integrity. In addition, the effects of an established sphingolipid inhibitor (PDMP) turn out to be unrelated to sphingolipid metabolism, but instead involve a PDMP-specific effect on the cell cycle machinery upstream of CDKs. On the other hand, the results reveal that MRP1 localisation in membrane domains, as well as its efflux function depends on cholesterol. This may explain the localisation of MRP1 in membrane domains, which are enriched in cholesterol. Future research could focus on the exact role of cholesterol in MRP1 function, which could be either dependent on membrane domain localisation per se, or a direct interaction of MRP1 with cholesterol. Concerning the role of sphingolipids in MDR, other hypotheses than sphingolipid modulation of ABC transporters and involvement
of Cer-mediated apoptosis now deserve attention. A tempting hypothesis to investigate in the near future concerns the role of sphingolipids as structural components of the plasma membrane. As such, they may provide the plasma membrane with physical characteristics that overall make the membrane less permeable to cytostatics. This in turn would lead to reduced intracellular accumulation of cytostatics and hence increased drug resistance. Manipulation of sphingolipid metabolism would then provide a way to sensitise tumour cells to cytotoxic drugs, exploiting influx rather than efflux of these drugs.