Chapter 8

Summary and Perspectives
Summary

Overexpression of the multidrug resistance protein MRP1 confer multidrug resistance (MDR) to cancer cells. The contents of this thesis describe the involvement of MRP1 in MDR and its importance as transporter for physiological phase II detoxification conjugates.

Chapter 1 provides an overview of the (patho)physiological function of MRP1 and its recently cloned homologues MRP2-MRP6. It gives a detailed description how MRP1 can confer drug resistance and discusses current knowledge about substrate specificity and localization of the MRP family with emphasis on drug resistance. The differences in substrate specificity and localization between MRP1-MRP6 suggest that this family may function as a complex system, individually or in concert, to maintain cellular homeostasis in metabolism, immune responses and signal transduction.

Resistance to anthracyclines is frequently observed in MDR1 and MRP1 overexpressing MDR cells. Anthracyclines are widely used in chemotherapy and the observed resistance aimed to search for modified antracyclines with novel modes of actions to circumvent MDR1 and MRP1-mediated resistance. Chapter 2 describes the toxicity of the doxorubicin analogue methoxymorpholino doxorubicin (MMRDX) in MDR1 and MRP1 overexpressing cells. Compared to sensitive parental cells the toxicity of MMRDX is decreased in MDR cells overexpressing MDR1. This corresponded with decreased intracellular MMRDX levels. Overexpression of MRP1 did not reduce MMRDX cytotoxicity. Due to the fluorescent nature of both compounds, intracellular distribution of MMRDX and doxorubicin could be examined with confocal scanning laser microscopy (CSLM). In contrast to doxorubicin, the distribution of MMRDX was not different between the MDR1 and MRP1 overexpressing cells and their parental cell lines. Doxorubicin showed a shift from nucleus to cytoplasm in MDR1 and MRP1 overexpressing cells. In MRP1 overexpressing cells this shift could be restored with the MRP1 inhibitor MK571. Results from this study showed that MMRDX can overcome MRP1-mediated resistance. In addition, a direct link between MRP1 overexpression and intracellular doxorubicin distribution was demonstrated. This suggests that MRP1 is active in preventing entry of drugs into the nucleus where several anticancer drugs exert their cytotoxic activity.

Because distribution of anticancer drugs appeared different in MRP1-overexpressing cells, intracellular activity and localization of MRP1 was examined further in GLC4 and GLC4/Adr cells (Chapter 3). Intracellular transport of glutathione S-conjugates (GS S-conjugates) and doxorubicin was measured with fluorescence microscopy and CSLM. The non-fluorescent monochlorobimane (MCB) and 5-chloromethyl fluorescein diacetate (CMFDA) are intracellularly conjugated to GSH resulting in the fluorescent compounds glutathione S-bimane (GS-B) and glutathione S-methylfluorescein (GS-MF). Because of its suitability, CMFDA, but not MCB, was used for CSLM analysis. In MDR GLC4/Adr cells GS-B is accumulated into intracellular vesicular structures, an effect which was much less present in the parental GLC4 cells. A more precise examination of intracellular transport of fluorescent compounds was performed with CSLM. Compared to GLC4 cells, GLC4/Adr cells
showed more vesicular structures containing GS-MF or doxorubicin and no accumulation of these compounds into the nucleus. However, preincubation of GLC₄/Adr cells with brefeldin A, an inhibitor of vesicle formation from the Trans Golgi Network, resulted in accumulation of GS-MF and doxorubicin in only one spot close to the nucleus. This suggested that the vesicular structures accumulating GS-MF and doxorubicin derive from the Golgi apparatus. Morphological analysis by electron microscopy showed a more extensive Golgi apparatus in GLC₄/Adr cells compared to GLC₄ cells. Immuno electron microscopy showed MRP1 localisation on the plasma membrane and on membranes from intracellular vesicular structures and Golgi apparatus. In addition, colocalisation of MRP1 and its substrate dinitrophenyl-glutathione was observed on similar intracellular vesicles. These results demonstrate that MRP1 is intracellularly active and that in MRP1-overexpressing cells GS S-conjugates and doxorubicin are accumulated into intracellular vesicular structures which derive from the Golgi apparatus and contain MRP1.

The first transport experiments identified MRP1 as GS S-conjugate transporter, remaining the question whether MRP1 could confer MDR by transporting anticancer drugs. Chapter 4 describes how MRP1 can transport basic anticancer drugs. Membrane vesicles were isolated from MDR1 and MRP1 overexpressing cells and transport of tritium labelled substrates was measured with a rapid filtration technique. In contrast to MDR1, MRP1 is not a transporter for basic anticancer drugs per se. However, MRP1 is a transporter for vincristine and daunorubicin only in the presence of GSH. This GSH-dependent transport was inhibited by MK571 and a MRP1-specific monoclonal antibody QCRL-3, showing MRP1-specific transport. MRP1-mediated daunorubicin was dose-dependently stimulated by GSH, while MK571 inhibited MRP1-mediated GSH-dependent daunorubicin transport in a dose-dependent manner. This study demonstrate that MRP1 is a transporter for basic anticancer drugs but only in the presence of GSH.

In Chapter 5 the specificity of MRP1-mediated transport of basic anticancer drugs was examined. Methyl-glutathione, a short chain alkyl derivate of GSH also stimulated MRP1-mediated vincristine and daunorubicin transport in contrast to cysteiny1-glycine a dipeptide fragment of GSH. This suggests that MRP1-mediated transport of basic anticancer drugs requires an intact GSH molecule. Because glucuronides are also MRP1 substrates, the ability of glucuronic acid to stimulate MRP1-mediated drug transport was tested. Glucuronic acid did not induce MRP1-mediated transport of vincristine or daunorubicin, suggesting that MRP1-mediated transport of basic anticancer drugs is specifically dependent on GSH.

Mutations in the p53 gene are the most frequent genetic alterations in malignancies. Loss of functionality of the p53 protein has been associated with MDR. Chapter 6 describes the relation between overexpression of mutant p53 and MRP1 and other MDR genes. Increased expression of MRP1 and MRP2 was found in cells overexpressing p53 mutated at codons 273 (m273) or 248 (m248), respectively. These are two of the most frequently observed p53 mutations in malignancies. Cytotoxicity tests demonstrated that m273 cells, in contrast to m248 cells, were resistant to doxorubicin, mitoxantrone and vincristine. MK571 significantly modulated the resistance to vincristine. It was hypothesized that loss of p53 function can induce
MRP1 expression and that this may contribute to the intrinsic MDR phenotype of tumour cells.

The potential physiological function of MRP1 is extrusion of phase II detoxification conjugates. In Chapter 7 the protective role of MRP1 against the toxicity of 4-hydroxynonenal (4HNE) is presented. 4HNE is the most prevalent lipid peroxidation product generated during oxidative stress. MRP1, together with GSH, protects against 4HNE toxicity. A major detoxification mechanism for 4HNE is conjugation to GSH. However, extrusion of this 4HNE glutathione conjugate (GS-4HNE) is required to prevent inhibition of the GSH-detoxification mechanism. GS-4HNE was identified as a novel MRP1 substrate. Characteristics of MRP1-mediated GS-4HNE transport were further investigated using an insect cell system overexpressing recombinant MRP1. GS-4HNE appeared to be a high affinity MRP1 substrate. Based on these results it was proposed that MRP1, together with GSH, play a role in the defence against oxidative stress.

Perspectives

Multidrug resistance (MDR) is a major problem in successful treatment of malignancies. Although conclusive data concerning the clinical relevance of MRP1 has not been presented yet, in vitro data clearly demonstrate that overexpression of MRP1 confer MDR. Therefore, a role of MRP1 in clinical MDR can not be excluded. Together with MRP1 several MDR mechanisms are identified. Extensive studies to each of these mechanisms are necessary to elucidate their exact contribution to MDR.

To circumvent the deleterious effects of MRP1 overexpression, either novel anticancer drugs should be developed to overcome MRP1-mediated MDR and/or inhibitors that specifically block the function of MRP1 as drug transporter. At least 5 homologues of MRP1 have been identified (MRP2-MRP6) and at least 2 of them have the capacity to confer MDR (MRP2 and MRP3). Thus during development of novel anticancer drugs or inhibitors the function of these transporters must be taken into account. Novel anticancer drugs should be tested for being potential substrates for MRP family members and MDR1. MDR1 has clearly been demonstrated to confer MDR in vitro and in vivo and it has a broad substrate specificity. High throughput screening assays would be helpful for this as the speed of drug development is increasing.

MRP1 transports basic anticancer drugs with the aid of GSH and anionic anticancer drugs without further modification in an ATP-dependent manner across cell membranes. To specifically interfere with this transport process one should know the molecular mechanism of MRP1-mediated drug transport. This requires further knowledge and integration of substrate binding, ATPase activity and translocation processes. Site-directed mutagenesis has already been proven to further elucidate the function of specific parts of the MRP1 molecule. Since MRP1 is a large (190 kD) molecule, step-by-step site-directed mutagenesis will be difficult. Development of high throughput site-directed mutagenesis and structural analysis of (parts of) the MRP1
molecule would be a step forward in understanding the molecular mechanism of drug transport.

Mrp1 knockout mice have been developed to study the physiological function of MRP1. Although these mice are viable, they show a poor response to inflammation. In addition Mrp1 protects several tissues against drug-induced damage and prevents drug entry into the brain. The effects of (long term) inhibition of human MRP1 are not clear. MRP1 protects against 4-hydroxynonenal, a major lipid peroxidation product and the potential physiological function of MRP1 is extrusion of GSH-dependent detoxification products. Inhibition of the function of MRP1 as phase II conjugate transporter may have hazardous effects on protection of cells against oxidative stress products. The function of MRP1 and other MRP family members in normal metabolism in the human body should be elucidated before malignancies can be treated safely with MRP1 inhibitors. Uncontrolled inhibition of MRP1 and other MRP family members may have severe consequences for the putative important physiological functions of the MRP family members in the human body.