A STUDY OF MRP1-DRUG INTERACTIONS: IDENTIFICATION OF THE DRUG BINDING SITE(S)

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ABSTRACT

Over-expression of either P-gp1 and/or MRP1 in tumor cell lines confers resistance to structurally diverse anti-cancer drugs. Although the role of these two proteins in clinical drug resistance remains to be confirmed, the use of Pgp1-specific inhibitors in combination with standard anti-cancer drugs have demonstrated significant improvement in clinical response. However, evidence exists that reversal of P-gp1 alone is not sufficient. Therefore, while no drugs are currently available that can efficiently reverse MRP1 drug efflux in tumor cells, there is an urgent need to develop MRP1-specific blockers. In an effort to gain a better understanding of MRP1-drug interactions and to identify sequences within MRP1 that interact directly with drugs, we developed two structurally diverse photosensitive drug analogues, a quinoline-based compound (IACI) and a xanthone-derivative (IAARh123). Both compounds photolabeled MRP1 and showed a direct and specific interaction with the protein at physiologically relevant sites. Initial mapping of photolabeled sequences in MRP1 (Chapters 2 and 3), identified multiple IACI- or IAARh123-photolabeled peptides (~4-7 kDa) derived from both the N-terminal (MSD_0+MSD_1+NBD_1) and C-terminal (MSD_2+NBD_2) domains of MRP1. A subsequent study (Chapter 4), using MRP1 variants with hemagglutinin (HA) epitopes inserted at eight different locations, led to a higher resolution mapping of the previously identified IACI- or IAARh123-labeled peptides. Specifically, two photolabeled peptides (~6-7 kDa), derived from variants with insertions at positions 574 and 1222, were immunoprecipitated with anti-HA monoclonal antibody. Based on the location of the HA epitopes in the latter variants together with molecular masses of the two peptides, the photolabeled amino acid residues were localized to MRP1 sequences encoding transmembranes 10 and 11 of MSD_1 and transmembranes 16 and 17 of MSD_2. Interestingly, the same sequences were photolabeled with both IACI and IAARh123, confirming the significance of transmembranes 10, 11, 16 and 17 in MRP1 drug binding. Collectively, the work of this thesis led to the characterization of two sensitive and specific probes that should facilitate future analysis of MRP1-drug interactions. More importantly, our results identified two domains within MRP1 that are involved in drug binding.

RÉSUMÉ

La sur-expression de la P-glycoprotéine (Pgp1) et de la protéine MRP1 dans les cellules tumorales confère la résistance croisée aux agents anti-cancéreux. Quoique le rôle de ces deux protéines dans le phénomène de la résistance chez les patients reste à déterminer, l'utilisation d'inhibiteurs de la Pgp1 en combinaison avec les agents anti-cancéreux a prouvé son efficacité dans les essais cliniques. Des évidences suggèrent toutefois que l'inhibition du transport par la Pgp1 seulement n'est pas suffisant. De plus, aucune drogue inhibe complètement l'expulsion des agents anti-tumoraux médiée par MRP1 dans les cellules tumorales, nécessitant donc le développement d'agents de réversion spécifiques. Afin de mieux comprendre les interactions entre MRP1 et ses substrats et d'identifier les sites de liaison des médicaments, nous avons développé deux analogues photoactivables, dont un est basé sur la quinoline (IACI) et l'autre dérivé de la xanthone (IAARh123). Nos résultats démontrent que ces deux composés photoactivables se lient d'une façon directe et spécifique à des sites physiologiquement importants de la protéine MRP1 (chapitres 2 et 3). De plus, la résolution préliminaire des sites de la MRP1, photomarqués par IACI et IAARh123, a identifié plusieurs peptides (~4-7 kDa chacun) qui correspondent aux domaines N-terminal (MSD_0+MSD_1+NBD_2) et C-terminal (MSD2+NBD2) de la MRP1. Une étude subséquente (chapitre 4) utilisant des variants de MRP1 modifiés avec des épitopes de l'hémagglutinine (HA) insérés à huit sites différents dans MRP1, a mené à une meilleure résolution des peptides photomarqués. Deux peptides photomarqués (~6-7 kDa chacun), dérivants des variants dont les insertions se trouvaient aux positions 574 et 1222, ont été immunoprécipités avec l'anticorps monoclonal anti-HA. Selon la position des insertions HA, ainsi que les masses moléculaires des deux peptides, les acides aminés photomarqués sont localisés dans des séquences comprenant les domaines transmembranaires 10-11 de la MSD1 et 16-17 de la MSD2. Il est intéressant de noter que les mêmes fragments ont été photomarqués par le IACI et le IAARh123. En résumé, nous avons développé deux sondes spécifiques et sensibles qui seront très utiles pour élucider les interactions entre MRP1 et ses substrats. En plus, l'utilisation des composés IACI et IAARh123 nous a permis d'identifier deux domaines dans MRP1 impliqués dans la liaison aux drogues.

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This thesis would not have been possible without the inspirational energy, support and encouragement of Dr. Elias Georges. Elias gave me the freedom to grow in my research, and was always there for very enriching and constructive discussions. Working with Elias will always be an experience to remember.

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Last but not least, my family in Beirut...Nidal, Salwa, Dani and Rania. Despite being thousands of miles away, their love and trust never failed to inspire and support me during my studies. This thesis is dedicated to them.

THESIS OFFICE STATEMENT

Candidates have the option, **subject to the approval of their Department**, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s) provided that these copies are bound as an integral part of the thesis.

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Additional material (procedural and design data, as well as descriptions of the equipment used) must be provided where appropriate and in sufficient detail (*e.g.* in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent;** supervisors must attest to the accuracy of such claims at the Ph.D. Oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

STATEMENT OF CONTRIBUTIONS

All the experimental work presented in this thesis was designed and performed by the author, under the supervision of Dr. Elias Georges. In **manuscript I** the IACI was synthesized by Jose Desneves, under the supervision of Dr. Leslie W. Deady and Dr. Leann Tilley, from the School of Chemistry and the School of Biochemistry, La Trobe University, Bundoora, Victoria, Australia. The iodination of the drug however, was carried by the author. In **manuscript I and II**, the monoclonal antibodies MRPr1 and MRPm6 were provided by Dr. Rik J. Scheper from the Department of Pathology, Free University Hospital, Amsterdam, the Netherlands. The MRP1-transfected HeLa cells (**manuscript II**), the plasma membranes from the different HA-tagged MRP1 variants and the anti-HA hybridoma (**manuscript III**) were prepared in Dr. Philippe Gros' laboratory from the School of Biochemistry, McGill University, Montreal, Quebec, Canada. Christina Kast has previously prepared the different HA-tagged MRP1 variants (Kast et al, 1997, 1998) and Michel Julien subsequently prepared the plasma membranes from the cells expressing these variants.

STATEMENT OF ORIGINALITY

The following findings are considered original contributions to the field:

Manuscript I: Daoud, R., J. Desneves, et al. (2000). "The Multidrug Resistance Protein is photoaffinity labeled by a quinoline-based drug at multiple sites" Biochemistry **39**(20): 6094-102.

The first study provides a sensitive and specific probe to study MRP1-drug interactions. In brief, we have identified a photoreactive quinoline-based drug, N-(hydrocinchonidin-8'-yl)-4-azido-2-hydroxybenzamide (IACI) to show the photoaffinity labeling of MRP. The photolabeling of MRP1 suggests direct binding between MRP1 and unmodified IACI. Transport results show IACI as a substrate for MRP1 drug efflux. Furthermore, the inhibition of MRP1 photolabeling by IACI with molar excess of LTC_4 indicates that IACI binds to MRP1 at physiologically relevant site. Thus we speculate that IACI binds to the same or an overlapping domain as that of LTC_4 . In addition, the study draws a line between the role of GSH in drug transport and drug binding. Our data suggest that GSH is not necessary for drug binding, though important for drug transport. Finally, we show for the first time that the N- terminal (MSD_0+ MSD_1+ NBD_1) and the C-terminal (MSD_2+NBD_2) domains of MRP1 are involved in drug binding with a total of three domains photolabeled within the protein.

Manuscript II: Roni Daoud, Philippe Gros and Elias Georges. "Rhodamine 123 binds to multiple sites in the Multidrug Resistance Protein 1 (MRP1)" (Biochemistry, in press)

In the second study, we report on the synthesis of another MRP1-specific photosensitive drug analogue, a derivative of the fluorescent dye Rhodamine 123 (IAARh123), which specifically labels MRP1. IAARh123 extends MRP1 specificity by providing further evidence for direct interaction between MRP1 and a new class of compounds. Again, the inhibition of IAARh123-labeling by LTC_4 is supportive of an overlap of binding sites in MRP1 for structurally dissimilar compounds. Similar to quinoline-based IACI, xanthone-based Rhodamine 123 binds to both N- and C-terminal domains of MRP1. Furthermore, the study shows again that binding does not require

GSH and involve multiple domains within the protein. Most importantly, the same domains that are involved in IACI binding are also involved in IAARh123 binding. Interestingly, IAARh123 is an excellent Pgp1 substrate, while other Pgp1-specific photoactive drugs are not MRP1 substrates. Therefore, IAARh123 will serve as a unique tool to study MRP1-drug interactions as well as similarities between Pgp1 and MRP1 binding sites.

Manuscript III: Roni Daoud, Philippe Gros, and Elias Georges. "Major Photoaffinity Drug Binding Sites in MRP1 are Within TM10-11 and TM16-17" (Submitted to Journal of Biological Chemistry).

The current study revealed, for the first time, the identity of the domains involved in drug binding in MRP1. By bringing into play eight MRP1 variants, which had hemagglutinin A (HA) epitopes inserted at eight different locations, we mapped the photolabeled residues in MRP1 to two sequences that include TM10-11 in MSD_1 and TM 16-17 in MSD_2. The same sequences were photolabeled with IACI and IAARh123. This is the first evidence showing that structurally diverse MRP1 substrates share common binding site(s). In addition, our discovery brought to light the different domains involved in drug binding, setting by that the stage for a dimensional analysis of the binding site(s). This will lead to a better design of reversing agents possibly without a major disruption of the physiological role(s) of MRP1.

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CHAPTER I: LITERATURE REVIEW

LIST OF ABBREVIATIONS

ABC	ATP-Binding Cassette
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSO	Buthionine sulfoxime
COL	Colchicine
CQ	Chloroquine
DOX	Doxorubicin
GSH	glutathione
GST	Glutathione *S*-transferase
LTC_4	Leukotriene C_4
MDR	Multidrug resistance
MRP	Multidrug Resistance Protein
MSD	Membrane spanning domain
NBD	Nucleotide binding domain
PBS	Phosphate-buffered saline solution
Pgp	P-glycoprotein
Rh123	Rhodamine 123
Rh6G	Rhodamine 6G
SDS	Sodium dodecyl sulfate
TM	Transmembrane domain
VP-16	Etoposide

INTRODUCTION

Although cancer has become increasingly prominent as a disease in modern times, it is not a modern disease. Malignant tumors were described in pictures and writings from ancient civilizations, including those of Asia, South America, and Egypt. Bone cancers (osteosarcomas) have also been diagnosed in Egyptian mummies. Cancer also occurs in all known species of higher animals. Early cultures attributed the cause of cancer to various gods, and this belief was held until the Middle Ages. One of the first scientific inquiries into the causes of cancer dates from 1775, when Sir Percival Pott, an English physician, observed that young men in their twenties who had been chimney sweeps as boys had a high rate of death due to cancer of the scrotum. He suggested that the causative agent might be chimney soot (now known to be tar) and recommended frequent washing and changing of clothing that trapped the soot so as to reduce exposure to the "carcinogen" (Tannock and Hill, 1998).

The recognition that the growth of cancer results from a disordered proliferation of cells only followed after the development of the microscope. Recently the most important advances in knowledge about the biology of cancer have come from our increased understanding of molecular genetics. The rapid evolution of techniques of molecular biology has led to the characterization, cloning, and sequencing of a variety of genes where mutation can lead to malignant transformation. Thus the current model of initial development envisions cells undergoing a series of mutations and/or alterations, brought about in various ways, which result in their inability to respond normally to intracellular and/or extracellular signals that control proliferation, differentiation, and, ultimately, death. Genetic alterations may arise directly or indirectly from such factors as inherited gene mutations, chemical- or radiation-induced DNA damage, incorporation of certain viruses into the cell, or random errors during DNA synthesis.

In current clinical practice, chemotherapy is used primarily as the major curative option for few types of malignancies, or as adjuvant treatment before, during, or after local treatment (surgery and/or radiotherapy). Such treatments usually involve a combination of drugs. The most important factors underlying the successful use of drugs in combination are 1) the ability to combine drugs at close to full tolerated doses with

additive effects against tumors and less than additive toxicities to normal tissues, 2) and the expectation that drug combinations will include at least one drug to which the tumor is sensitive.

Unfortunately, many types of cancer that occur commonly in humans (e.g., colon cancer, lung cancer other than the small cell-type) have a relatively low probability of response to treatment with anticancer drugs (*intrinsic resistance*). Other human tumors (e.g., breast cancer, ovarian cancer, and small-cell cancer of the lung) often respond to initial treatment, but acquired resistance to further therapy usually prevents drug treatment from being curative. The selection of a drug-resistant population in human tumors is probably the major factor limiting the efficacy of clinical chemotherapy. Even if drug-resistant cells are present initially only at low frequency (e.g., one drug-resistant cell per 10^5 drug-sensitive cells), their selective advantage during drug treatment will lead to their rapid emergence as the dominant cell population, giving the clinical impression of *acquired resistance.*

One form of acquired resistance is Multidrug resistance (MDR). The MDR phenotype is a well studied experimental phenomenon that may play a role in clinical resistance to certain antineoplastic agents. As is well known, tumor cells selected for resistance to a particular drug in a class of natural product anticancer drugs display cross-resistance to these and other agents that have in common the following features: they are in general lipophilic, they range in molecular weight from around 300 to 900 daltons, and they appear to enter cells by passive diffusion. The accumulation and retention of these drugs is lower in the MDR cells than in the drug sensitive cells from which they were derived, resulting in insufficient intracellular drug levels to mediate cytotoxicity. This alteration in cellular pharmacology was shown over the past two decades to be mediated in part, by P-glycoprotein (Pgp) (Juliano and Ling, 1976) and the Multidrug resistance-associated protein (MRP) (Cole et al., 1992). Both proteins are large integral membrane proteins that appear to bind to anticancer drugs and efflux them from the cell.

The introduction of photoaffinity probes allowed rapid progress in characterizing Pgp in multidrug resistant cells. Ten years after the discovery of Pgp, Photoaffinity labeling provided the first evidence that anticancer drugs bind *directly* to and are *substrates* for the protein (Safa et al., 1986). These photoactive probes allowed the

identification of Pgp as a specific drug acceptor, thereby increasing our understanding of the function of Pgp in MDR cells and its involvement in the reversal of multidrug resistance by modulators. Later on, biochemical analysis of Pgp fragments that bind photoactive drug analogs suggested that the hydrophobic portions of the protein may be involved in drug binding. In addition, it helped in mapping the binding sites to specific transmembrane domains within the protein, a finding that is a cornerstone for understanding the mechanism of action of Pgp, and developing new reversing agents.

In 1997, research in our laboratory led to the identification of IAAQ (Vezmar et al., 1997), the first photoactive compound to recognize MRP as a specific substrate. To give more insight into MRP-drug interactions, two other photoactive compounds were identified and characterized. The first, a quinoline-based drug (IACI), and the second, a photoactive analog of the fluorescent dye Rhodamine 123 (IAARh123). Once these drugs were characterized with respect to their interaction with MRP, our growing interest was to identify their binding location(s) within MRP. The results of our work are the subject of the current Ph.D. thesis.

Briefly, the first chapter will be an introductory review to cancer as a disease, and chemotherapy as a treatment, in addition to the mechanisms of resistance to chemotherapy with a particular emphasis on multidrug resistance. The key players in the multidrug resistance phenotype, namely Pgp and MRP, are also reviewed. The second chapter, will describe our published results concerning the characterization of IACI. The third chapter will address the synthesis and characterization of IAARh123. And Finally the fourth chapter will present the findings on mapping the drug binding sites in MRP.

CHAPTER I

Literature Review

PART I: CANCER

Most normal cells are differentiated; that is they have developed specific morphology and function. During the process of differentiation, normal cells lose their ability to proliferate. However, many tissues in the body undergo a process of renewal in which the loss of mature, fully differentiated cells is replaced by the proliferation of less-mature precursors, known as stem cells. Most cancers originate from these precursor stem cells, which proliferate to form a clone.

The ability of cancer cells to invade other tissues where they can generate new tumors (metastasis) is an indication of the malignant nature of cancer. Once a diagnosis of cancer is established, the urgent and fearful question is whether it is localized or has already spread to regional lymph nodes and visceral organs. This fear is well justified. Despite improvements in early diagnosis, surgical techniques, general patient care, and local and systemic adjuvant therapies, most deaths of cancer patients result from the relentless growth of metastases that are resistant to conventional therapies.

CANCER TREATMENT

The absence of specific identifiable differences between normal and malignant cells is a major barrier that has limited the development of specific anti-cancer therapy. Treatment has had to rely on spatial separation of tumor from normal tissue (surgery or radiotherapy) or on minor and empirical differences in the responses of tumors and normal tissues to systemic treatments such as chemotherapy. If a cancer is diagnosed before metastasis has occurred, it is often curable by local treatment with surgery and/or radiotherapy. **Surgical removal** of a tumor must include an adequate margin of normal tissue to allow for local invasive spread. **Radiation treatment** kills mammalian cells mainly by causing damage to DNA, but it is not specific for malignant cells. A major aim of research in experimental radiotherapy thus is to maximize the effect of radiation treatment on the tumor while minimizing the damage to the surrounding normal tissue.

Many localized treatments fail because of the growth of metastases. Systemic **treatment with cytotoxic drugs** is often the only treatment that may influence all sites of metastatic disease. A major limitation to the success of drug treatment is the presence in

the tumor of drug-resistant cells that convey either initial resistance to treatment (*intrinsic resistance*), or subsequent resistance after the tumor has initially responded (*acquired resistance*). There is considerable research interest in understanding the mechanisms involved in drug resistance; this research may lead to the design of new and better drugs or to the use of available drugs in more effective combinations.

Chemotherapy

The first documented clinical use of chemotherapy was in 1942 when the alkylating agent nitrogen mustard was used to obtain a brief clinical remission in a patient with lymphoma (Goodman et al., 1984). Following the initial demonstration that a chemical agent might cause remission of human malignancy many drugs have been developed and tested for potential clinical activity. About 40 cytotoxic drugs (excluding hormonal agents) are currently licensed for use in North America and several new agents are undergoing clinical trials. Some of the most powerful are the so-called natural product drugs. As their name implies, these drugs were originally isolated from plants, bacteria, and fungi. Semi-synthetic or synthetic derivatives of these naturally occurring toxins, which are typically complex multi-heterocyclic structures, have become the mainstays of many commonly used and effective chemotherapeutic protocols. Key among them are the anthracyclines (e.g., doxorubicin and daunorubicin), the *vinca* alkaloids (e.g., vincristine and vinblastine), the epipodophyllotoxins (e.g., VP-16, VM-26), and the taxanes (e.g., paclitaxel). These drugs exert their lethal effects by interacting with multiple targets within the tumor cell. They are classified by their currently accepted mechanisms of action into four major categories:

1) DNA-interactive agents, which alter DNA structure and interfere with its template functions. The broad category of DNA-interactive agents includes the alkylating agents (e.g., cisplatin, cyclophosphamide); the DNA strand-breakage agent bleomycin; the intercalating topoisomerase II inhibitors (e.g., doxorubicin); the non-intercalating topoisomerase II inhibitors etoposide (VP-16) and teniposide; and the DNA minor groove binder plicamycin.

2) The anti-metabolites, are structural analogues of naturally occurring metabolites. They interfere with the normal synthesis of nucleic acids by falsely substituting for biosynthetic precursors or other intermediates in metabolic pathways. This group of drugs is subdivided into the folate antagonists (e.g., methotrexate), the purine antagonists (e.g., 6-thioguanine), and the pyrimidine antagonists (e.g., fluorouracil).

3) Tubulin-interactive agents, such as the *vinca* alkaloids (vincristine and vinblastine) and paclitaxel, commonly known as taxol. Both *vincas* appear to act by binding to specific sites on tubulin, a protein that polymerizes to form cellular microtubules. Their binding inhibits microtubule formation, consequently leading to a block in mitosis. On the other hand, paclitaxel is capable of blocking cells in mitosis by over-stabilizing microtubules rather than by inhibiting their assembly.

4) Hormonal agents. A number of human tumors arise from tissues normally sensitive to hormonal growth controls. Therapy of these hormonally sensitive tumors involve endocrine ablation (removal of a particular hormone-secreting tissue that stimulates tumor growth) or the administration of natural or synthetic hormonal substances that downregulate tumor growth. Chemically, many of these substances are natural steroids or steroidal derivatives. Hormonal therapies, in general, lack direct cytotoxicity and, therefore, offer little curative potential.

Mechanisms of resistance to chemotherapy

Despite advances in the design and use of chemotherapeutic drugs, the majority of human cancers are resistant to therapy at presentation, or become resistant after an initial partial response. This suggests that resistance may be inherent in a tumor cell or may evolve under the selective pressure of drug administration. A number of possible molecular explanations for drug resistance exist. There may be a) failure to activate the prodrug to its active form, b) increased detoxification, c) alteration in the drug target, d) enhanced repair capability of the cell after injury, e) failure to engage apoptosis, f) or exclusion of drug from the cell.

Failure of prodrug activation - While some chemotherapeutic agents such as doxorubicin are reactive molecules, others such as cyclophosphamide, require metabolic activation before gaining biological efficacy. Many of the activation reactions are dependent on the action of members of the cytochrome P450 gene superfamily (Nebert et al., 1991). Cyclophosphamide is 4-hydroxylated primarily by one P450, *CYP2B6*. Like many members of the superfamily, this enzyme is present at high levels in liver hepatocytes, and at lower levels in other tissues. In many human tumors, mainly those in the liver, the level of *CYP2B6* is reduced (Hayes and Wolf, 1990). Thus the activation of cyclophosphamide in the actual tumor is minimal.

Increased detoxification - Many chemotherapeutic agents are electrophiles and thus subject to conjugation with molecules such as glutathione (GSH) and glucuronic acid in order to facilitate transport, further metabolism, and ultimately excretion. Therefore, the amount of glutathione and glucuronic acid available for detoxification, as well as the activity of glutathione- and glucuronate-dependent enzymes, may be critical in determining drug resistance to certain agents. In fact, many human cancers show overexpression of conjugation enzymes, amongst which the glutathione *S*-transferases (GSTs). Clinical studies using ethacrinic acid, an inhibitor of GST π, have shown a significant increase in drug sensitivity to chemotherapy (for review, Tew et al., 1998). Chlorambucil and melphalan are examples of drugs susceptible to GSH detoxification.

Alteration in drug target - A number of anti-cancer drugs target topoisomerases. These enzymes are involved in transcription, DNA repair, and cell proliferation by virtue of their ability to cause single- or double-strand DNA breaks, thus allowing strand separation to occur during DNA replication. Topoisomerase II increases during the cell cycle and exists as two distinct isoforms, IIα and IIβ. Intercalating agents such as doxorubicin, and mitoxantrone, as well as non-intercalating agents such as etoposide, inhibit topoisomerase II. In some cancer resistant cells, it is believed that topoisomerase II shifts from IIα to the IIβ form, which is less sensitive to drug induced damage (for review, Larsen and Skladanowski, 1998).

Enhanced DNA repair - A number of chemotherapeutic agents, such as platinum drugs, nitrogen mustards, and chloroethylnitrosoureas, act by forming bifunctional DNA adducts. The exact mechanism by which these adducts cause cell death is not known. However, it is most likely that abortive attempts to replicate and/or repair the damaged DNA cause the formation of double-strand breaks or single-strand gaps in the DNA, which lead to chromosome aberrations and breakage during mitosis. Ultimately, either the DNA adducts themselves or the damage to the DNA caused by adducts triggers apoptosis. Thus, any substantial increase in the repair of bifunctional adducts would cause resistance to the chemotherapeutic agents. In fact, many signs of enhanced repair have been observed. In particular, it has been demonstrated that individual repair enzymes are present at higher levels in resistant cell lines (for review Chaney and Sancar, 1996).

Failure to enter apoptosis - The primary effect of many anti-cancer drugs and of radiation therapy is to induce apoptotic cell death in the damaged cell. This is an active process requiring the interaction and cooperation of a large number of genes, many of which are involved in cell cycle control. Failure of a tumor cell to enter apoptosis would therefore cause drug resistance. For example, overexpression of *bcl2* oncogene in lymphoma cell lines confers drug resistance and is of proven clinical significance. Rather than causing an increased rate of proliferation, the primary role of this gene is its ability to stop a cell from undergoing apoptosis even after sustaining damage. The classical tumor suppressor p53 acts as a sensor in cells, detecting DNA strand breakage and preventing a cell from proliferating while bearing damaged or mutated DNA. In some systems p53 causes cell cycle arrest at the G1-S interface, but in other situations p53 commits a cell to die by apoptosis. Since many human tumors have effectively lost p53 function by mutation or deletion, this raises the possibility that this gene may be of major importance in clinical drug resistance (for review Schmitt and Lowe, 1999).

Exclusion of drugs - Soon after the advent of chemotherapeutic drugs, it became obvious that tumor cells which were resistant to one drug often showed cross-resistance to a wide variety of other, structurally unrelated drugs. For example, a cell resistant to

doxorubicin could show cross-resistance to diverse drugs, to which it had never been exposed, including *vinca* alkaloids but not to other drugs such as alkylating agents. This phenomenon of **multidrug resistance (MDR)** is currently associated with overexpression of two mebrane proteins, **P-glycoprotein (Pgp[1])** and the **Multidrug Resistance Protein (MRP[2])**. Briefly, the mechanism underlying this type of drug resistance is an energy-dependent efflux, which extrudes a variety of unrelated compounds outside the cell away from their sites of action. Parts III and IV of this review will discuss multidrug resistance and its causative agents in further details.

PART II: ATP-BINDING CASSETTES

ABC (ATP-Binding Cassette) proteins form one of the largest protein families, whose members have been traced to all organisms examined so far. ABC transporters contain two types of structural domains: hydrophobic Membrane Spanning Domains (**MSD**), and hydrophilic cytosolic Nucleotide Binding Domains (**NBD**). The typical functional ABC transporter has two MSDs and two NBDs, and the majority of identified eukaryotic ABC transporters are composed of tandemly arrayed pairs of subunits (MSD-NBD or NBD-MSD) expressed as one continuous or two separate polypeptides (figure 1) (Decottignies and Goffeau, 1997). In most ABC transporters, the binding and subsequent hydrolysis of ATP by the NBDs is believed to be coupled to, and provide the energy for, substrate transport (Higgins, 1992). These domains are highly conserved, typically showing 30-40% identity among different superfamily members in a core region of about 200-250 amino acids. The NBDs of ABC superfamily members share two sequence motifs, designated "Walker A" and "Walker B", with many other nucleotide binding proteins (Walker et al., 1982). Mutational analysis of a number of ABC proteins indicates that these two regions are critical for ATPase function (Schneider and Hunke, 1998). In addition, most ABC transporters possess a characteristic conserved fourteen amino acid signature (or "C motif") located between the Walker A and B motifs.

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[1] Used interchangeably throughout the thesis as Pgp, Pgp1, or MDR1.
[2] Used interchangeably throughout the thesis as MRP or MRP1.

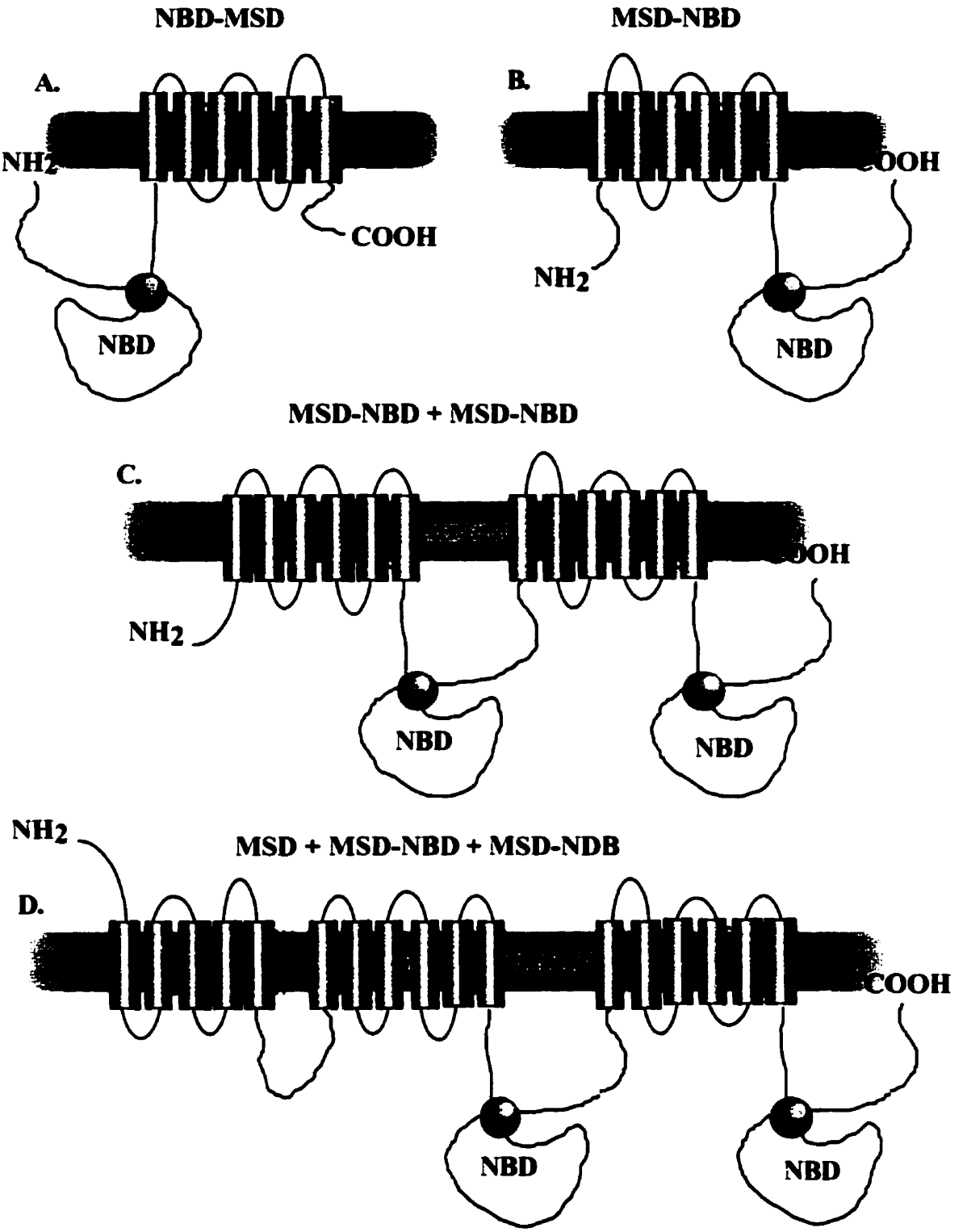

Figure 1. Domain arrangement prototypes of human ABC protiens. Vertical cylinders represent transmembrane helices within the membrane spanning domain (MSD). Areas labeled as NBD, represent nucleotide binding domains. The upper surface of the transmembrane domains represents extracellular medium or the lumen of a cellular compartment (e.g., endoplasmic reticulum, peroxisome, or mitochondrium), while the lower surface represents intracellular medium. (Adopted from (Klein et al., 1999), with modification).

In contrast to the NBDs, the MSDs of ABC transporters are highly divergent. Current evidence suggests that the majority of these MSDs possess either four or six membrane-spanning helices; however, the MSDs of different family members generally show little sequence identity to one another. This sequence divergence is consistent with the notion that the MSDs are important determinants of the different substrate specificities of various ABC transporters, and in cases where it has been examined, this appears to be true (Akabas et al., 1997; Ueda et al., 1997; Hrycyna et al., 1998).

SUBFAMILIES OF ABC PROTEINS

Analysis of the human ABC protein sequences revealed eight different subfamilies, five of which will be briefly described in the following section. However, for a an extensive review on all human ABC subfamilies, readers are advised to consult a recent review by Klein et.al. (Klein et al., 1999).

The **MDR/TAP** subfamily - Members of this family are either full transporters (MDR1, MDR3, and Sister-Pgp) or half transporters (TAP1, TAP2, M-ABC1, and ABC7). MDR1, commonly known as P-glycoprotein, was the first human ABC cloned (Chen et al., 1986). It is one of the most intensively studied proteins because of its involvement in multidrug resistance in cancer (P-glycoprotein will be described in part III in more details). sPgp or 'bile salt export pump' (BSEP) and MDR3 are full transporters, with 75% and 50% amino acid identity respectively, when compared to Pgp. sPgp was first described by Ling and his co-workers (Childs et al., 1995), and was found later to be an ATP-dependent bile salt transporter, expressed exclusively in the canalicular microvilli of the liver (Gerloff et al., 1998). MDR3 is a phospholipid translocator (van Helvoort et al., 1996), with a highly specific expression pattern in the canalicular membrane of the liver (Smith et al., 1994). TAP1 and TAP2 are 'half-transporters' (MSD-NBD arrangement), localized in the endoplasmic reticulum membrane. By forming heterodimers, TAP proteins actively transport degradation peptides from the cytosol into the ER lumen. Finally, the M-ABC1 and ABC7 are transporter proteins localized to the mitochondrial membrane. Both proteins are half-transporters and function as dimers. While ABC7 is likely to play a role in iron homeostasis of the cell (Csere et al., 1998; Allikmets et al., 1999), the physiological role of M-ABC1 requires further

investigation. It is not known whether ABC-7 and M-ABC1 could dimerize together or not.

The **MRP/CFTR** subfamily - It is currently the largest in the human ABC family, consisting of nine members. Similar to Pgp, MRP was a cornerstone in our understanding of the molecular basis of the MDR phenotype (Cole et al., 1992b). Sequence analysis (Bakos et al., 1996; Stride et al., 1996), which was supported by epitope insertion (Kast and Gros, 1997, 1998), and analysis of glycosylation sites (Hipfner et al., 1997) revealed a particular arrangement of MRP. The protein contains an N-terminal segment of about 230 amino acids forming five transmembrane domains (MSD_0 or TMD_0), and a 'six+six' MDR1-like core. Five other family members - MRP2, MRP3, MRP6, SUR1, and SUR2 - share the same topological organization of MRP1, whereas MRP4, MRP5, and CFTR are characterized by an MDR1-like organization since they lack the MSD_0 domain. Members of this family will be discussed in further detail at a later point.

The **White** subfamily - The subfamily is named after the White gene of *Drosophila melanogaster*, the first gene ever mapped. It is characterized by a unique NBD-MSD arrangement, and is involved in the transport of pigment precursors into pigment cells responsible for eye color. Two human ABC transporter genes, belonging to the White subfamily have been recently identified. The first member, ABC8/hwhite, was identified by screening a retina library (Chen et al., 1996). It is probable that the protein forms either homodimers or heterodimers with other half-transporters to create active transporters. The physiological role of the human white gene is not known. The second member of the white subfamily was discovered in different multidrug resistant cells and in placenta. The 'breast cancer resistance protein' (BCRP) (Doyle et al., 1998) was identified in a human breast cancer subline MCF-7/AdrVp, that shows anthracycline resistance without the overexpression of MDR1 or MRP1. The same protein was also identified in a human colon carcinoma line S1-M1-80, and was given the name 'mitoxantrone resistance-associated protein 1' (MXR1) (Miyake et al., 1999). At the same time, the transcript was detected in placenta and was identified as 'placenta-specific ABC gene' (ABCP) (Allikmets et al., 1998). Several experimental evidence support that the protein is involved in multidrug resistance. Transfection of BCRP cDNA in breast cancer MCF-7 sensitive cells, resulted in resistance to mitoxantrone, daunorubicin,

doxorubicin, and manifested an ATP-dependent increase in the efflux of rhodamine 123 (Doyle et al., 1998). It is still not clear whether this transporter functions as a homo- or heterodimer.

The **ABC1** subfamily - Four members of this family have been discovered, namely ABC1, ABCR, ABC3, and KIAA0822. All four members are full-size transporters, with ABC1 (2201 a.a.) and ABCR (2273 a.a.) being the largest known ABC proteins. The highest levels of ABC1 expression were observed in placenta, liver, lung, adrenal gland, and various fetal tissues. This high level of expression in fetal tissues is believed to be correlated with clearing apoptotic cells by macrophages (Langmann et al., 1999). In fact, the level of identity of ABC1 in humans with Ced-7 in nematodes, which functions in translocating molecules that mediate adhesion between cells that are dying by apoptosis and cells that are engulfing them, might suggest so. ABC3 is also believed to play a role in humans parallel to ced-7 in nematodes (Wu and Horvitz, 1998). ABCR is a retinal transporter (Allikmets et al., 1997). Its physiological substrate is currently unknown, but it is most probably a molecule(s) involved in photoreceptor homeostasis. Finally, KIAA0822 was identified in 1998 (Nagase et al., 1998), and its function is currently unidentified.

The **ALD** subfamily - This subfamily includes four half-transporters, ALDP, PMP70, ALDPR, and PMP69, with MSD-NBD arrangement. They are localized to the peroxisomal membrane and their mutant forms are involved in different peroxisomal disorders.

The MDR/TAP, the MRP/CFTR, and the White subfamilies include some members that are closely associated with the MDR phenotype in a variety of human tumors. In particular, the role of MDR1 and MRP1 in this phenotype is now well established. A review of the current understanding of both proteins, with an emphasis on MRP1, is presented in parts III and IV, respectively.

PART III: P-GLYCOPROTEIN

A search for the causes of multidrug resistance has occupied the attention of cancer researchers for more than four decades. The primary approach to this problem has

been to isolate lines of cultured cells selected for resistance to various anti-cancer drugs. The isolation of tissue cultured cell lines with a broad pattern of cross-resistance to many natural product anti-cancer drugs was frequently reported (Kessel et al., 1968; Biedler and Riehm, 1970; Dano, 1973; Ling and Thompson, 1974; Beck et al., 1979; Tsuruo et al., 1983; Akiyama et al., 1985; Bhalla et al., 1985; Shen et al., 1986; Beck and Danks, 1991; Sugimoto et al., 1991).The analyses of the biochemistry of MDR cell lines suggested that one or more major alterations involved plasma membrane proteins (Biedler et al., 1975; Juliano and Ling, 1976; Beck et al., 1979; Shen et al., 1986). One of these alterations, an increased expression of a cell surface phospho-glycoprotein, termed P-glycoprotein by Ling and his colleagues (Juliano and Ling, 1976; Riordan and Ling, 1979), has been shown to be encoded by the *mdr* gene in both rodents and humans (Chen et al., 1986; Gros et al., 1986; Ueda et al., 1986).

GENE FAMILIES OF PGP

Three distinct isoforms encode the Pgp gene family in mammals (classes I, II, and III). Classes I and III are present in humans, while classes I, II and III exist in rodents (table 1) (Van der Bliek et al., 1987; Ng et al., 1989). Interestingly, only classes I and II were shown to confer the MDR phenotype. Chromosome mapping studies have localized human *mdr* gene on chromosome 7q 21-31 (Fojo et al., 1986; Trent and Witkowski, 1987). The *mdr* gene includes 28 exons with a total span of greater than 100 kb (Chen et al., 1990). Its downstream promoter contains no TATA element, but does contain a GC-rich region, a CAAT box, a heat-shock consensus element, and an AP1-like element (Chin et al., 1993).

Table 1. Classification of Pgp genes in rodents and humans

Species	P-glycoprotein		
	Class I	Class II	Class III
Hamster	*Pgp1*	*Pgp2*	*Pgp3*
Mouse	*mdr3(mdr1a)*	*mdr1 (mdr1b)*	*mdr2*
Human	*MDR1*	-	*MDR3(MDR2)*

TOPOLOGY OF PGP

Mammalian P-glycoprotein is a single chain protein, which consists of 1280 amino acid residues. The Pgp sequence consists of a tandem duplication of approximately 600 amino acids, with an additional loop of approximately 60 amino acids which serves as a linker region (629-686) connecting the two halves of the protein (figure 2). Each half of Pgp encodes a hydrophobic N-terminal domain, and a more hydrophilic C-terminal region. The hydrophobic domain encodes six putative transmembrane regions, while the hydrophilic domain contains a consensus sequence for an ATP-binding motif (Gerlach et al., 1986; Gros et al., 1986). In other words, two ATP-binding domains are present in Pgp; one in the intracellular loop connecting the two halves, and one at the C-terminal end following the second half of the protein.

POST-TRANSLATIONAL MODIFICATIONS OF PGP

GLYCOSYLATION - Pgp is highly glycosylated, with carbohydrates accounting for approximately 20-40 kDa of its apparent molecular mass (Kartner et al., 1985; Greenberger et al., 1987). It has three *N*-linked glycosylations at Asn^{91}, Asn^{94}, and Asn^{99}

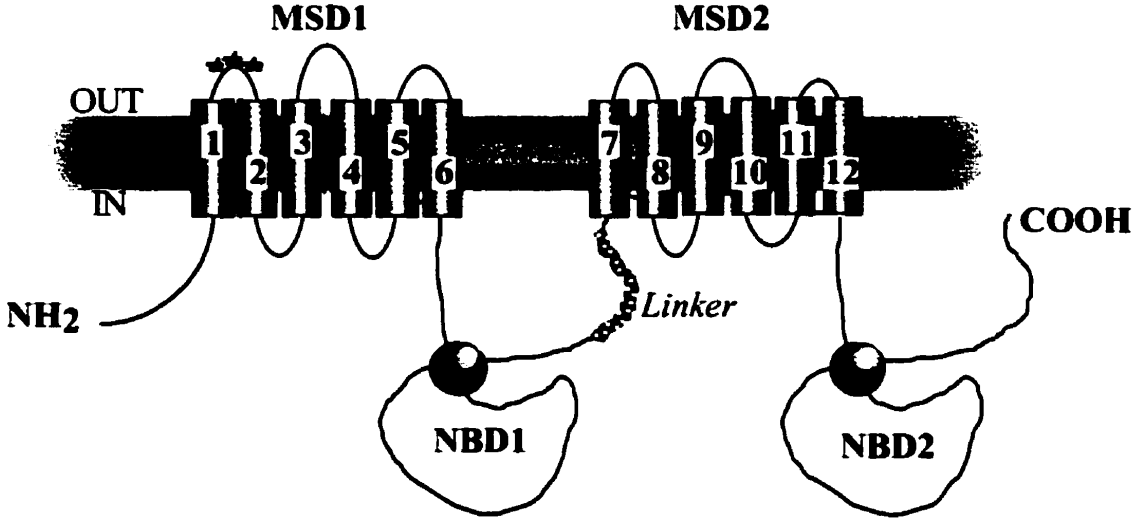

Figure 2. Topology of P-glycoprotein. P-glycoprotein is a phospho-glycoprotein containing twelve transmembrane domains. Both the N- and C-terminals are cytosolic. The dotted area represent the Linker domain, rich in phosphorylation sites. The stars represent the glycosylation sites.

located within the first extracellular loop from the N-terminal. Several studies have indicated that glycosylation of Pgp is not pivotal for its drug transport function (Beck and

Cirtain, 1982; Ling et al., 1983; Ichikawa et al., 1991). Recently, It was reported that mutations of Asn^{91}, Asn^{94}, and Asn^{99} to glutamine did not affect the transport function of Pgp as determined by its ability to efflux a variety of fluorescent compounds (Gribar et al., 2000). It was suggested that glycosylation may contribute to targeting Pgp to the cell surface, and stabilizing the membrane-associated polypeptide chain against proteolytic digestion (Schinkel et al., 1993; Gribar et al., 2000).

PHOSPHORYLATION - In contrast to glycosylation as a post-translational modification of Pgp, the role of phosphorylation remains unclear to date, and has been the subject of some controversy (Idriss et al., 2000). Analysis of the primary amino acid sequence of Pgp revealed several potential protein kinase A (PKA) and protein kinase C (PKC) phosphorylation sites (Kennelly and Krebs, 1991). The linker region of Pgp has been characterized by its high content of charged amino acids clustered into two regions with predominantly acidic (629-658) and basic (659-686) domains. Early studies identified several serine residues in the **basic domain** of the linker region of human Pgp that are phosphorylated by PKC and/or PKA (serines 661, 667, 671, and 683) *in vitro*, with certain residues being exclusively phosphorylated by PKC (serine 661) or PKA (serine 683). Three of these residues have been shown to be phosphorylated *in vivo* (Chambers et al., 1993; Orr et al., 1993; Chambers et al., 1994; Chambers et al., 1995; Chambers, 1998). More recently, serine and threonine amino acids in the **acidic domain** of the linker region were shown to be phosphorylated by casein kinase II, a prototypic acidic-directed protein kinase (Glavy et al., 1997). Many believe that Pgp phosphorylation can contribute to the regulation of its drug efflux and ATPase activity (Bates et al., 1992; Bates et al., 1993; Blobe et al., 1993; Ahmad et al., 1994; Ratnasinghe et al., 1998), as well as its chloride channel activity (Hardy et al., 1995; Goodfellow et al., 1996; Vanoye et al., 1999). It is interesting to note that the linker region of human Pgp is present as a putative regulatory domain at an analogous location within the polypeptide chain of another ABC transporter, the R-domain of the cystic fibrosis transmembrane conductance regulator (CFTR). The R domain of CFTR contains a large number of potential phosphorylation sites, some of which contribute to the regulation of its cAMP-dependent chloride channel (Riordan et al., 1989; Chang et al., 1993). In an analogy with the R-domain of the CFTR protein, the linker region of Pgp has been

hypothesized to represent a "mini-R-domain" that may function to regulate its biological activity (Chambers et al., 1993).

ATPASE ACTIVITY OF PGP

Functional studies using a photoactive analogueue of ATP (8-azido-alpha-^{32}P-ATP) established that the postulated ATP-binding sites do bind ATP (Cornwell et al., 1987; Schurr et al., 1989). Furthermore, the role of Pgp ATPase activity was demonstrated by cDNA transfection studies using full length Pgp containing a single point mutation in either one of the two NBDs. It was suggested that both ATP binding sites are required for the function of Pgp as indicated by the inability of the mutated Pgp to confer multidrug resistance (Azzaria et al., 1989).

Purified and reconstituted Pgp shows a drug-stimulated ATPase activity of approximately 5–22 μmol/min per mg of protein (Ambudkar et al., 1992; Shapiro and Ling, 1994; Urbatsch et al., 1994; Ambudkar, 1995; Ambudkar et al., 1998; Ramachandra et al., 1998), which is equivalent to that observed for other ion-translocating ATPases. Pgp ATPase activity is Mg^{2+} dependent, although other divalent cations support the activity to a lesser extent. Among the nucleotides, ATP is preferred, with a K_m for Mg-ATP hydrolysis ranging from 0.3 to 1.4 mM, whereas ADP inhibits the activity in a competitive manner with a K_i of approximately 0.3 mM. Because of the high K_m for Mg-ATP and the high K_i for Mg-ADP, it has been suggested that drug transport could be sensitive to ATP depletion under certain conditions (Senior et al., 1995).

ATPase activity of Pgp is inhibited by sulfhydryl reagents such as *N*-ethylmaleimide, indicating the presence of important cysteine residue(s) in the catalytic domains. Inhibition by *N*-ethylmaleimide can be blocked by ATP and is not reversible with dithiothreitol. The cysteines that are modified by *N*-ethylmaleimide within human Pgp have been identified as Cys-431 and Cys-1074 in the Walker A regions of ATP binding sites in N- and C-terminal halves, respectively (Loo and Clarke, 1995).

PHYSIOLOGICAL ROLES OF PGP

The identification of Pgp as an energy-dependent drug efflux pump, which confers resistance to hydrophobic compounds cytotoxic to cancer cells, raised the

question of the normal functions of Pgp. The first hint as to what these functions might be, came from studies in which monoclonal antibodies to Pgp were used to localize the protein in frozen sections of human tissues. All positive tissues show plasma membrane localization of positive cell types. In epithelial cells of the **lower gastrointestinal tract** (jejunum, ileum, and colon), high levels of Pgp are located only on the mucosal surface of these tissues, which suggests a function to prevent uptake of substrates and perhaps to facilitate excretion across the mucosa of the GI tract (Thiebaut et al., 1987). In **kidney** and **liver**, Pgp is present on the brush border and biliary face, respectively, of proximal tubule cells and hepatocytes, consistent with a role for Pgp in excretion of xenobiotics and endogenous metabolites into the urine and bile (Thiebaut et al., 1987). Some Pgp is also found on the apical surface of **pancreatic ductules**. One of the most interesting localization for Pgp is on the luminal surface of capillary endothelial cells in the **brain** and **testes**, consistent with a role for Pgp in forming the blood-brain barrier (Cordon-Cardo et al., 1989; Thiebaut et al., 1989).

Several other cell types and tissues express Pgp. There is Pgp in the **placenta**, probably in more than one cell type (Willingham et al., 1987; Sugawara et al., 1988), which suggests a role for Pgp in protecting the fetus from toxic xenobiotics. In rodents, the pregnant **endometrium** has glands that are very positive for Pgp (Arceci et al., 1988; Bradley et al., 1990), and human **adrenal cortex** is rich in Pgp. The localization of Pgp in steroid-secreting glands suggest that Pgp might be involved in secretion of steroids, or in protecting the plasma membranes of steroid-secreting cells from the toxic effects of high steroid concentrations. One observation consistent with this result is that progesterone is a Pgp inhibitor (Yang et al., 1989), and other steroids, specifically corticosterone, are transported by epithelial monolayers expressing Pgp (Ueda et al., 1992).

KNOCKOUT MICE - Firm evidence for the function of Pgp in mice came from studies in which mdr genes were inactivated by insertional mutagenesis (Schinkel et al., 1994; Schinkel et al., 1995; Borst and Schinkel, 1996). Rodents have two *mdr1* genes, termed *mdr1a* and *mdr1b*, both of which have been inactivated individually and simultaneously (Schinkel et al., 1997). Loss of either or both genes has no effect on viability, fecundity, or life span of mice. In contrast, the related gene, *mdr2*, is essential for transport of phosphatidylcholine from hepatocyte membranes into bile, and *mdr2*

knockout mice develop progressive cirrhosis because of inadequate formation of bile micelles (Smit et al., 1993). The viability of the complete *mdr1* knockout makes a strong argument for the feasibility of pharmacological strategies to improve cancer therapy in which Pgp is mostly, or completely, inhibited in the human.

PGP AND MULTIDRUG RESISTANCE

Cell lines which are highly resistant to a variety of anti-cancer agents have been generated by slowly increasing the concentration of a cytotoxic agent in a step-wise fashion. Initial analysis of the cell lines indicated decreased accumulation of cytotoxic compounds (Dano, 1973; Skovsgaard, 1978), consistent with gene amplification (Baskin et al., 1981; Grund et al., 1983) and overexpression of a 170 kDa membrane glycoprotein, termed P-glycoprotein (Juliano and Ling, 1976). However, interpretation of the results from such studies had been a subject of contention because of the pleiotropic effects of drug selection on cellular functions, including possible activation of endogenous drug resistance mechanisms. Therefore, cDNA expression systems that do not involve drug selection have been adapted for functional expression of Pgp. Transfection of the full length cDNAs isolated from libraries of either non-resistant (Gros et al., 1986; Croop et al., 1987; Lincke et al., 1990) or drug resistant (Ueda et al., 1987) cell lines into drug sensitive cells were effective in conveying drug resistance. Drug resistance correlated with overexpression of the recombinant RNA and a 170 kDa protein as well as decreased accumulation of cytotoxic compounds.

Mice lacking functional mdr1 gene(s) show a striking sensitivity to some drugs in the central nervous system, which suggests in some cases almost complete abrogation of the blood-brain barrier for these drugs and in other cases partial elimination of this barrier. These results are entirely consistent with the previously observed high level of expression of MDR1 on the luminal surface of capillary endothelial cells in the human brain (Cordon-Cardo et al., 1989).

Anti-cancer Pgp substrates

Pgp confers resistance against a wide spectrum of compounds that are hydrophobic, amphipathic natural product drugs. These compounds include not only anti-

cancer drugs, but also therapeutic agents such as HIV-protease inhibitors (Kim et al., 1998; Lee et al., 1998a). These compounds are chemically diverse, some of them may carry a positive charge at physiological pH and, because all are hydrophobic, they enter cells by passive diffusion. Table 2 lists classes of agents in clinical or laboratory use to which MDR cells are resistant, or that are thought to interact with Pgp.

Table 2. Selected substrates of P-glycoprotein[3]

Anti-cancer drugs	**Cyclic and linear peptides**
Vinca alkaloids (vincristine, vinblastine)	Gramicidin D
Anthracyclines (doxorubicin, daunorubicin)	Valinomycin
Epipodophyllotoxins (Etoposide, Teniposide)	*N*-Acetyl-leucyl-leucyl-norleucine
Paclitaxel (taxol)	Yeast a-factor pheromone
Actinomycin D	**HIV protease inhibitors**
Topotecan	Ritonavir
Mithramycin	Indinavir
Mitomycin C	Saquinavir
Other cytotoxic agents	**Other compounds**
Colchicine	Hoechst 33342
Emetine	Rhodamine 123
Ethidium bromide	Calcein-AM
Puromycin	

Reversal of Pgp-mediated multidrug resistance

A large number of non-cytotoxic compounds known as chemosensitizers or MDR modulators sensitize resistant cells to the action of cytotoxic drugs. Chemosensitizers include calcium channel blockers, calmodulin antagonists, steroids, cyclic peptides, and drug analogues (see table 3). Efforts are ongoing in several laboratories to understand the structural and functional basis for the inhibition of Pgp-mediated transport by chemosensitizers or modulators. It is believed that a clear understanding of *drug binding sites* and the mechanism by which modulators inhibit Pgp function will aid in the development of better chemosensitizers for clinical use. Inhibition of drug transport could potentially result from the blockage of specific recognition of the substrate, of ATP hydrolysis, or of coupling ATP hydrolysis to translocation of the substrate. Most reversing agents block drug transport by acting as competitive or noncompetitive inhibitors (Garrigos et al., 1997) through binding either to drug interaction sites (Dey et

[3] Adopted from Ambudkar SV, Dey S, et.al. (1999)

al., 1997) or to other modulator binding sites (Dey et al., 1997), leading to allosteric changes. Modulators such as verapamil are substrates of the transporter and hence inhibit the transport function in a competitive manner without interrupting the catalytic cycle of Pgp (Ford, 1996). Reversing agents such as cyclosporin A inhibit transport function by interfering with both substrate recognition (Tamai and Safa, 1991) and ATP hydrolysis (Ramachandra et al., 1998). Because ATP hydrolysis is required for transport (Azzaria et al., 1989; Loo and Clarke, 1995), modulators that inhibit ATPase activity are unlikely to be transported by Pgp. In addition to a direct interaction with Pgp resulting in inhibition of transport, it has also been postulated that some of the modulators, such as safingol (Sachs et al., 1995), may regulate Pgp function by affecting such post-translational modifications as phosphorylation.

Table 3. Selected reversing agents of P-glycoprotein[4]

Calcium channel blockers	**Immunosuppressants**	**Steroid hormones**
Verapamil	Cyclosporine A	Progesterone
Nifedipine	FK506	**Modified steroids**
Azidopine	Rapamycin	Tamoxifen
Dihydroperidines	**Antihistamines**	Tirilazard
Anti-arrhythmics	Terfenadine	**Antidepressants**
Quinidine	**Lipophilic cations**	Tioperidone
Amiodarone	Tetraphenylphosphonium	**Antibiotics**
Anti-hypertensive	**Diterpenes**	Hydrophoboc
Reserpine	Forskolin	cephalosporins
Detergents	**Antipsychotics**	
Tween-80	Phenothiazines	

DRUG BINDING SITES OF PGP

The identification of protein domains and/or amino acid residues implicated in recognition, binding, transport, and release of drugs or MDR modulators by Pgp is a necessary prerequisite to understanding its structure, function, and mechanisms of action. Major questions include the number, location and nature of the drug interaction sites. Are both halves of Pgp involved in drug binding? If so, can the halves of Pgp operate independently of each other? Do different drugs and/or modulators bind to the same site within Pgp or do they bind to different sites? If drugs bind to different sites, are there

[4] Adopted from Gottesman MM, Pastan I (1993)

allosteric interactions between these sites? Are there central amino acid regions and/or residues within the Pgp polypeptide chain that are essential for the interaction with all substrates for transport? Two major approaches to answer these questions have involved 1) classical biochemical analyses of Pgp peptides labeled with analogues of MDR drugs and modulators that can be photoactivated, and 2) genetic analyses of Pgp mutants that demonstrate altered substrate specificity.

Photoaffinity labeling - The use of photoaffinity labeling as a tool for mapping binding site(s) is based on the assumption that a reversible complex occurs between the photoactive drug analogue and the protein binding site(s). UV irradiation then converts the photoactive analogue into a reactive nitrene intermediate, which covalently attaches at or near the drug binding site(s) (Safa, 1998). Many different radioactive photoaffinity analogues of drugs and MDR-reversing agents have been used to prove the drug-binding capacity of Pgp, including derivatives of vinblastine (Cornwell et al., 1986), colchicine (Safa et al., 1989; Safa et al., 1990) verapamil (Safa, 1988), daunomycin (Busche et al., 1989), azidopine (Safa et al., 1987; Akiyama et al., 1988; Bruggemann et al., 1989; Yoshimura et al., 1989b; Greenberger et al., 1990; Bruggemann et al., 1992), prazosin (Greenberger et al., 1991), Rhodamine 123 (Nare et al., 1994a), and others (Nare et al., 1994b; Safa, 1998). Moreover, competition assays involving a photoaffinity analogue and a non-radioactive drug or MDR-reversing agent added in excess are a common experimental approach used to identify compounds that interact directly with Pgp. Data from such analyses have suggested that different drugs, such as vinblastine and colchicine, bind to separate, possibly overlapping or allosterically coupled sites, or alternatively, Pgp harbors a common binding site that displays variable affinity for different agents (for review, Safa, 1993, 1998).

Attempts to map putative drug-binding sites within the primary structure of Pgp have involved the immunological identification of radioactive Pgp peptides obtained by enzymatic or chemical degradation of Pgp labeled with [^{3}H]-azidopine, [^{125}I]-forskolin, or [^{125}I]-aryl azidoprazosin (IAAP) (Bruggemann et al., 1989; Yoshimura et al., 1989b; Greenberger et al., 1990; Greenberger et al., 1991; Greenberger, 1993; Morris et al., 1994). These studies showed that both halves of Pgp contribute to drug binding. For [^{125}I]-AAP two major labeling sites have been identified within the mouse *mdr1b* Pgp:

one in a region near or within TM6 in the N-terminal half, and the other at an analogous position near or within TM12 in the C-terminal half of Pgp (Greenberger, 1993). Interestingly, highly related, if not identical regions within the human Pgp are labeled by [^{3}H]-azidopine (Bruggemann et al., 1989) and [^{125}I]-forskolin (Morris et al., 1994).[^{3}H]-azidopine equally labels both halves of human Pgp, and vinblastine equally inhibits labeling of the two [^{3}H]-azidopine sites within Pgp (Bruggemann et al., 1992). Based on this latter observation, it has been suggested that amino acid residues from the N- and C-terminal halves of Pgp interact and cooperate to form one major drug interaction site (Bruggemann et al., 1992). However, there is no evidence that this domain defines the only drug-binding site. In fact, a vast amount of evidence suggests the presence of three binding sites (Fleming et al., 1992; Shapiro et al., 1999).

Mutational analysis – Experimental manipulation of the drug-binding sites in Pgp relied heavily on the results obtained from photoaffinity labeling. Pgp mutational analyses have suggested that both halves of Pgp are involved in determining its drug substrate and/or MDR modulator specificity and that crucial amino acid residues are generally localized within or near TM regions. The different types of Pgp mutants analyzed include insertion and deletion mutants (Currier et al., 1989), mutants with one or more amino acid substitution(s) (Choi et al., 1988; Kioka et al., 1989; Gros et al., 1991; Currier et al., 1992; Devine et al., 1992; Loo and Clarke, 1993a, b, 1994a, b), and hybrids between different MDR gene products (e.g. mouse *mdr1* and *mdr2* (Buschman and Gros, 1991; Beaudet and Gros, 1995), mouse *mdr1* and *mdr3* (Dhir and Gros, 1992), human *MDR1* and *MDR2* (Currier et al., 1992; Zhang et al., 1995)).

Mutational analysis has provided further evidence for the importance of TM5-6 and TM11-12 regions for drug interactions. Next are few selected examples from the large number of mutations that have been carried-out so far within these regions.

- Substitution of Phe335 in TM6 by Ala or Ser strongly altered the substrate specificity of human Pgp and impaired its ability to confer resistance to vinblastine and actinomycin D, while the ability to confer resistance to colchicine and doxorubicin was retained (Loo and Clarke, 1993b).
- In contrast, a Gly341 to Val mutation in TM6 reduced the ability to confer resistance to colchicine and doxorubicin, while mutation of Ala342 to Leu in TM6 had a quite

general effect and reduced the ability of human Pgp to confer resistance to colchicine, doxorubicin, vinblastine, as well as actinomycin D (Loo and Clarke, 1994a).

- More dramatically, mutation of Ser 344 in TM6 to Ala, Thr, Cys, or Tyr completely abrogated the ability of human Pgp to confer drug resistance (Loo and Clarke, 1994a).
- Mutations of Ser941 in TM11 in mouse *mdr1* Pgp and of Ser939 in TM11 in mouse *mdr3* Pgp to Phe lowered the capacity to confer colchicine and doxorubicin resistance (Gros et al., 1991).
- A human Pgp mutant carrying a Phe to Ala or Ser mutation at position 978 in TM12 had lowered capacity to confer resistance to vinblastine and actinomycin D and conferred no detectable resistance to colchicine and doxorubicin (Loo and Clarke, 1993b).

Thus several key amino acid residues are present within TM5-6 and TM11-12 regions, which when altered, change the drug substrate specificity of Pgp.

Additional amino acid residues within other membrane-spanning segments or in close proximity to it, may also play a role in determining the cross-resistance pattern conferred by the multidrug transporter. One particular functionally significant mutation located at position 185 near TM3 in human Pgp involved a Gly to Val mutation. The MDR1-Val185 product conferred enhanced resistance to colchicine, vinblastine, vincristine, actinomycin D, doxorubicin, and paclitaxel (Choi et al., 1988; Safa et al., 1990).

Taken together, point mutations that are scattered throughout the primary structure, but reside within or are in close proximity of membrane-spanning segments, contribute to drug substrate specificity of Pgp. In fact, cysteine-scanning mutagenesis and inhibition of drug-stimulated ATPase activity by dibromobimane, a thiol-reactive substrate, suggested a number of residues in TM6, TM11, and TM12 that are involved in binding of drug substrates (verapamil, vinblastine, and colchicine) (Loo and Clarke, 1997, 1999). Residues in TM11 (Phe942 and Thr945) (Loo and Clarke, 1999) , together with residues in TM6 (Leu339 and Ala342) and TM12 (Leu975, Val982, and Ala985) (Loo and Clarke, 1997) are believed to form part of the drug-binding domain of Pgp.

PART IV: MULTIDRUG RESISTANCE PROTEIN 1

For some time after its discovery, it was widely believed that P-glycoprotein was the exclusive cause of multidrug resistance. However, increasing evidence in mid to late 1980s suggested that this was not the case. Several cell lines were isolated that displayed a multidrug resistance phenotype in the absence of detectable P-gp expression, despite having undergone drug selection by protocols similar to those that often result in the elevated expression of this protein (Cole, 1986; McGrath and Center, 1987; Slovak et al., 1988; Marquardt et al., 1990). One such non-P-glycoprotein multidrug resistant cell line was H69/AR, which was derived from the parental NCI-H69 small cell lung cancer cell line by repeated, transient exposure to Doxorubicin (Cole, 1986). H69/AR cells displayed moderately high levels of resistance (10-100 fold) to the *vinca* alkaloids, epipodophyllotoxins, doxorubicin, and mitoxantrone. In addition, these cells were highly resistant to daunorubicin, epirubicin, and colchicine (Mirski et al., 1987; Cole, 1992). Prolonged growth of H69/AR cells in the absence of drugs resulted in a revertant cell line, H69/PR, that had only 1.5-2.5-fold resistance to most of these drugs (Cole et al., 1992a). The first clues to the resistance mechanism came from Center and colleagues (Marquardt et al., 1990). They derived a series of 15 synthetic peptides from the deduced amino acid sequence of Pgp. Polyclonal rabbit antisera were then raised to each of the peptides and used to probe immunoblots membranes prepared from both Pgp-expressing cell lines and also a human leukemia cell line (HL60/AR) in which Pgp could not be detected. Each of the antisera recognized a 170 kDa band in Pgp positive cells but, in addition, one also recognized a band around 190 kDa in HL60/AR cells. The antisera in question had been raised against a 15 amino acid sequence in the C-terminal ATP-binding site of Pgp, a region known to be highly conserved in ABC transporters. It was clear that an alternative route to an MDR phenotype involved overexpression of a transporter protein, which shared some sequence with Pgp, but which was somewhat larger in size. Several groups attempted to clone the gene coding for the 190 kDa protein using a variety of strategies. However, the group of Cole and Deely was the first to isolate the full length cDNA of a gene that coded the 190 kDa protein or the Multidrug Resistance-associated Protein (MRP) (Cole et al., 1992a). Cole's group used a differential

hybridization screen aimed at identifying mRNA species whose levels changed in association with the gain and loss of the multidrug resistance phenotype in the previous panel of lung cancer cell lines. MRP mRNA was overexpressed 100-200-fold in H69AR cells relative to H69 cells, and was downregulated 20-fold in the revertant H69PR cells relative to H69AR cells (Cole et al., 1992a).

MRP IN HUMAN TUMORS

Since its discovery in the human small cell line H69/AR, MRP has been identified in non-Pgp multidrug resistant cell lines from a variety of tumor types, including leukemia, fibrosarcoma, non-small cell and small cell lung carcinoma, as well as breast, cervix, prostate, and bladder carcinoma (Loe et al., 1996a). Although many of these cells have been selected with doxorubicin or other anthracylines, others have been selected with etoposide (VP16) or vincristine. In addition to *in vitro* drug-selected cell lines, MRP expression has been reported in a number of human tumors. The expression of MRP protein and/or mRNA has been detected in almost every tumor type examined, including both solid tumors (lung, gastrointestinal and urothelial carcinomas, neuroblastoma, glioma, retinoblastoma, melanoma, cancers of the breast, endometrium, ovary, prostate, and thyroid) (Endo et al., 1996; Loe et al., 1996a; Campling et al., 1997; Chan et al., 1997a; Chan et al., 1997b; Fillpits et al., 1997; Nooter et al., 1998; Takebayashi et al., 1998; Wright et al., 1998) and in hematological malignancies (Filipits et al., 1997a; Filipits et al., 1997b; Zhan et al., 1997; den Boer et al., 1998).

GENETICS OF MRP

The human MRP gene has been mapped to chromosome 16 at band 13.13-13.12 (Cole et al., 1992b; Slovak et al., 1993; Kuss et al., 1994). The coding sequence is made up of 4596 bases. The MRP promoter is GC-rich and similar to promoters of many housekeeping genes in that it lacks both TATA and CAAT motifs (Zhu and Center, 1994). The region encoding the 5' untranslated leader sequence of MRP mRNA is also exceptionally GC-rich and contains a GCC triplet repeat (Zhu and Center, 1994). The MRP gene is amplified relatively frequently in drug-selected human cell lines that overexpress MRP mRNA (Cole et al., 1992b; Slovak et al., 1993; Slapak et al., 1994; Eijdems et al., 1995). However, the amplification varies considerably between different

cell lines. Evidence also exists that overexpression of MRP in drug-selected cells can occur by mechanisms other than gene amplification. Elevated levels of MRP mRNA have been detected in revertant H69/PR, SW-1573-30.3, and in murine myeloid leukemia cell lines without an increase in MRP copy number (Cole et al., 1992b; Slovak et al., 1993; Slapak et al., 1994; Eijdems et al., 1995).

TOPOLOGY OF MRP

When MRP was first identified, it was proposed that the protein had eight and four transmembrane segments in the NH_2- and COOH-proximal halves, respectively (Cole et al., 1992b). Subsequent investigations led to a substantial revision of the originally proposed topology. One approach involved studying post-translational modification of the protein by limited proteolysis (Bakos et al., 1996; Hipfner et al., 1997). These experiments revealed that MRP1 is glycosylated at Asn^{19}, Asn^{23} suggesting an extracytosolic NH_2-terminal and Asn^{1006} in the COOH-proximal membrane-spanning domain 2 (MSD_2). The topological model generated using PredictProtein algorithm is depicted in figure 3a. MRP was found to possess five, six and four transmembrane segments commonly referred to as MSD_0, MSD_1, and MSD_2. The topology of MRP has also been examined using epitope insertion methodology (Kast and Gros, 1997, 1998). In that study, the membrane orientation of hemagglutinin (HA) epitope tags inserted at various locations throughout MRP1 was determined in permeabilized and non-permeabilized cells expressing the mutant molecules. With the exception of a molecule containing an HA tag at amino acid position 1222, the immuno-localization of the various HA-tagged MRP1 molecules tested is consistent with the PredictProtein-generated model shown in Figure 3a. The extracytoplasmic localization of the HA tag inserted at amino acid 1222 indicates that MSD_2 of MRP1 exists in a conformation with six transmembrane segments (figure 3b) rather than four, an arrangement that is more typical of MSDs of ABC transporters. The topology of the comparable region of P-glycoprotein (MSD_2) is still a matter of some debate. Although it is widely accepted that MSD_2 of P-glycoprotein possesses six transmembrane segments, there is some experimental evidence which suggest that it may also exist in mammalian cells in a conformation with only four transmembrane segments (Zhang et al., 1996). By analogy, MSD_2 of MRP1 may also

exist in two conformations, and could conceivably shift between the two states as part of its catalytic cycle.

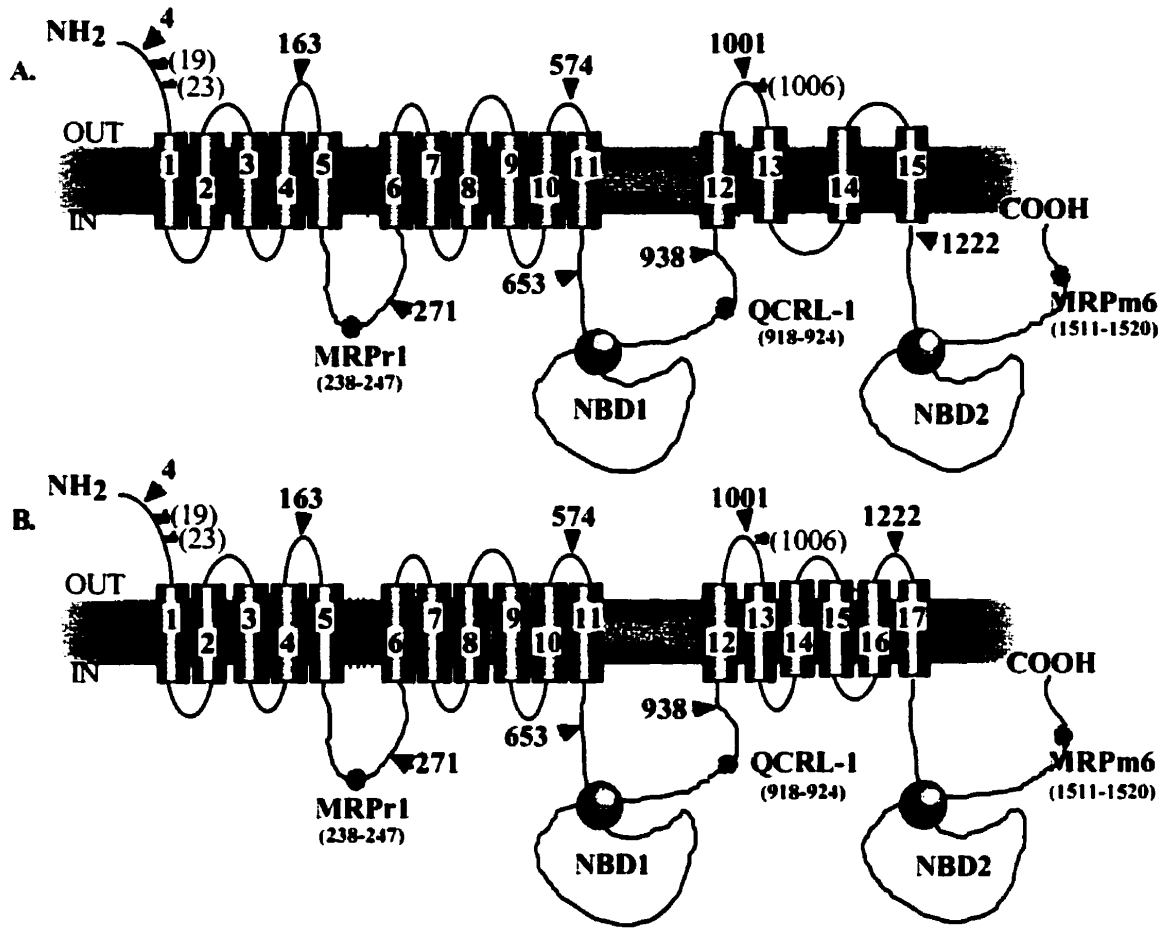

Figure 3. Proposed membrane topologies of MRP1. (A) Topological model of MRP1 with four transmembrane segments for MSD_2. This was generated using sequence analysis, which was supported by epitope insertion, and analysis of glycosylation sites. Seven amino acids in MRP have been localized to the extracellular side of the membrane by *N*-glycosylation sites analysis (N19, N23 and N1006) (stars) or HA epitope insertion (after amino acid positions 4, 163, 574, and 1001) (arrows). Additional amino acids or regions have been localized to the cytosolic side of the membrane by epitope mapping of MRP1-specific mAbs MRPr1 (238-248), QCRL-1 (918-924) and MRPm6 (1511-1520) (filled circles) or by HA epitope insertion (after amino acid positions 271, 653, 938) (arrows). (B) Topological model of MRP with six transmembrane segments for MSD_2. This was supported by epitope insertion analysis at amino acid position 1222 (Kast and Gros, 1998). (Adopted from Hipfner et al. (1999a), with modification).

POST-TRANSLATIONAL MODIFICATIONS OF MRP

GLYCOSYLATION - On the basis of its deduced amino acid sequence, MRP has 1531 amino acids with a polypeptide molecular weight of 171.548 kDa. Analysis of MRP primary amino acid sequence predicts an integral *N*-glycosylated ATP-binding phosphoprotein. Evidence has shown MRP to be immunologically detected as a 190 kDa

protein in selected cell lines and MRP-transfected HeLa cells (Abbaszadegan et al., 1994; Grant et al., 1994). The additional mass in the protein is a result of three *N*-linked glycosylations of high mannose oligosaccharides at Asn^{19}, Asn^{23} and Asn^{1006} (Bakos et al., 1996; Hipfner et al., 1997). Pulse chase studies on the processing of MRP indicate that it takes approximately 90 min to process the newly synthesized 170 kDa MRP polypeptide to the mature *N*-glycosylated 190 kDa form. The mature protein has a half-life of approximately 20 hours in both cell types which is within the range typical of plasma membrane proteins subject to internalization by endocytosis (Hare, 1990) and similar to that observed by Pgp (Yoshimura et al., 1989a; Cohen et al., 1990).

PHOSPHORYLATION - Another post-translational modification of MRP is phosphorylation. Multiple sequence motifs suggest the protein could be a target for several different kinases. Metabolic labeling of MRP with $^{32}P_i$ showed MRP to be highly phosphorylated. In addition, the phosphate groups are metabolically active and undergo cycles of phosphorylation and dephosphorylation in the cell. Serine is the single amino acid phosphorylated in MRP and the phosphate groups are contained in nine tryptic peptides. It has also been shown that treatment of HL60/AR cells with protein kinase inhibitors H-7, staurosporine, and chelerythrine reduces the phosphorylation of MRP and increases drug accumulation (Ma et al., 1995). Whether any of the phosphorylated sites in MRP are actually involved in regulating drug accumulation remains to be directly demonstrated.

ATPASE ACTIVITY OF MRP

When the sequence of MRP was analyzed, the two most closely related members were the cystic fibrosis transmembrane regulator (CFTR) chloride channel (19% identity) and *Leishmania tarentolae* PgpA (LtPgpA) (30% identity) (Cole et al., 1992b). The latter is a protein that was thought to be a P-glycoprotein homolog (Ouellette et al., 1990). The sequence predicted two potential nucleotide-binding domains (NBD), which indicated that MRP was a member of the ATP Binding Cassette (ABC) superfamily. Alignment of the primary sequences of MRP, LtPgpA, and CFTR with the human P-glycoprotein encoded by the *MDR1* gene revealed that, in comparison to P-glycoprotein, these transporters all contain a deletion of 13 amino acids located between the Walker A and B

motifs of NBD1. The corresponding 13 amino acids are present in the COOH-proximal NBDs of these proteins and in both NBDs of P-glycoprotein and most other eukaryotic ABC transporters (Cole et al., 1992b). This highly conserved deletion alters the spacing between the Walker A and B motifs in NBD1. Recent studies in which the 13 amino acids of NBD1 of human Pgp were inserted into NBD1 of MRP have demonstrated that this deletion affects the folding and activity of this domain (Hipfner et al., 1999b). It is present in most of the MRP-related proteins, including the murine mrp1 ortholog and all five of the more recently identified human MRPs as well as CFTR . The absence of these 13 amino acids in CFTR and in the majority of MRP1-related proteins contributes to the relatively low sequence identity (~30%) between the two NBDs within each of these proteins. There is strong evidence that the two NBDs of CFTR are functionally *dissimilar* (Ramjeesingh et al., 1999; Szabo et al., 1999). In light of the shared structural features of the NBDs of CFTR and the MRP-like transporters, it is likely that the two NBDs of the latter proteins will also be functionally distinct. Recent analyses of purified recombinant MRP1 suggest the presence of two types of nucleotide binding sites on MRP, a *catalytic site* to which ATP preferentially binds and is hydrolyzed and a *regulatory site* to which ADP preferentially binds and stimulates hydrolysis. ATP was hydrolyzed in proportion to the amount of purified protein assayed, and typical Michaelis-Menten behavior was exhibited, yielding estimations of K_m of approximately 3.0 mM and V_{max} of 0.46 $\mu mol.mg^{-1}.min^{-1}$ (Chang et al., 1997, 1998).

PHYSIOLOGICAL ROLES OF MRP

TISSUE DISTRIBUTION OF MRP - Analysis of the tissue distribution of the human MRP detected low levels of mRNA in all tissues examined including peripheral blood, the endocrine glands (adrenal and thyroid), striated muscle, the lymphoreticular system (spleen and tonsil), the digestive tract (salivary gland, esophagus, liver, gall bladder, pancreas, and colon), the respiratory tract (lung), and the urogenital tract (kidney, bladder, testis, and ovary) (Cole et al., 1992b; Zaman et al., 1993; Nooter et al., 1995). A similar tissue profile expression has been reported for murine mRNA (Stride et al., 1996). Cell fractionation studies and immunocytochemistry indicated the protein was predominantly localized to the plasma membrane in drug-resistant cells, with detectable

levels present in intracellular membrane compartments of some cell types (Flens et al., 1994; Hipfner et al., 1994; Almquist et al., 1995; Barrand et al., 1995).

KNOCKOUT MICE - To assist in the understanding of the physiological mechanism(s) of action of MRP, *in vitro* and *in vivo* knockout models were generated (Lorico et al., 1996; Lorico et al., 1997; Rappa et al., 1997; Wijnholds et al., 1997). Human and murine MRPs have 88% identity, and they both can induce multidrug resistance when their respective cDNAs are transfected into drug-sensitive cells (Grant et al., 1994; Zaman et al., 1994; Breuninger et al., 1995; Stride et al., 1997). Therefore it is conceivable that the results obtained in the murine mrp knockout systems can be extrapolated to humans. The development of mrp knockout mice has shown that this mutation does not interfere with the viability or fertility. The heterozygous and homozygous MRP knockout mice were not distinguishable from wild-type animals in appearance or mortality rate up to one year of age (Lorico et al., 1997; Wijnholds et al., 1997).

While knockout mice did not suggest a crucial role for MRP, it is clear that MRP is ubiquitously expressed, although at very low levels in the liver and some other organs (Cole et al., 1992b; Nooter et al., 1995; Stride et al., 1996), suggesting that it may play certain physiological role(s). In fact, several physiological functions of MRP have been suggested: a) protection against environmentally present heavy metal oxyanions, b) modulation of the activity of ion channels, c) transport of leukotriene C_4 and other glutathione, glucuronate, and sulfate conjugates, and d) transport of GSH through a co-transport mechanism.

A) Transport of heavy metal oxyanions - Humans are exposed to inorganic as well as to organic arsenicals in the environment. Double knockout cell lines of mrp (-/-) have been found hypersensitive to sodium arsenite (3-fold), sodium arsenate (2-fold), antimony potassium tartrate (5- to 6-fold) but not to cadmium chloride (Rappa et al., 1997). These findings are consistent with resistance patterns in MRP-transfected cell lines (Cole et al., 1994). Interestingly, in *S. cerevisiae*, the relationship between ABC transporters and arsenite resistance is clear; Ycf1p (yeast cadmium resistance factor), which is 63% identical to MRP, provides a pathway for the removal of arsenite from the cytosol (Ghosh et al., 1999). YCF1 has been shown to confer resistance to Cd(II), and the

gene product (Ycf1p) pumps GS-conjugates of cadmium and other compounds into the yeast vacuole. Disruption of YCF1 results in sensitivity to both arsenite and arsenate (Ghosh et al., 1999). Similarly, four MRP homologues were identified in the soil nematode *Caenorhabditis elegans*. In particular, mrp1 was found highly expressed in cells of the pharynx, the anterior intestinal cells, and the epithelial cells of the vulva. Targeted inactivation of mrp1 resulted in increased sensitivity to the heavy metal ions cadmium and arsenite, to which wild-type worms are highly tolerant (Broeks et al., 1996).

B) Regulation of K^+ channels - In addition to its ligand transport function, MRP may be involved in the regulation of endogenous ion channels. ABC transporters have been shown to modulate the activity of ion channels, including outwardly rectifying Cl^- channels (cystic fibrosis transmembrane regulator), volume-regulated Cl^- channels (P-glycoprotein), and inwardly rectifying K^+ channels (sulfonylurea receptor) (Gabriel et al., 1993; Hardy et al., 1995; Higgins, 1995; Inagaki et al., 1995; Jovov et al., 1995). The overexpression of MRP was accompanied by increases in both K^+ channel and volume-regulated Cl^- channel currents (Jirsch et al., 1993). Changes in the level of MRP expression were also closely correlated with the appearance of an inwardly rectifying K^+ channel activity in resistant H69/AR cells and its subsequent loss in revertant H69/PR cells (Jirsch et al., 1993).

C) Transport of Leukotriene C_4 and other GSH, glucuronate and sulfate conjugates - In membrane vesicles isolated from MRP-transfected or -overexpressing cell lines, MRP has been reported to be a high affinity transporter of cysteinyl leukotrienes (table 4). Following the demonstration in 1994 that MRP functions as a unidirectional ATP-dependent export pump for the endogenous glutathione *S*-conjugate leukotriene C_4 (LTC_4), (Jedlitschky et al., 1994; Leier et al., 1994b; Muller et al., 1994), the substrate specificity of this transport protein was studied intensively using inside-out membrane vesicles from cells expressing high levels of the recombinant protein (Jedlitschky et al., 1996; Leier et al., 1996; Loe et al., 1996b; Loe et al., 1996c; Stride et al., 1997). LTC_4 is involved in the control of smooth muscle contraction and vascular

permeability and is synthesized from leukotriene A_4 by conjugation with GSH. It remains the highest affinity substrate known for MRP, with a K_m of approximately 100 nM.

Table 4. Substrates of MRP1[5]

(Some endogenous metabolites and conjugates of natural products toxins)

Leukotriene D_4		(Leier et al., 1994b), (Jedlitschky et al., 1996)
N-acetyl- Leukotriene E_4		(Jedlitschky et al., 1996)
S-glutathionyl 2,4 dinitrobenzene	3.6	(Jedlitschky et al., 1996)
S-glutathionyl ethacrinic acid	28	(Zaman et al., 1996)
Chlorambucil		
- monohydroxy-monoglutathionyl		(Barnouin et al., 1998)
Melphalan		
- monohydroxy-monoglutathionyl		(Barnouin et al., 1998)
Biliribin		
- bisglucuronosyl		(Jedlitschky et al., 1997)
	2.5	(Loe et al., 1996c)
6α-Glucuronosyl hyodeoxycholate		(Jedlitschky et al., 1996)
3α-Sulfatolithocholyltaurine		(Jedlitschky et al., 1994)
Folate		(Keppler et al., 1998)
Fluo-3	12	(Keppler et al., 1999)
Vincristine+GSH		(Loe et al., 1996b), (Stride et al., 1997), (Renes et al., 1999), (Loe et al., 1998)
Aflatoxin B1+GSH		(Loe et al., 1997)

[5] Adopted from Hipfner, Deely, et.al. (1999).

In addition to LTC_4, other endogenous MRP substrates were identified such as bilirubin glucuronides, glucuronide and sulfate conjugated bile salts, glutathione disulfide, 17β-estradiol 17-(β-D-glucuronide), GSH conjugates of prostaglandin A_2, as well as GSH conjugates of alkylating agents (table 4). Glutathione *S*-conjugation of alkylating agents is a well known detoxification pathway. Increases in expression of glutathione-*S*-transferase (GST) π, Ya or Yb_1 following transfection of the respective GST cDNAs impart resistance to many alkylating agents, including chlorambucil, cisplatin, and melphalan (Puchalski and Fahl, 1990). The export of the conjugated drugs by MRP, is believed to play a role in the detoxification pathway.

D) MRP-mediated co-transport of GSH - Eukaryotic cells maintain high intracellular GSH levels (0.1 to 10 mM). Export of GSH from eukaryotic cells in culture has been documented extensively in studies carried out in the presence of buthionine sulfoxime (BSO) (Meister, 1985; Kaplowitz et al., 1996). BSO is a potent irreversible inhibitor of γ-glutamylcysteine synthetase, the enzyme that catalyzes the first, rate-limiting step in de novo GSH biosynthesis. In 1995 Zaman and colleagues (Zaman et al., 1995) reported that MRP-transfected cells export two times more GSH into the extracellular medium than parental cells. It is believed that MRP-mediated GSH export may contribute to fueling the production of cysteine by extracellular enzymatic hydrolysis of GSH (Kaplowitz et al., 1996). In fact, a variety of cells have an absolute requirement for a constant supply of cysteine, an amino acid that is exceedingly toxic when present at high concentrations (Kaplowitz et al., 1996). GSH export may be important in protecting the surface of cells from oxidants (Kaplowitz et al., 1996).

To clarify the relationship between MRP and GSH, *mrp* gene knockout systems were used. Parental embryonic stem cells exported GSH into the extracellular medium at approximately double the rate that occurred in two *mrp* knockout embryonic stem cell lines (Rappa et al., 1997). Thus the expression of the *mrp* gene appears to account for one-half of the baseline GSH export, which suggests that there is at least one other mrp-independent system responsible for GSH release into the extracellular medium. Similarly, in *mrp* knockout mice, tissue levels of GSH were increased markedly (Lorico et al., 1997), especially in tissues such as lung, kidney, and muscle, where mrp is highly expressed. On the other hand, tissue levels were unchanged in the small intestine and

liver, which are known to express very little, if any, mrp. Interestingly, in purified membrane vesicles, **GSH alone does not appear to be a substrate for MRP** (Loe et al., 1996c; Jedlitschky et al., 1996; Muller et al., 1996). *Thus it is conceivable that the mrp-mediated basal release of GSH occurs in association with a presently unknown endogenous metabolite(s) possibly through a co-transport mechanism.*

Although MRP appears to have several physiological roles, accumulating evidence suggest that the cell could compensate for the "services" provided by MRP through alternative routes. In fact, a number of MRP family members could perform similar functions to MRP. However, the role of MRP becomes accentuated in tumors that overexpress the protein. MRP-overexpressing cell lines are similar, and include moderate to high level resistance to various natural product toxins used in cancer chemotherapy.

ROLE OF MRP IN MULTIDRUG RESISTANCE

Definitive proof that MRP causes multidrug resistance was provided by transfection studies. HeLa cells transfected with MRP cDNA displayed multidrug resistance, accompanied by reduced drug accumulation, relative to cells transfected with vector alone (Cole et al., 1994; Grant et al., 1994). Similar findings were reported by several independent laboratories using different cell types (Kruh et al., 1994; Zaman et al., 1994; Chang et al., 1997). It was also shown that MRP antisense oligonucleotides can decrease MRP1 levels and reverse drug resistance in transfected and drug-selected cells (Stewart et al., 1996).

In acute toxicity experiments, using lethality as an end-point, mrp double knockout mice were found to be 2-fold more hypersensitive to etoposide (Lorico et al., 1997; Wijnholds et al., 1997). It is important to stress that a 2-fold hypersensitivity can be extremely important clinically, where even a small increase in the dosage of a chemotherapeutic agent, such as etoposide, may result in toxicity to normal tissues. These results have established the importance of mrp in protecting normal tissues from the toxicity of anti-cancer drugs. However, the fact that total block of mrp is compatible with life suggests that MRP inhibitors can be safely used in the treatment of cancer patients. Thus it is conceivable that MRP overexpressing tumors could lose their advantage over

normal tissues and re-acquire sensitivity to chemotherapeutic agents, without major effects on human physiology.

Resistance profile of MRP

In general, the resistance profiles of the various MRP-overexpressing cell lines are similar, and typically include moderate to high level resistance to the anthracyclines, *vinca* alkaloids, and VP-16 (Cole et al., 1994; Zaman et al., 1994; Breuninger et al., 1995; Chang et al., 1997), and no resistance to platinum-based drugs (Kruh et al., 1994). MRP1-transfected cells accumulate less vincristine and daunorubicin than control transfectants (Cole et al., 1994; Zaman et al., 1994). However, unlike Pgp-mediated resistance, MRP confers low levels of resistance to taxol and colchicine (Cole et al., 1994; Zaman et al., 1994; Breuninger et al., 1995). On the other hand, no resistance to cisplatin, was detected in transfected cells (Kruh et al., 1994).

Reversal of MRP-mediated drug resistance

A number of compounds modulating MRP-associated MDR have been characterized. Initially, it was found that BSO, an inhibitor of GSH synthesis, could block MRP-mediated MDR (Zaman et al., 1995). This was followed by the identification of other drugs that also affect GSH levels and reverse MRP-mediated MDR (Manzano et al., 1996). Several reports have demonstrated that verapamil can reverse MRP-mediated MDR (Abe et al., 1995; Binaschi et al., 1995). However, the latter findings has been controversial (Grant et al., 1994; Loe et al., 1996a; Gaj et al., 1998). Other compounds including MK571, a *quinoline-based* LTD_4 receptor antagonist (Gekeler et al., 1995), the pyridine analogue PAK-104P (Sumizawa et al., 1997), dihydropropyridine derivative NIK250 (Tasaki et al., 1995), imizothiazole derivatives (such as N276-12, (Naito et al., 1998)), and the pipecolinate derivative VX-710 (Germann et al., 1997). However, with the exception of VX-710 (Germann et al., 1997), it is not known if the above MRP-mediated MDR modulators interact with MRP. More recently, MS209 another *quinoline derivative* was found to reverse MRP-mediated MDR of gastric cancer (Nakamura et al., 1999).

Role of GSH in MRP drug resistance

Given MRP endogenous natural substrates, many proposed that the protein might transport drug conjugates rather than free drugs. In support of this proposal, transport studies using MRP1-enriched membrane vesicles have failed to demonstrate that MRP1 directly transports natural product chemotherapeutic agents (Jedlitschky et al., 1996; Loe et al., 1996c; Heijn et al., 1997; Loe et al., 1998). Early clues to the reason why transport of these drugs was not detectable came from studies using BSO. Treatment of cells with this agent resulted in reduction of intracellular GSH levels by as much as 90% within 24 hours. Several studies showed that BSO treatment could enhance drug accumulation and toxicity in MRP1-overexpressing cell lines (Schneider et al., 1995; Versantvoort et al., 1995; Lautier et al., 1996; Draper et al., 1997; Vanhoefer et al., 1997). In addition, treatment with the membrane permeable GSH ethyl-ester restored cytoplasmic GSH and decreased daunorubicin accumulation in two BSO-treated MRP-overexpressing cell lines (Versantvoort et al., 1995).

The apparent inability of MRP to directly transport natural product chemotherapeutic agents is in agreement with the observation that these xenobiotics are poor inhibitors of MRP1-mediated transport of other substrates. Even at high drug concentrations (100 μM), little inhibition of LTC_4 transport was observed with doxorubicin, daunorubicin, vincristine, vinblastine, VP-16, or paclitaxel (Muller et al., 1994; Loe et al., 1996c). However, the inhibitory potency of some of these drugs was markedly increased in the presence of reduced GSH with the most dramatic enhancement observed with the *vinca* alkaloids, vincristine and vinblastine (Loe et al., 1996c). It was subsequently demonstrated that vincristine uptake into membrane vesicles in the absence of reduced GSH was extremely low, and then increased with increasing concentrations of GSH (up to 5 mM) (Loe et al., 1996c; Loe et al., 1998). Similarly, GSH stimulated the active transport of unmodified aflatoxin B1 by MRP (Loe et al., 1998), and of unmodified daunorubicin into membrane vesicles from MRP-overexpressing cells (Renes et al., 1999).

The finding that MRP-mediated transport could be stimulated by GSH suggests that either MRP co-transports drugs with GSH or that GSH in some way activates MRP, facilitating substrate binding and/or transport. However, examining the transport of GSH

in vitro provided support for a co-transport mechanism (Loe et al., 1998). The study found that ATP-dependent uptake of [^{3}H]-GSH into membrane vesicles from MRP-transfected cells was stimulated by vincristine in a dose-dependent manner (Loe et al., 1998).

STRUCTURE-FUNCTION ANALYSIS OF MRP

While the role of MRP in conferring drug resistance has been established, the molecular mechanism responsible for this phenotype is not entirely understood. In an attempt to clarify these mechanisms, there has been considerable interest in structure-function analyses of the protein. One approach has been to express intact MRP1 and its constituent domains in a baculovirus system. Although under-glycosylated, full-length MRP is expressed at high levels in insect cells and has kinetic parameters for LTC_4 transport and competitive inhibition profiles that are similar to the protein expressed in mammalian cells (Gao et al., 1996; Bakos et al., 1998). Two fragments of MRP1 composed of amino acids 1-932 or 932-1531 had no transport activity when expressed independently (figure 4a, 4b). However, when co-expressed, the two fragments formed a fully functional LTC_4 transporter, demonstrating that they do not need to be covalently attached to associate properly (figure 4c) (Gao et al., 1996). MRP1 fragments that are lacking most of the linker region (amino acids 1-858 and 932-1531) can also associate to form a functional transporter, indicating that this region is dispensable for LTC_4 transport activity (figure 4d) (Gao et al., 1998).

Efforts to understand the role of the unique NH_2-terminal MSD in the function of MRP1 suggested that this region was essential for transport (Gao et al., 1998). Thus, truncated MRP1 molecules containing amino acids 229-1531 or 281-1531 expressed in insect cells were inactive (figure 4e, 4f) but LTC_4 transport activity could be reconstituted when either polypeptide was co-expressed with a fragment containing amino acids 1-281 (figure 4g, 4h). On the other hand, co-expression of amino acids 1-227 and 281-1531 did not result in detectable transport activity, suggesting that at least a portion of the cytoplasmic region joining MSD_0 and MSD_1 was critical for activity (figure 4i) (Gao et al., 1998). In a separate study, it was reported that an NH_2-terminally truncated MRP1 molecule containing amino acids 204-1531 (lacking the five transmembrane segments of

MSD0) transported LTC_4 slightly less efficiently than the intact protein when expressed in both mammalian and insect cells (figure 4j) (Bakos et al., 1998).

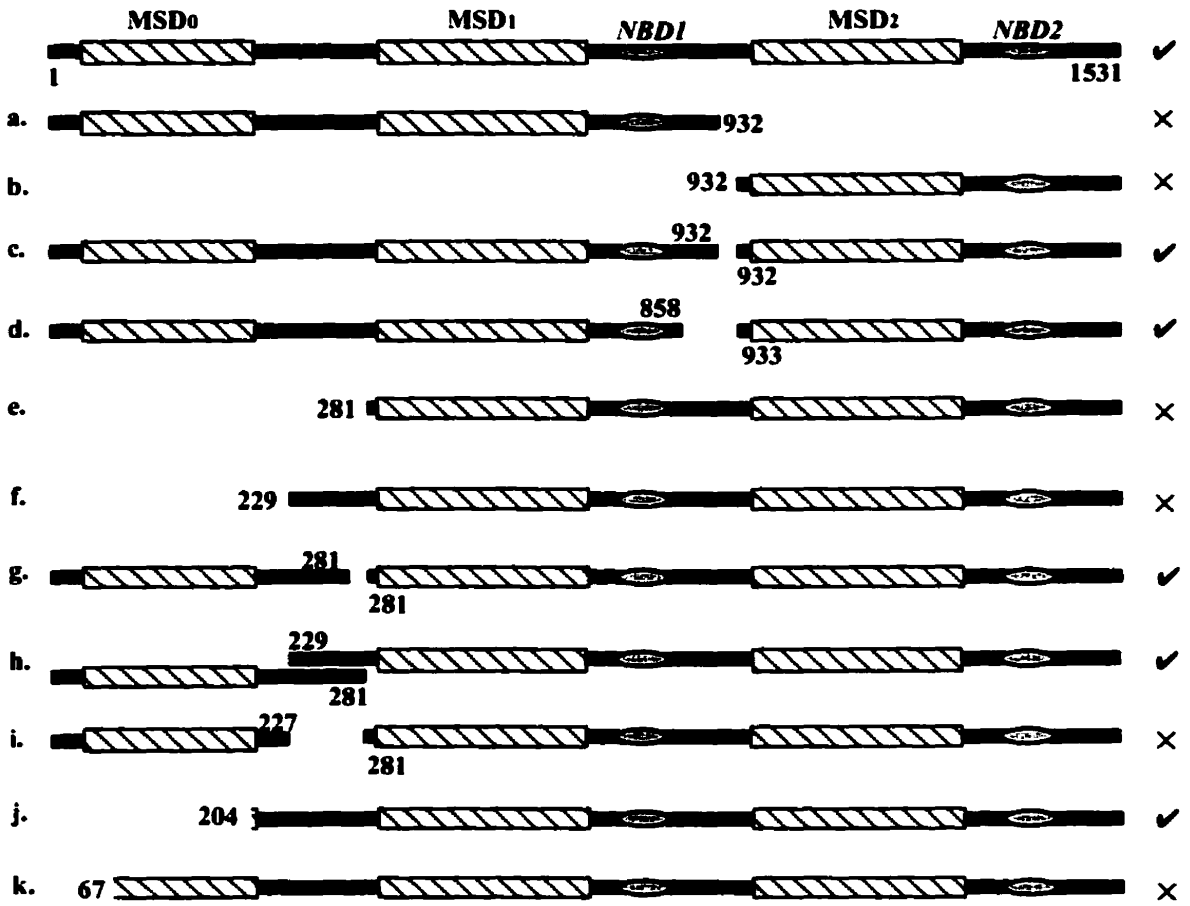

Figure 4. Summary of various MRP structure-function analyses. This figure summarizes the MRP structure-function analyses that have been done so far. A representative model of MRP is used: dashed rectangles represent the membrane spanning domains (MSDs), and the dotted ovals represent the nucleotide binding domains (NBDs). The check mark (✔) or the cross (✕) describe whether the corresponding MRP-variant carries LTC_4 transport or not, respectively (see text for more details).

Taken together, these results suggest that determinants located between amino acids 204 and 229 in the intracellular loop connecting MSD0 to MSD1, rather than the entire MSD0 region itself, are essential for transport. Unlike most of MSD0, this region contains a peptide segment that is relatively highly conserved among MRP-like proteins (Tusnady et al., 1997; Bakos et al., 1998) and may therefore be important for the transport activity of all proteins in this branch of the ABC superfamily. Interestingly, MRP lacking its extracytosolic NH_2-terminal and first transmembrane α-helix (amino acids 1-66) was inactive for reasons which are presently unclear (figure 4k) (Gao et al.,

1998). On the other hand, MRP1 lacking its NH_2-terminal 203 amino acids properly localized to the basolateral membranes of polarized MDCKII cells, suggesting that MSD_0 is also not essential for trafficking (Bakos et al., 1998).

PHOTOAFFINITY LABELING OF MRP

Photoaffinity labeling allows for covalent crosslinking of a biologically active molecule directly to the receptor protein that normally acts on it. Photoaffinity labeling of protein receptors with photoactive drug analogues has been extensively used to provide insights into the mechanisms of protein-drug interactions. The absence of MRP-specific photoactive drugs has been a major obstacle in elucidating *direct binding* to the protein. Previous attempts to photoaffinity label MRP with Pgp-specific photoactive drugs have failed (Cole et al., 1994; Vezmar et al., 1997). One interesting feature of LTC_4 is its endogenous molecular photosensitivity. In 1994, a number of studies took advantage of this characteristic (Leier et al., 1994b; Leier et al., 1994a; Loe et al., 1996c) and demonstrated the photoaffinity labeling of MRP with [^{3}H]-LTC_4. The specificity of [^{3}H]-LTC_4 photolabeling was confirmed by competition experiments with non-radiolabeled LTC_4 and MK571, a quinoline-based LTD_4 receptor antagonist which reverses MRP-mediated MDR (Jones et al., 1989; Gekeler et al., 1995). However, MRP photolabeling with [^{3}H]-LTC_4 suffered from a weak efficiency, requiring large amounts of membrane and long exposure periods (minimum 2 weeks). This imposed many restrictions on studying MRP1-drug interactions.

In 1997, Vezmar and Georges (Vezmar et al., 1997) reported the first clues showing direct binding of a photoactive drug, IAAQ, to MRP. IAAQ (the radio-labeled form of ***N*-{4[1-hydroxy-2-(dibutyl-amino)ethyl]quinolin-8-yl}-4-azidosalicylamide**) is a quinoline-based photoactive analogue of chloroquine, that has been previously used to probe for chloroquine-binding proteins in malaria-infected erythrocytes (Foley et al., 1994). The photoaffinity labeling of MRP with IAAQ was specific. Cell growth and accumulation studies showed H69/AR resistant cells to be less sensitive to and to accumulate less IAAQ than the parental H69 cells. In addition, a molar excess of LTC_4 and MK571 inhibited photolabeling of MRP1. This led to speculations that the inhibition of IAAQ-photolabeling of MRP by MK571 is due to the quinoline moiety, which is found in both

IAAQ and MK571. Therefore, IAAQ could be binding to the same or overlapping domains as MK571 and LTC_4.

As previously described, it is believed that GSH plays an important role in MRP's multidrug resistance phenotype. However, whether GSH is needed for *drug binding* and/or *drug transport* requires investigation. Using HPLC analysis, it was observed that IAAQ incubation with H69/AR cells did not alter its elution time when compared to unmodified drug (Vezmar et al., 1997). In addition, photolabeling MRP from plasma membrane samples, with IAAQ, was an example of direct binding to the protein in a GSH free milieu (Vezmar et al., 1997). The previous observations provide good evidence that no GSH modification is required for IAAQ binding to MRP. Further, investigation is required to determine whether this applies to other MRP classes of substrates.

MRP FAMILY MEMBERS

Soon after its discovery in 1992, MRP started to acquire new members into its family. MRP2 was introduced in 1996 (Buchler et al., 1996; Flens et al., 1996; Taniguchi et al., 1996), followed by three more in 1997, commonly known as MRP3, MRP4, and MRP5. Finally, MRP6 has been recently added in 1998. Based on the complete sequences of MRP1 family members, two subgroups can be recognized. One group that includes MRP1, MRP2, MRP3, and MRP6, sharing 45-58% amino acid identity, is characterized by the presence of the NH_2 terminal membrane-bound extension of about 200 amino acids, with five putative transmembrane segments in addition to an MDR1-like core (Tusnady et al., 1997). The N-terminal domain is present in the yeast MRP1 homolog YCF1 and the SUR proteins SUR1 and SUR2, but not in the other MRP homologs, MRP4 and MRP5. MRP4 and MRP5 have less homology to MRP1 (34-39% amino acid identity), and their predicted structure is more similar to CFTR and MDR1.

MRP2, also known as cMOAT, is mainly localized to the canalicular membranes of hepatocytes (Mayer et al., 1995; Buchler et al., 1996; Paulusma et al., 1996; Evers et al., 1998). Mutations of the cMOAT are known to cause the Dubin-johnson syndrome (DJS). DJS is an inherited defect in the secretion of amphiphilic anionic conjugates from hepatocytes into the bile. MRP2 and MRP1 share a similar substrate spectrum despite differences in cellular localization and kinetic properties (Keppler et al., 1998; Cui et al.,

1999). In fact, increasing evidence are correlating MRP2 expression with multidrug resistance (Mayer et al., 1995; Buchler et al., 1996; Paulusma et al., 1996; Taniguchi et al., 1996; Kool et al., 1997; Minemura et al., 1999).

Of all the MRP family members, **MRP3** is closest to MRP1 (58% amino acid identity). Similar to MRP2, MRP3 is highly expressed in the liver, colon, intestine, and adrenal gland and on a lower scale in the kidney (Kool et al., 1997; Ballatori and Rebbeor, 1998; Konig et al., 1999). MRP3 was mainly localized in bile duct epithelial cells and to a lesser extent in the basolateral membrane of hepatocytes as determined by immunofluorescence microscopy (Konig et al., 1999; Kool et al., 1999b). Similar to MRP1 and MRP2, MRP3 is an organic anion transporter and is able to confer resistance to anti-cancer drugs (e.g. methotrexate, etoposide, and teniposide). The most striking difference found thus far is that cells overexpressing MRP3 do not excrete more GSH than the parental cells, in contrast to cells transfected with MRP1 or MRP2 (Paulusma et al., 1999).

The physiological functions of MRP4, MRP5, and MRP6 are not known. **MRP4** (MOAT-B) is widely expressed, with high level in the prostate, but it is not found in the liver (Lee et al., 1998b). **MRP5** (MOAT-C) is expressed at the highest level in skeletal muscle and at intermediate levels in kidney, testis, heart, and brain. Low levels of expression of MRP5 was detected in liver (Belinsky et al., 1998). **MRP6** is specifically expressed in liver and kidney, and it was suggested that MRP6 is not involved in conferring multidrug resistance to tumor cells (Kool et al., 1999a).

Other members of the human MRP family are the sulfonylurea receptor (SUR1) and the cystic fibrosis transmembrane regulator (CFTR). SUR1 has a similar topology to MRP. Sulfonylureas have been shown to promote insulin secretion, interact with the sulfonylurea receptor of pancreatic β cells and inhibit the conductance of ATP-dependent potassium channels (Aguilar-Bryan et al., 1995). Mutations of SUR1 has been found to cause a rare genetic disease called persistent hyperinsulinemic hypoglycemia of infancy (PHHI). PHHI is due to defective negative feedback regulation of insulin secretion by low glucose levels, resulting in excess insulin secretion and hypoglycemia. The CFTR, with a topological arrangement similar to P-glycoprotein, is responsible for cystic

fibrosis, one of the most frequent inherited diseases with a prevalence of 1 in 2500 births within Caucasian populations (Rommens et al., 1989).

CHAPTER II

The Multidrug Resistance Protein 1 is photoaffinity labeled by a quinoline-based drug at multiple sites

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ABBREVIATIONS

MDR, multidrug resistance; P-gp, P-glycoprotein; MRP, multidrug resistance protein; SCLC, Small cell lung cancer; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; ABC, ATP-binding cassette.

ABSTRACT

Tumor cells overcome cytotoxic drug pressure by the overexpression of either or both transmembrane proteins, the P-glycoprotein (P-gp) and the Multidrug Resistance Protein (MRP). MRP has been shown to mediate the transport of cytotoxic natural products, in addition to glutathione, glucuronate and sulfate conjugated cell metabolites. However, the mechanism of MRP drug binding and transport is presently not clear. In this study we have used a photoreactive quinoline-based drug *N*-(hydrocinchonidin-8′-yl)-4-azido-2-hydroxybenzamide (IACI) to show the photoaffinity labeling of 190 kDa protein in membranes from the drug resistant SCLC H69/AR cells. The photoaffinity labeling of the 190 kDa protein by IACI was saturable and specific. The identity of the IACI-photolabeled protein as MRP was confirmed by immunoprecipitation with the monoclonal antibody QCRL-1. Furthermore, molar excess of leukotriene C_4, doxorubicin, colchicine and other quinoline-based drugs, including MK571, inhibited the photoaffinity labeling of MRP. Drug transport studies showed lower IACI accumulation in MRP-expressing cells which was reversed by depleting ATP levels in H69/AR cells. Mild digestion of purified IACI photolabeled MRP with trypsin showed two large polypeptides (~111 kDa and ~85 kDa). The 85 kDa polypeptide which contains the QCRL-1 and MRPm6 monoclonal antibodies epitopes correspond to the C-terminal half of MRP (~900 to 1531) containing the 3rd multiple spanning domain (MSD_2) and the 2nd nucleotide binding site. The 111 kDa polypeptide which contains the epitope sequence of MRPr1 monoclonal antibody encodes for the remainder of the MRP sequence (1 - 900) containing the MSD_0 and MSD_1 plus the 1st nucleotide binding domain. Cleveland maps of purified IACI-labeled 85 kDa and 111 kDa polypeptides revealed a 6 kDa and ~6 kDa plus 4 kDa photolabeled peptides, respectively. In addition, resolution of exhaustively digested IACI photolabeled MRP by hplc showed two major and one minor radiolabeled peaks that eluted late in the gradient (60-72% acetonitrile). Taken together, the results of this study show direct binding of IACI to MRP at physiologically relevant sites. Moreover, IACI photolabels three small peptides which localize to the N- and C-halves of MRP. Finally, IACI provides a sensitive and specific probe to study MRP-drug interactions.

INTRODUCTION

Treatment of cancer patients with chemotherapeutic drugs is often unsuccessful due to the emergence of drug resistant tumors. Similarly, tumor cell lines selected *in vitro* with anticancer drugs become resistant to multiple drugs with concurrent overexpression of either or both transmembrane proteins, the P-glycoprotein (P-gp1) and the multidrug resistance protein (MRP) (*1-3*). Gene transfer studies with human P-gp1 or MRP cDNA were shown to confer resistance to similar anticancer drugs onto previously drug susceptible cells (*4, 5*). Furthermore, disruption of P-gp1 or MRP genes in mice led to increased sensitivity to natural-product toxins and elevated glutathione levels in MRP-expressing tissues (*6-8*). Although both proteins are likely to mediate several physiological functions, P-gp1 appears to function as a non-specific efflux pump at the blood brain barrier (*6*), while MRP functions include mediation of inflammatory responses (*8*). Moreover, both proteins have been shown to function as "flipases" of short chain lipids (*9, 10*) and to mediate the transport of normal cell metabolites and xenobiotics (*5, 11-13*).

P-gp1 and MRP are members of a large family of membrane trafficking proteins that couple ATP hydrolysis to ligand transport across the cell membrane (*14*); however the two proteins share only 15% amino acid identity (*15*). MRP primary structure encodes an MDR-like core of two membrane spanning domains (MSD_1 and MSD_2) and two nucleotide-binding domains (NBD_1 and NBD_2); in addition to a 220 amino acid N-terminal membrane spanning domain (MSD_0) (*16-18*). Although the role of MSD_0 remains to be clarified, it is thought to contain five transmembrane helices with an extracytosolic N-terminus (*17, 18*). Furthermore, deletion of the first transmembrane helix from MSD_0 or the entire MSD_0 plus the linker sequence between MSD_0 and MSD_1 was shown to inhibit MRP-mediated transport of LTC_4 (*19*). More recently, Bakos et al. (*20*) showed that the deletion of all transmembrane helices of MSD_0 had no effect on MRP-mediated LTC_4 transport. Interestingly, deletion of the linker domain between MSD_0 and MSD_1 abolished LTC_4 transport (*20*).

Several studies have shown MRP-mediated transport of glutathione, glucuronate and sulfate conjugated drugs (*11, 21-23*). The glutathione conjugated eicosanoid,

leukotriene C_4 (LTC_4) is the highest affinity substrate for MRP transporter (*11, 21, 22*). Indeed, MRP appears to function as a co-transporter of glutathione, hence MRP-like proteins are known as GS-X pumps or multispecific organic anion transporters (*24, 25*). The ability of MRP to bind and transport unmodified drugs remains to be resolved. MRP was shown to transport several unmodified drugs and natural products (*26, 27*). For example, direct binding and transport of unmodified quinoline-based drugs in MRP-expressing MDR cells has been previously described (*27-29*). However, Loe *et al.* (*30*) showed MRP-mediated active transport of unmodified vincristine only in the presence of GSH.

Photoreactive drug analogues have been previously employed to study protein receptors interactions with small ligand molecules (*31*). The use of photoreactive drug analogues that photoaffinity label P-gp have demonstrated the presence of at least two drug binding sites which map to sequences in transmembranes 5-6 and 11-12 (*32*). The drug binding domains identified by photoaffinity labeling were later confirmed by mutational analyses of P-gp1 TMs sequences (*33*). Several attempts to photoaffinity label MRP with commercially available P-gp-specific photoreactive drugs have not been successful (*28, 34*). Leier *et al.* (*11*) demonstrated the photoaffinity labeling of MRP with [^{3}H] LTC_4. The specificity of LTC_4 photoaffinity labeling was confirmed by competition experiments with non-radiolabeled LTC_4 and MK571, a quinoline-based antagonist of the LTD_4 receptor which reverses MRP-mediated MDR (*35*). However, photoaffinity labeling of MRP by [^{3}H]-LTC_4 suffers from weak photolabeling efficiency which limits its usefulness in studying MRP-drug interactions. Furthermore, with respect to MRP binding to anticancer drugs, it is not known if LTC_4 and unmodified anticancer drugs bind to the same sites. In this report, we demonstrate specific photoaffinity labeling of MRP by a quinoline-based photoreactive drug. Moreover, our results show for the first time photoaffinity labeling of three sites in the N- and C-halves of MRP.

MATERIALS AND METHODS

Materials - Iodine-125 (100.7 mCi/ml) was purchased from Amersham Biochemical Inc., (Mississauga, Ontario, Canada). Protein-A coupled Sepharose was purchased from Pharmacia Inc. (Montreal, Quebec, Canada). The LTD_4 receptor antagonist MK 571 was kindly provided by Dr. A.W. Ford-Hutchinson (Merck-Frost Centre for Therapeutic Research, Point Claire-Dorval, Quebec, Canada; (*36*)). Leukotriene C_4 (LTC_4) was purchased from Cayman Chemical Co. (Ann Arbor, MI). The Small Cell Lung Cancer cells (H69 and H69/AR) and the MRP-specific monoclonal antibody (QCRL-1) were kind gifts from Dr. S.P.C. Cole (Cancer Research Laboratories, Queen's University, Kingston, Ontario, Canada). All other chemicals were of the highest commercial grade available.

Cell culture and plasma membrane preparations - Drug sensitive (H69) and resistant (H69/AR) cells were grown in RPMI 1640 media containing 4 mM glutamine and 5% fetal calf serum (Hyclone). Resistant cells were cultured continuously in the presence of 0.8 μM of doxorubicin; however, cells used for drug transport studies were grown in drug-free media for ten days prior to the date of the experiment. Plasma membranes from H69 and H69/AR cells were prepared as described by Lin *et al.*, (*37*). In brief, cells were collected by low speed centrifugation and washed three times with ice-cold phosphate buffered saline (PBS), pH 7.4. Cells were homogenized in 50 mM mannitol, 5 mM Hepes and 10 mM Tris-HCl, pH 7.4 (containing 2 mM PMSF and 3 μg/ml leupeptin) in a Dounce glass homogenizer. Calcium chloride solution was then added to the homogenate to a final concentration of 10 mM and mixed by stirring to ensure even distribution of the cation. The slightly turbid supernatant solution that contains plasmalemma vesicles was pelleted by high-speed centrifugation at 100,000 xg for 1 hour at 4°C using Beckman SW28 rotor. For sucrose gradient purification, membrane suspension was made to a final concentration of 45% sucrose with the addition of sucrose powder. The gradient was setup in a 14 ml polycarbonate tubes using 1ml of 60% sucrose; 5ml of membranes in 45% sucrose; 2.5ml of 35% sucrose and 2.5ml of 30% sucrose. Samples were spun for 3 hours at 100,000 xg at 4°C. Membranes floating at the 30%, 35% and 45% interfaces were harvested and washed with 10 mM

Tris-HCl, pH 7.4. The enriched plasma membrane fraction was resuspended in the same buffer containing 250 mM sucrose. Membranes were stored at -80°C if not immediately used. Protein concentrations were determined by the Lowry method (*38*).

Radio-iodination and photoaffinity labeling - The coupling method used in the synthesis of photoreactive drugs has been previously described (*39*). Details of the synthesis of IACI in particular will be described elsewhere. Iodination of IACI was carried out in the dark. Briefly, IACI (10 nM) was dissolved in 20 µl of dimethylsulfoxide (DMSO) and mixed with 10 µl of carrier-free $Na^{125}I$ (1 mCi, 0.5 nmol) and 10 µl of chloramine T (10 nmole) in 1 M K_2HPO_4, pH 7.4. The reaction was allowed to continue for 5 minutes and was stopped by the addition of sodium metabisulphite (50 µl of 5% (w/v) solution). The reaction mixture was loaded onto a C_{18} cartridge (Sep-Pak, Waters-Millipore) prewashed with 10 mM K_2HPO_4, pH 7.4. The column was washed with 5 ml aliquots of 10 mM K_2HPO_4, pH 7.4 containing 10% (v/v) methanol until no significant radiolabel was detected. IACI was eluted with 2.5 ml methanol and vacuum-dried in the dark. The dried residue was resuspended in DMSO and the concentration of the radioactive-photoactive drug determined by hplc.

Either plasma membranes (10-20 µg) or intact cells ($5X10^6$ cells) were photoaffinity labeled with IACI. Briefly, membranes or cells were photoaffinity labeled by IACI (0.20 – 1.0 µM) in the absence or the presence of molar excess of colchicine, chloroquine, doxorubicin, LTC_4 or MK 571. Membranes or cells were incubated at room temperature in the dark for 30 minutes and then transferred to ice for 10 minutes. Following the latter incubation, cells were irradiated for 10 minutes on ice with a *UV* source at 254 nm (Stratagene *UV* crosslinker, Stratagene, La Jolla, CA). The free photoactive drug was removed by centrifugation and cells were lysed in 20 µl of 50 mM Tris (pH 7.4) containing 1% Nonidet P-40 (NP40), 5 mM $MgCl_2$ and protease inhibitors (3 µg/ml of leupeptin and 2 mM PMSF). Photoaffinity labeled proteins were isolated by brief centrifugation at 4°C and resolved on SDS-PAGE. It should be mentioned that incubation of cells or membranes with IACI but without *UV* irradiation did not result in the photoaffinity labeling of proteins (data not shown).

Immunoprecipitation and SDS gel electrophoresis - IACI photoaffinity labeled cells were lysed in 50 mM Tris-HCl, pH 7.4, containing 0.5% CHAPS, 0.5% sodium deoxycholate, 150 mM NaCl and protease inhibitors (3 μg/ml of leupeptin and 2 mM PMSF). The cell lysates were clarified by centrifugation at 12,000 xg at 4°C. Equal amounts of cell lysate proteins were separately incubated overnight at 4°C with 10 μg of QCRL-1, MRPr1 or MRPm6 monoclonal antibodies or an irrelevant IgG_{2a}. Protein-A coupled sepharose was added to cell lysates and allowed to incubate for one hour at room temperature. After several washes in lysis buffer, proteins were released from the sepharose beads with buffer I (10 mM Tris-HCl, pH 8.0 containing 2% SDS, 50 mM dithiothreitol (DTT), 1 mM ethylene diaminetetraacetate (EDTA)) and II (2X buffer I and 9 M urea). Immuno-purified proteins were eluted and resolved by SDS PAGE using the Laemmli or the Fairbanks gel systems (*40,41*). Gel slabs containing the immunoprecipitated proteins were fixed in 50% methanol, dried and exposed to XAR Kodak film at -70°C for 2 – 12 hours. Alternatively, proteins were visualized by silver staining, using the NOVEX SilverXpress™ Silver Staining Kit.

Proteolytic digestion and HPLC - Immuno-purified photoaffinity labeled MRP bands were cut-out of dried SDS-PAGE gels and digested with increasing concentrations of *Staphylococcus aureus* V8 protease (1-20 μg/gel slice) in the well of a 15% Laemmli gel (*41*) according to the method of Cleveland *et al.* (*42*). For partial digestion of IACI photoaffinity labeled MRP, 100 μg of H69 and H69/AR plasma membrane samples were photolabeled with 0.2 μM IACI and immunoprecipitated overnight with QCRL-1 Mab as previously described (*43*). Protein A sepharose beads were washed in buffer A (0.1% TX-100, 0.03% SDS, 0.05 M Tris-HCl pH 7.4, 5 mg/ml fraction V bovine serum albumin (BSA), 150 mM NaCl) containing proteases inhibitors (0.1 mM PMSF, 3 μg/ml leupeptin, pepstatin A and aprotinin) followed by several washes without protease inhibitors. Mild trypsin digestion was carried out in the presence of 8 or 16 ng of trypsin at 37°C for 5 minutes. Digestion was stopped with 10 μg/ml leupeptin, pepstatin A, aprotinin and 1 mM PMSF followed by 5 min incubation at 65°C in SDS-PAGE sample buffer. Samples were resolved on Fairbanks gels, transferred to nitrocellulose membrane and probed with QCRL-1, MRPm6 and MRPr1 monoclonal antibodies. Alternatively,

partially digested fragments were immunoprecipitated separately with MRPr1 or MRPm6. Following an overnight immunoprecipitation, protein-A sepharose-beads with MRP halves (85 kDa and 111 kDa polypeptides) were washed and incubated with 40 μg of V8 protease in 50 mM Na_2HPO_4, pH 7.4. Digestion was allowed for 16 hrs at 37°C and samples were then resolved on Fairbanks gels (*41*).

For a complete trypsin digestion, gel slices were rehydrated for 5 minutes in water prior to the elution of IACI photoaffinity labeled MRP, using the GE200 SixPacGel Eluter (Hoefer Scientific Instruments). The buffer of the eluted protein was changed to 50 mM ammonium bicarbonate, pH 8.0 by repeated washing using the Spin-X UF concentrators with a 100,000 kDa cut-off. The digestion was commenced at 37°C with the addition of 2 μg and 1 μg of trypsin for 14 hours and 4 hours, respectively. The digested sample was vacuum dried, resuspended into 250 μl of 1% trifluoroacetic acid in water and resolved by reverse phase hplc (Vydac 201HS54 C_{18} RP column). The chromatographic procedure consisted of an 80-minute gradient of 0-100% acetonitrile with 1% trifluoroacetic acid at a flow rate of 1 ml/min. Fractions were collected and checked for radioactivity.

Drug accumulation - Drug sensitive and resistant cells ($1x10^6$ cells) were washed three times with phosphate buffer saline (PBS) pH 7.4 and incubated at 37° with 10 mM D-glucose or 10 mM 2-deoxy-glucose and 100 nM sodium azide. One micromole of IACI was added and drug accumulation in cells was determined at 0 minutes and 60 minutes incubations as previously described (*44*).

RESULTS

Photoaffinity labeling of MRP with IACI - MRP has been shown to mediate the transport of conjugated cell metabolites and natural product toxins (*22, 23, 45*). Several studies have now demonstrated a direct binding between one such glutathione containing compound, cysteinyl leukotriene, LTC_4 and MRP (*11, 46*). However, MRP interaction with unmodified compounds remain unclear. In this study, we examined the photoaffinity labeling of plasma membrane proteins from MRP-expressing cell line (H69/AR) by a photoactive quinoline-derived drug (IACI; Figure 1). To determine if

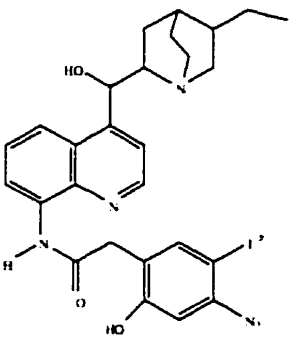

Figure 1. The organic structure of *N*-(hydrocinchonidin-8′-yl)-4-azido-2-hydroxybenzamide or IACI.

IACI binds directly to MRP, plasma membranes from drug sensitive (H69) and resistant (H69/AR) SCLC cells were incubated in the presence of 0.20 μM of IACI and *UV* irradiated (see experimental procedures). The results in Figure 2 show a 190 kDa protein photolabeled by IACI in H69/AR, but not in H69 membranes. When similar membrane samples were photoaffinity labeled by ([^{125}I]-iodoarylazidoprazosin) or [^{3}H] azidopine, shown previously to photolabel P-glycoprotein (*47*), no 190 kDa protein was photoaffinity labeled (results not shown and (*28*)). These results suggest that neither iodoarylazidoprazosin nor azidopine photoaffinity label the above 190 kDa protein in H69/AR membranes. The identity of the 190 kDa protein as MRP was confirmed by immunoprecipitation of IACI photolabeled plasma membranes from H69/AR cells with the MRP-specific monoclonal antibody, QCRL-1. Figure 2A shows that the IACI photolabeled 190 kDa protein can specifically immunoprecipitate with QCRL-1 from H69/AR, but not from H69 membranes. Moreover, no IACI photoaffinity labeled 190 kDa protein was immunoprecipitated with an irrelevant antibody (Figure 2A).

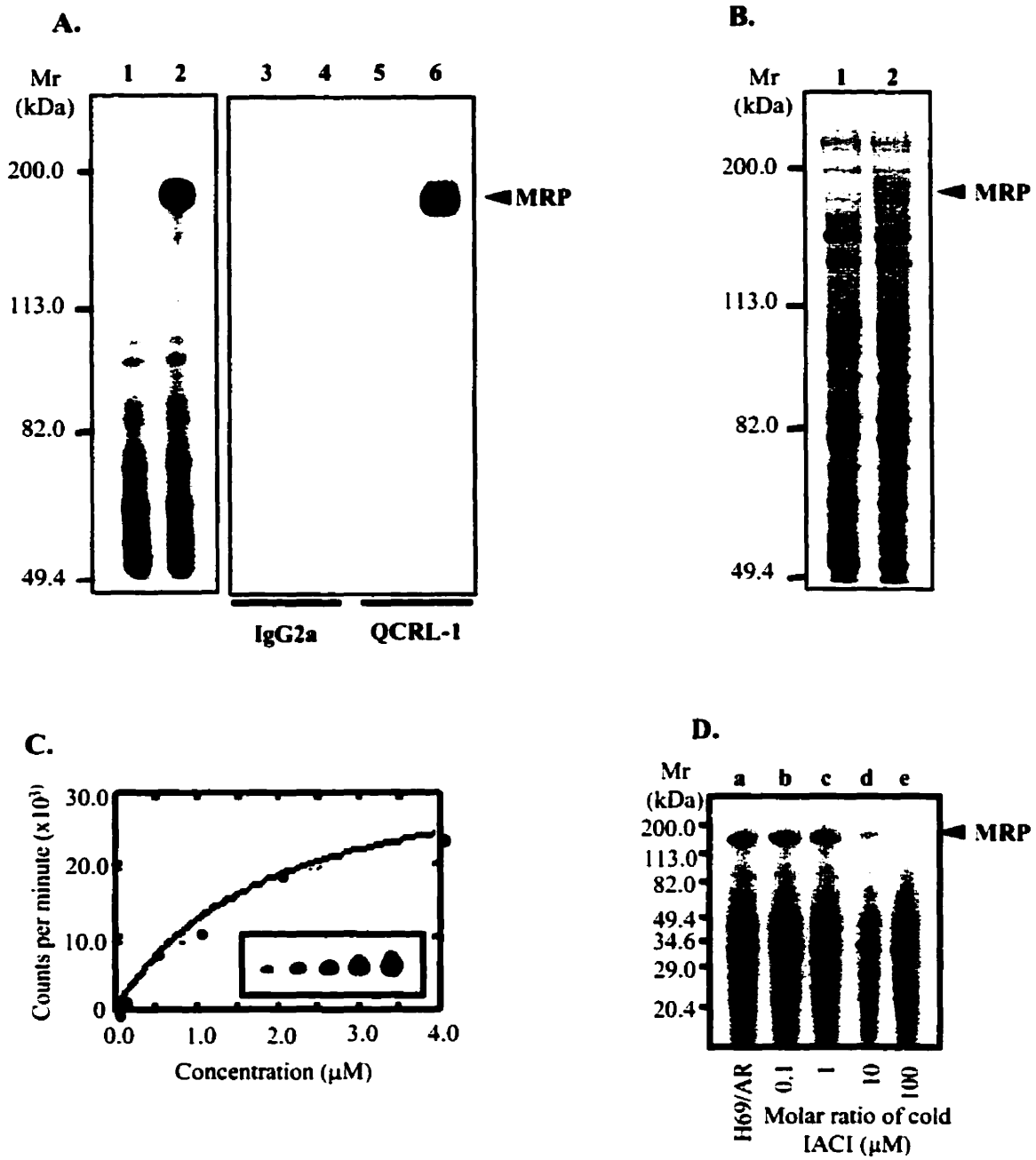

Figure 2. Photoaffinity labeling of MRP by IACI. Plasma membranes from drug sensitive (H69) and resistant (H69/AR) cells were photoaffinity labeled with 0.20 μM IACI and resolved on SDS PAGE (2A). Figure 2A also shows IACI photoaffinity labeled membranes from H69 and H69/AR cells immunoprecipitated with MRP-specific Mab (QCRL-1) or an irrelevant IgG_{2a}. Figure 2B shows total membranes from H69 and H69/AR cells stained by Silver staining. Figure 2C shows the photoaffinity labeling of H69/AR membranes with increasing concentrations of IACI (0-4.0 μM). The inset in figure 2C shows the increase in the intensity of 190 kDa photolabeled protein which was excised and the radiolabel quantified. Figure 2D shows photoaffinity labeled proteins from H69/AR cells incubated in the absence or presence of excess (0.1 - 100 μM) non-iodinated IACI.

To determine if photolabeling of the 190 kDa protein (or MRP) in membranes from H69/AR cells is due to non-specific binding to an abundant protein, total membrane proteins from H69 and H69/AR cells were resolved by SDS-PAGE on a 6% Laemmli gel (41) and visualized by Silver staining. Comparison of H69 and H69/AR proteins did not show significant differences, except for the broad band at ~190 kDa (figure 2B). Interestingly, the region where MRP photoaffinity labeling is observed, between the 113 kDa and the 200 kDa (figure2A), shows no photoaffinity labeling of some abundantly expressed proteins. High levels of protein expression are seen for both H69 and H69/AR cells between the 113 kDa and 50 kDa molecular weight markers (figure 2B). This may account for some of the photolabeling of certain abundantly expressed proteins seen below the 113 kDa molecular weight marker. To further confirm the binding specificity of IACI towards the 190 kDa protein, H69/AR membranes were photoaffinity labeled with increasing concentrations (0.25 μM to 4.0 μM) of IACI. The inset of figure 2C shows the photoaffinity labeling of the 190 kDa protein is saturable at 4.0 μM of the drug. Furthermore, the specificity of IACI towards the 190 kDa protein was confirmed by photolabeling in the presence of molar excess of the un-iodinated IACI. Figure 2D, lanes d and e, show marked decrease in the photolabeling of the 190 kDa protein in the presence of molar excess of IACI. The photolabeling which is evident in some lower molecular mass proteins, was not significantly affected with excess un-iodinated IACI (figure 2D).

Inhibition of photoaffinity labeling of MRP - To determine if IACI binds to physiologically relevant site(s) in MRP, membranes from H69/AR cells were photolabeled with IACI in the presence of molar excess of colchicine, chloroquine, doxorubicin, MK571 and LTC_4. The photoaffinity labeling of MRP was inhibited at 160-fold molar excess of LTC_4 (Figure 3A). Similarly, molar excess (150 to 300-fold) of MK 571 also led to a dramatic decrease in the photolabeling of MRP with IACI. Colchicine, chloroquine and doxorubicin at 1000-folds molar excess were similar to MK571 at 300-fold and LTC_4 at 160-fold consistent with higher affinity of MK571 and LTC_4 for MRP (Figure 3A). The latter results are in accord with the previous findings that LTC_4 is the highest affinity substrate for MRP (*11, 21, 22*). Together, these results confirm the

specificity of IACI towards MRP and suggest that IACI binding to MRP occurs at the same or overlapping site(s) as MK 571 and LTC_4.

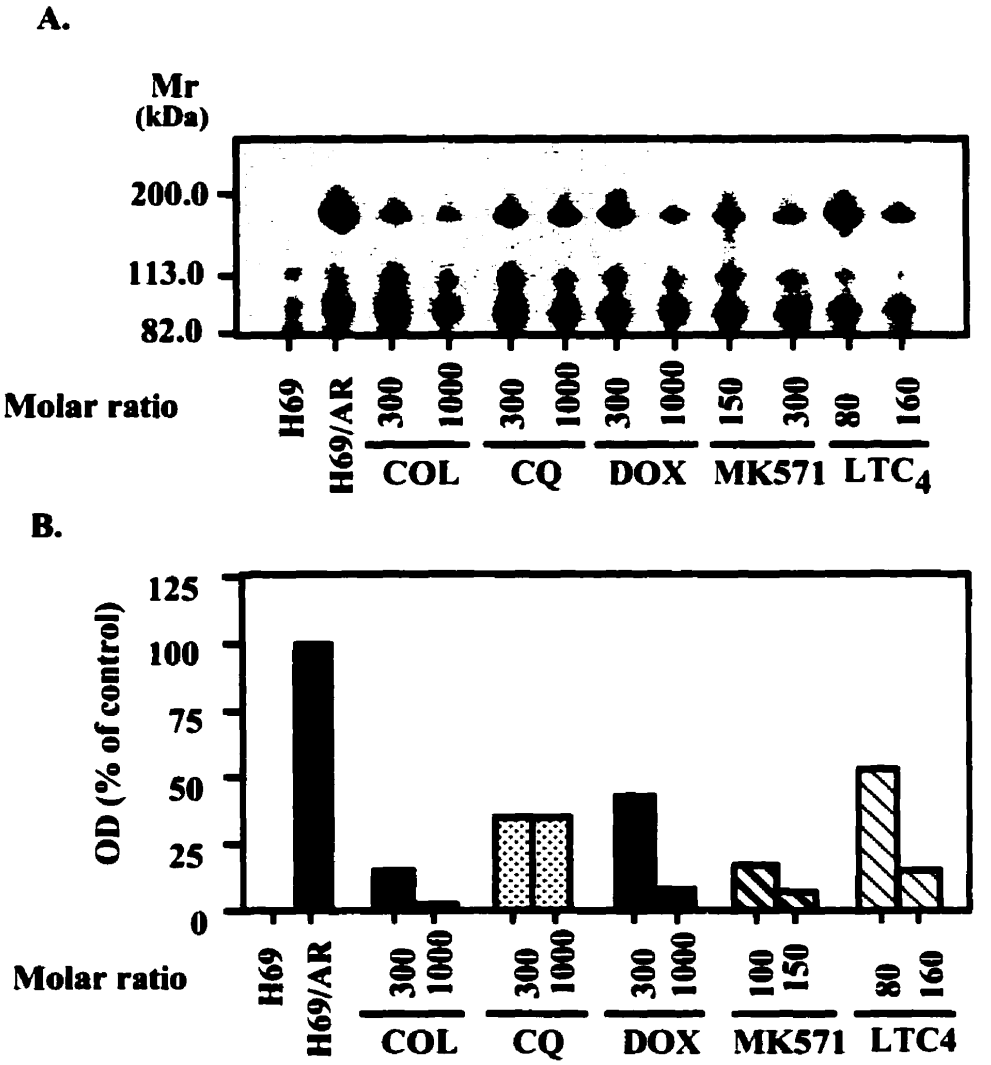

Figure 3. Effects of diverse drugs on the photoaffinity labeling of MRP by IACI. H69 or H69/AR cells were photoaffinity labeled with IACI in the absence or presence of molar excess (300 -1000-fold) of Colchicine (COL), Chloroquine (CQ), Doxorubicin (DOX), MK571 (150-300) and LTC_4 (80-160). Figure 3B shows a plot of the relative decrease in the photolabeling of MRP with IACI in the presence of the above drugs.

IACI accumulation in H69 and H69/AR cells - Given the above results, it was of interest to determine if IACI is a substrate for MRP transport function. Figure 4 shows the accumulation of IACI in H69 and H69/AR cells in the presence of 10 mM glucose. H69/AR cells show lower steady-state drug accumulation of IACI than H69 cells (figure 4). Moreover, preincubation of cells with 10 mM 2-deoxyglucose and 100 nM sodium azide, which depletes ATP levels, increased the accumulation of IACI in H69/AR cells to

the same level as the H69 parental cells (Figure 4). Taken together, these results show that the accumulation of IACI in MRP-expressing cells is ATP dependent.

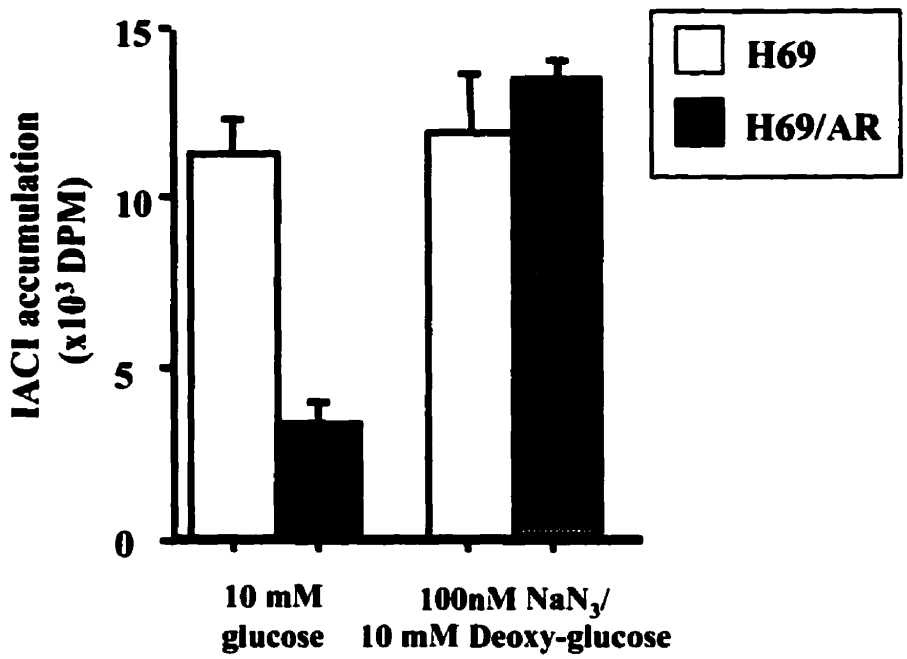

Figure 4. Drug accumulation in H69 and H69/AR cells. Cells were preincubated in 10 mM glucose or 2-deoxy-glucose and sodium azide prior to the addition of 1 μM IACI. Drug accumulation in cells was measured at 0 and 60 minutes following the addition of IACI. Cell were lysed and the amounts of accumulated radiolabel were determined by fluorography. Each value is mean ± SD of the two experiments in which triplicates were assayed.

Three peptides of MRP are photoaffinity labeled by IACI - Several reports on MRP secondary structure and topology have suggested the presence of an N-terminal five transmembrane helices (MSD_0), followed by an MDR-like core of duplicated six transmembrane domains and an ATP binding site (*16-18*). Limited proteolysis of MRP have revealed two trypsin sensitive sites (L_0 and L_1; see figure 5B) that connect MSD_0 to MSD_1+MSD_2 and MSD_0+MSD_1 to MSD_2 sequences (*48*). The L_1 sequence was shown to be more sensitive to trypsin cleavage than the L_0 (*48*). Therefore limited proteolysis of MRP with trypsin showed two polypeptides with molecular masses of 120 kDa and 75-80 kDa containing MSD_0+MSD_1+NBD_1 and MSD_2+NBD_2, respectively (*16-18*). However, further digestion with trypsin led to the cleavage of the 120 kDa polypeptide into two smaller peptides (40-60 kDa and 57 kDa) that correspond to MSD_0 and MSD_1+NBD_1, respectively (*16-18*). Taking advantage of the trypsin sensitive sites in MRP, it was of

interest to map IACI photoaffinity labeled peptides in MRP. Figure 5A shows the results of subjecting IACI photolabeled MRP to mild proteolysis with trypsin and the digested products resolved by SDS-PAGE. Lanes 3 and 4 of figure 5A show two major photoaffinity labeled polypeptides that migrate with apparent molecular masses of 111 kDa and 85 kDa. Figure 5C shows Western blots of the same samples as in figure 5A probed with MRPr1, QCRL-1 and MRPm6 Mabs, respectively. The Mabs QCRL-1

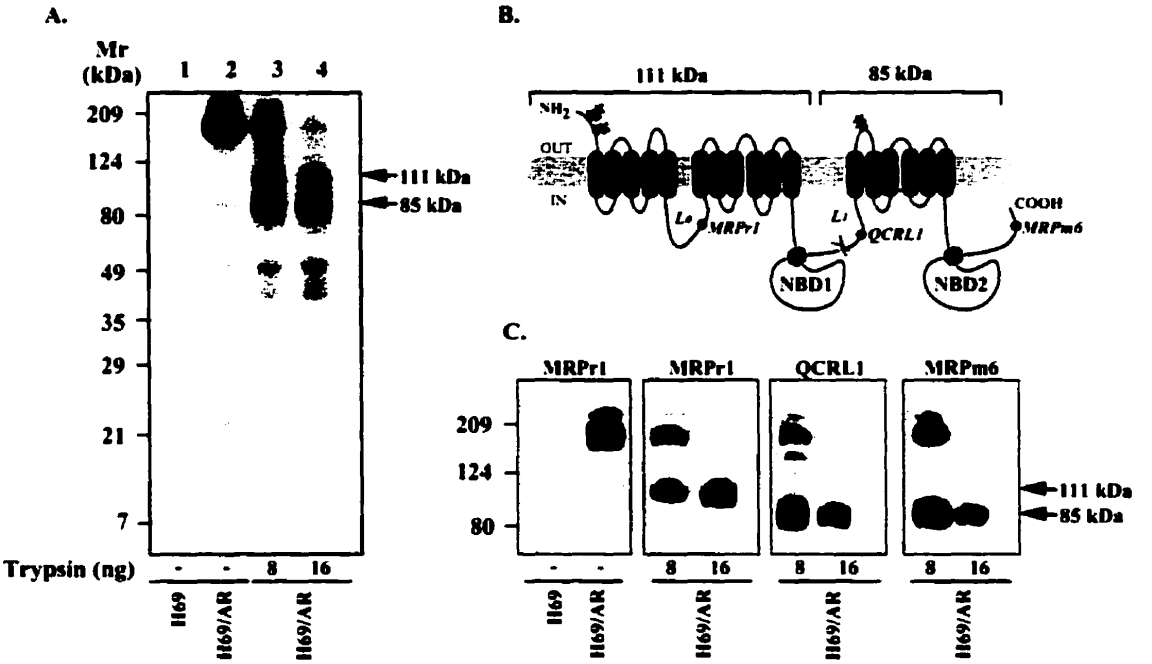

Figure 5. Photoaffinity labeling of two large polypeptides of MRP by IACI. MRP photoaffinity labeled by IACI was purified from H69/AR membranes and subjected to mild tryptic digestion. The photolabeled-radiolabeled products were split in two halves, one half was resolved by SDS-PAGE (figure 5A) while the second half was resolved by SDS-PAGE and transferred to nitrocellulose for Western blotting (Figure 5C). Lanes 2-4 of figure 5A show the signal from purified IACI-photolabeled MRP incubated in the absence and in the presence of 8 and 16 ng of trypsin, respectively. Figure 5C shows the Western blot of the IACI-photolabeled MRP tryptic digest probed separately with MRPr1, QCRL-1 and MRPm6 Mabs, respectively. Figure 5B shows a Schematic of MRP predicted topology with the above three monoclonal antibodies epitopes in MRP relative to the protease hypersensitive sites (L_0 and L_1). The nucleotide binding domains (NBD_1 and NBD_2) and the extracellular glycosylation sites (**) are indicated on the schematic of MRP secondary structure (Fig. 5B).

and MRPm6 recognized the 85 kDa polypeptide; while MRPr1 recognized the 111 kDa fragment (Figure 5C). These results are consistent with earlier proteolysis of MRP (*16-18*) whereby the 85 kDa polypeptide contains the QCRL-1 and MRPm6 Mabs epitopes while the 111 kDa polypeptide contains the MRPr1 epitope (*48*). Therefore, the two photoaffinity labeled polypeptides (111 kDa and 85 kDa) correspond to MRP sequence containing MSD_0+MSD_1+NBD_1 and MSD_2+NBD_2, respectively. Probing the same

nitrocellulose membrane with goat anti-mouse peroxidase second antibody alone did not show any reactive proteins (data not shown). Taken together, these results demonstrate the photoaffinity labeling of two different domains in MRP.

To determine the number of IACI-photolabeled sites in MRP, photoaffinity labeled MRP was immuno-purified with QCRL-1 and subjected to in-gel digestion with increasing concentrations of *Staph. aureus* V8 protease (1-20 μg/gel slice). Figure 6A

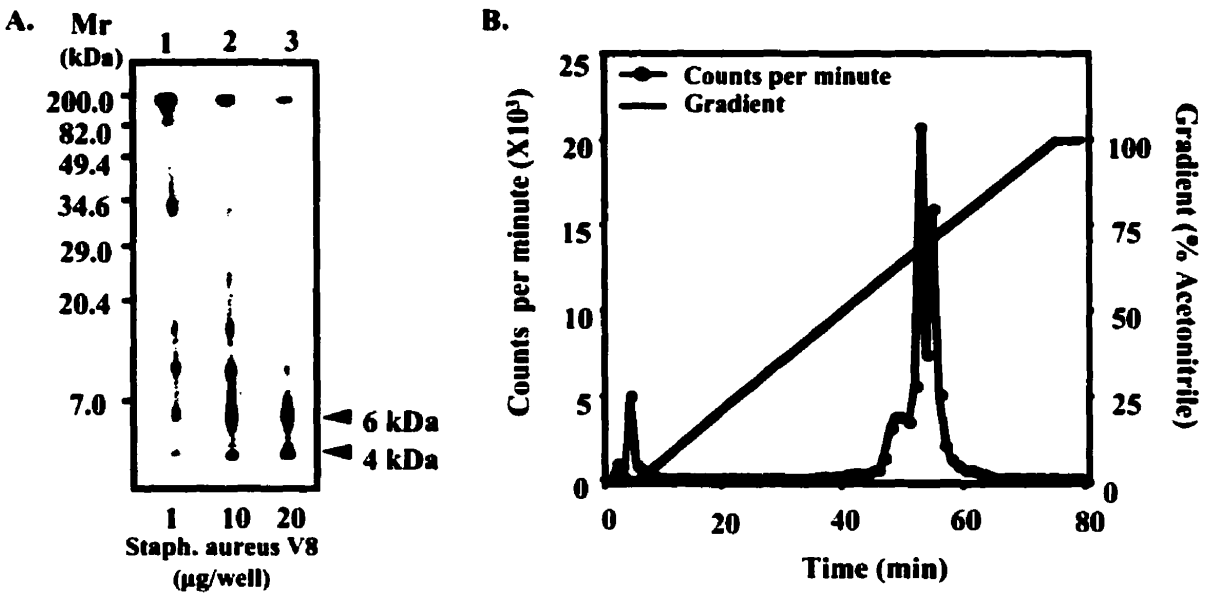

Figure 6. Complete proteolytic digestion of IACI photoaffinity labeled MRP yields two labeled peptides. Purified MRP photoaffinity labeled with IACI was subjected to complete in-gel or solution digestions. The in-gel digested MRP products were resolved on 15% acrylamide SDS-PAGE, while the solution digested products were resolved by reverse-phase chromatography. Lanes 1-3 of figure 6A show an in-gel digestion of IACI photolabeled MRP with increasing concentrations of *Staphylococcus aureus V8* (1 – 20 μg/well). Figure 6B shows the separation of MRP photoaffinity labeled tryptic peptides on a C18 reverse phase column using 0-100% gradient of acetonitrile. The amount of radiolabel in each eluted fraction was plotted versus time of elution in the gradient. The peak signal at the beginning of the gradient represents the void volume.

shows the resultant proteolytic fragments migrating with apparent molecular masses of 6 kDa and 4 kDa. Similarly, IACI photoaffinity labeled MRP was subjected to exhaustive trypsin digestion (see methods) and the resulting digest was resolved by hplc using reverse phase chromatography. Figure 6B shows the eluted IACI photolabeled-radiolabeled tryptic peptides resolved on a C_{18} reverse phase column with 0-100% acetonitrile gradient. The results in figure 6B show one minor peak eluting at 60% acetonitrile followed by two major peaks eluting at 65-72% acetonitrile.

To identify the origin of the 4 kDa and 6 kDa IACI-photolabeled peptides relative to the two large photolabeled domains of MRP, IACI-photolabeled 111 kDa and 85 kDa polypeptides were purified and digested with *Staphylococcus aureus* V8 protease. Lanes 2 and 3 of figure 7A show IACI-photolabeled 111 kDa and 85 kDa polypeptides following immunoprecipitation with MRPr1 and MRPm6 Mabs, respectively. Exhaustive

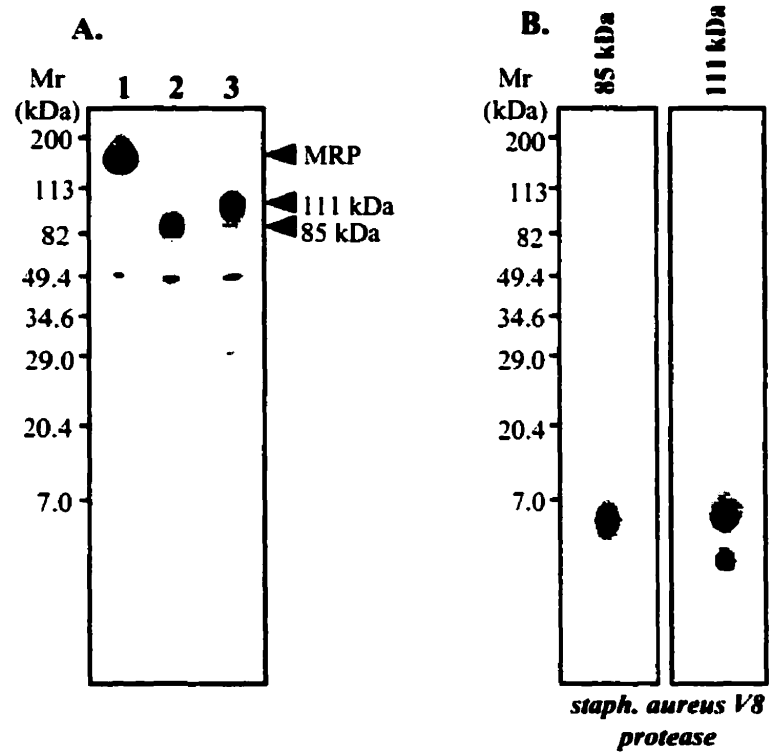

Figure 7. Proteolytic cleavage of immunopurified IACI-photolabeled N- and C-halves of MRP. MRP enriched membranes were photolabeled with IACI and subjected to mild tryptic cleavage. Lanes 1-3 of figure 7A show immunoprecipitation of native and trypsin digested MRP with MRPm6 and MRPr1 Mabs, respectively. Figure 7B shows complete digestion of immuno-purified IACI photolabeled 111 kDa and 85 kDa polypeptides with *Staphylococcus aureus V8*. The digested fragments were resolved on Fairbanks' gel system. The apparent molecular masses of standard proteins are indicated to the left of gels.

digestion of the 111 kDa polypeptide which corresponds to MSD_0+MSD_1+NBD_1 of MRP resulted in two photolabeled peptides which migrate with apparent molecular masses of 4 kDa and 6 kDa (figure 7B). However, digestion of the 85 kDa polypeptide, which corresponds to MSD_2+NBD_2 of MRP, resulted in only one photolabeled peptide of 6 kDa (figure 7B). Taken together, the results in figure 7 suggest the presence of three IACI labeled sites in MRP.

DISCUSSION

It is now believed that the overexpression of MRP in tumor cell lines can confer resistance to certain natural products toxins (*23, 49*). Moreover, MRP-mediated transport of normal cell metabolites have been demonstrated in intact cells and in MRP-enriched membrane vesicles (*11, 21-23*). However, the mechanism of MRP drug binding and transport remain unclear. For example; i) MRP's broad substrate specificity; ii) MRP's ability to bind and transport unmodified drugs; iii) the role of free GSH in MRP drug binding and transport; and iv) MRP drug binding site(s). In this report, we have made use of a photoreactive quinoline-based drug (IACI) to examine MRP-drug interactions. Our results show the photoaffinity labeling of a 190 kDa protein by IACI only in drug resistant cells (H69/AR). The identity of IACI photolabeled protein, as MRP, was confirmed by its binding to three MRP-specific monoclonal antibodies (QCRL-1, MRPr1 and MRPm6; (*48*)). The photolabeling of MRP in enriched membranes from H69/AR cells suggests direct binding between MRP and unmodified IACI. Moreover, the addition of free GSH (up to 5 mM) did not cause any significant change in MRP photoaffinity labeling (results not shown). With respect to the role of GSH in MRP drug transport, ATP-dependent transport of unmodified vincristine into membrane vesicles was shown only in the presence of GSH (*30*). Thus, although the authors of that study (*30*) did not examine vincristine binding to MRP; it is likely that drug transport but not drug binding required the presence of free GSH. Alternatively, GSH may be required for the binding and transport of certain classes of drugs. Future experiments will examine the effect of GSH and other MRP substrates on IACI transport in membrane vesicles from MRP expressing cells. Taken together, these findings demonstrate direct binding between MRP and an unmodified drug that is unaffected by GSH.

The question of whether IACI is a relevant substrate for MRP and whether the photoaffinity labeling of MRP by IACI occurs at a physiologically relevant site(s) was addressed by drug transport and drug competition experiments. Our transport results show IACI is a substrate for MRP drug efflux as it accumulates less in MRP-expressing cells (H69/AR) than in the parental cells (H69). Furthermore, depletion of ATP levels by preincubating cells with sodium azide and 2-deoxyglucose restored IACI accumulation in

H69/AR to the same level as H69 cells. The possibility that differences in IACI accumulation between H69/AR and H69 cells is due to other changes or ATP-dependent mechanisms than MRP can not be ruled out completely based on our drug transport data alone. However, coupled with the photoaffinity labeling results, described in this study, there is compelling evidence for MRP-mediated transport of IACI. The inhibition of MRP photolabeling by IACI with molar excess of LTC_4 indicates that IACI binds to MRP at a physiologically relevant site. Although little is known about MRP drug binding domain(s), we speculate that IACI binds to the same or an overlapping domain as that of MK571 or LTC_4. Alternatively, IACI may bind at another site that is allosterically linked to LTC_4 binding domain. In a recent study by Stride *et al.* (*50*), it was shown that substitution of the carboxyl third of human MRP with that of mouse mrp sequences modulated MRP specificity to the anthracycline, doxorubicin; while the LTC_4 transport was unaffected. Thus, confirming further the notion of more than one drug binding site in MRP. In addition, support for multiple drug binding sites in MRP comes from studies with another broad spectrum transporter (P-gp1) which is thought to encode for three drug binding sites (*51*).

Studies of MRP topology are in agreement with a "6+6" arrangement (MSD_1 and MSD_2) and two NBDs characteristic of many ABC transporters, in addition to the hydrophobic N-terminal domain predicted to encode five transmembrane helices (MSD_0) (*17, 18*). A previous limited proteolysis study (*48*) showed MRP to contain two hypersensitive regions (L_0 and L_1) connecting MSD_0 to MSD_1+MSD_2 and MSD_0+MSD_1 to MSD_2, respectively. In this study we took advantage of the protease hypersensitive regions in MRP linker domains together with the positions of three monoclonal antibodies with known epitope sequences in MRP to identify the photoaffinity labeling domains in MRP. Mild trypsin digestion of IACI photolabeled-MRP produced two large photolabeled polypeptides with apparent molecular masses of ~111 kDa and ~85 kDa on SDS-PAGE. Based on the locations of MRPr1, QCRL-1 and MRPm6 epitopes (at ^{238}GSDLWSLNKE^{247}, ^{918}SSYSGD^{924} and ^{1511}PSDLLQQRGL^{1520}, respectively; (*48*)) relative to one of the trypsin hypersensitive sites in MRP linker domain (L_1) (*17, 48*), the 111 kDa polypeptide corresponds to the N-terminal MSD_0+MSD_1 and NBD_1. Similarly, the 85 kDa polypeptide should encode for MSD_2+NBD_2 as it reacted only with QCRL-1

and MRPm6 Mabs (see figure 5). Exhaustive digestion of IACI photolabeled MRP with V8 protease revealed two photolabeled peptides with apparent molecular masses of ~6 kDa and ~4 kDa. However, digestion of purified 111 kDa and 85 kDa polypeptides with V8 protease revealed three IACI photolabeled peptides, two (6 kDa + 4 kDa) from the 111 kDa polypeptide and one (6 kDa) from the 85 kDa polypeptide. Although the possibility that the 6 kDa peptide, from the 111 kDa fragment, is an incomplete digestion can not be entirely ruled out, it is unlikely given the exhaustive nature of our digestion conditions. Furthermore, exhaustive digestion of IACI labeled MRP with trypsin showed two major and one minor peaks (figure 6B), consistent with a total of three IACI photolabeled peptides. The photoaffinity labeling of MRP at multiple sites is consistent with that observed with another membrane transporter proteins, P-gp1 (*32*). The exact amino acid sequence of P-gp1 drug binding sites is presently not know, however several reports support the role of transmembrane helices 5+6 and 11+12 in drug binding and transport (*33*). The photoaffinity labeling of three peptides in MRP with IACI suggests MRP drug binding may involve three domains. Given the hydrophobic nature of MRP substrates and our knowledge of P-gp1 drug binding domains, it is tempting to speculate that the three IACI photolabeled peptides correspond to the three membrane spanning domains MSD_0, MSD_1, and MSD_2 in MRP.

Although it is likely that the drug binding domains in MRP and the photoaffinity-labeled sites are different, earlier studies with P-gp1 have shown these sites to be the same or overlapping (32,33). Indeed, results from the photoaffinity labeling studies were used as a starting point for mutational analyses of P-gp1 sequences. Furthermore, results from a recent study to localize MRP specificity domains towards anthracyclines suggested a role for the carboxyl third of MRP (*50*). The latter findings are consistent with our results in this report which show the photoaffinity labeling of the carboxyl third of MRP (MSD_2+NBD_2) (*50*).

Molar excess of colchicine and doxorubicin also caused a significant decrease in MRP photoaffinity labeling by IACI. These finding are interesting since earlier analysis of cross-resistance of MRP expressing cells showed only low levels of cross-resistance to colchicine (*52*). Inhibition of photoaffinity labeling of IACI with MK571 and CQ is less surprising as both drugs share the quinoline moiety. The LTD_4 receptor antagonist, MK

571 (*36*), was recently shown to inhibit LTC_4 and S-(p-azidophenylacyl)-glutathione labeling of MRP (*11, 46*) and to reverse MRP-mediated MDR (*35*). Indeed, several quinoline-based drugs have been reported to interact directly and specifically with MRP (*27, 28, 53*). Hence, establishing this group as another class of compounds that interact with MRP. Collectively, these findings are important as many therapeutically important drugs are quinoline-based drugs (*54*). For example, quinoline based drugs are extensively used in the treatment of parasitic infections such as malaria (*55, 56*) and resistance to these drugs has rendered this first choice drug treatment ineffective (*55, 56*). Given these findings, we speculate about an MRP-like mechanism responsible for resistance in this parasite.

In conclusion, there is increasing evidence that MRP mediates the transport of structurally diverse drugs through direct binding. Although several studies have demonstrated direct binding between MRP and LTC_4 using a photoaffinity labeling assay, photoaffinity labeling of MRP by [^{3}H] LTC_4 was inefficient requiring large amounts of membrane (200 μg/sample) and extremely long exposure times (more than four weeks in our hands). By contrast, photoaffinity labeling of MRP by IACI was done using 20 μg of membranes/sample and exposure of 2-12 hours showed a strong signal for MRP. The availability of photoactive radio-iodinated drug that binds specifically to MRP should facilitate future analysis of MRP drug interactions. The photoaffinity labeling of MRP at multiple sites which map to two domains in MRP is consistent with protein-drug interactions seen with other members of the ABC family of drug transporters, such as P-gp1. Finally, we show that IACI binding to MRP is inhibited by known substrates of MRP, the LTC_4 and other natural product drugs. We speculate that IACI binds to the same or an overlapping domain(s) as that of LTC_4 or MK 571. Work is in progress to determine if IACI photoaffinity labels the same or different sequences as MK 571 and LTC_4.

ACKNOWLEDGMENTS

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CONNECTING STATEMENT I

In the previous study (Chapter 2), we have shown direct binding between MRP1 and the quinoline-based photosensitive drug, IACI. Using epitope specific monoclonal antibodies together with an MRP1-specific photoreactive drug, we provided the first direct evidence that MRP1 is photoaffinity labeled at multiple sites in the N- and C-terminal domains. However, given the broad substrate specificity of MRP1 and the possibility that it may encode more than one binding site, it was of interest to compare the IACI binding domains to those of other drugs. For example, it was not clear if the identified sites (results in Chapter 2) are specific to quinoline-based drugs or represent sites in MRP1 that interact with structurally diverse drugs. To address this possibility, we examined the interactions between MRP1 and Rhodamine 123, a xanthone-containing drug. Rhodamine 123 is an excellent substrate for P-gp1 and its transport occurs by direct binding to P-gp1. In addition, several studies have suggested that Rhodamine 123 is a substrate for MRP1, albeit MRP1 transports Rhodamine 123 less efficiently. In the next study, we report on the synthesis of a photoactive drug analogue of Rhodamine 123 and its interaction with MRP1.

CHAPTER III

Rhodamine 123 binds to multiple sites in the Multidrug Resistance Protein 1 (MRP1)

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ABBREVIATIONS

MDR, multidrug resistance; Pgp, P-glycoprotein; MRP1, multidrug resistance protein 1; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Rh123, Rhodamine 123; IAARh123, iodoaryl azido-Rhodamine 123; ABC, ATP-binding cassette; MSD, membrane spanning domain; NBD, nucleotide binding domain.

ABSTRACT

The Multidrug Resistance Protein (MRP1) is an ABC transmembrane protein shown to confer resistance to anti-cancer drugs and to mediate the transport of glutathione, sulfate and glucuronate modified metabolites. However, the mechanism of MRP drug binding and transport remains unclear. In this study, we have characterized the interaction between MRP1 and Rhodamine 123 using the iodinated photoreactive analogue, [^{125}I] iodoaryl azido-Rhodamine 123 (or IAARh123). Photoaffinity labeling of plasma membranes from HeLa cells transfected with MRP1 cDNA (HeLa-MRP1) with IAARh123 shows the photolabeling of a 190 kDa polypeptide not labeled in HeLa cells transfected with the vector alone. Immunoprecipitation of a 190 kDa photoaffinity labeled protein with MRP1 monoclonal antibodies (QCRL-1, MRPr1 and MRPm6) confirmed the identity of this protein as MRP1. Analysis of MRP1-IAARh123 interactions showed that photolabeling of membranes from HeLa-MRP1 cells with increasing concentrations of IAARh123 was saturable, and was inhibited with excess of the uniodinated analogue (AARh123). Furthermore, the photoaffinity labeling of MRP1 with IAARh123 was greatly reduced in the presence of excess Leukotreine C_4 or MK571, but to a lesser extent with excess doxorubicin, colchicine or chloroquine. Cell growth assays showed 5-fold and 14-fold increase in the IC_{50} of HeLa-MRP1 to Rhodamine 123 and the Etoposide VP16 relative to HeLa cells, respectively. Analysis of Rhodamine 123 fluorescence in HeLa and HeLa-MRP1 cells with or without ATP suggest that cross-resistance to Rhodamine 123 is in part due to reduced drug accumulation in the cytosol of HeLa-MRP1 cells. Mild digestion of purified IAARh123-photolabeled MRP1 with trypsin showed two large polypeptides (~111 kDa and ~85 kDa) resulting from cleavage in the linker domain (L_1) connecting the multiple spanning domains MSD_0 and MSD_1 to MSD_2. The identity of the 111 kDa and the 85 kDa polypeptides, which encode MSD_0+MSD_1 and MSD_2, was confirmed using two epitope-specific monoclonal antibodies, MRPr1 and MRPm6. Exhaustive proteolysis of purified IAARh123-labeled 85 kDa and 111 kDa polypeptides revealed one (~6 kDa) and two (~6 kDa plus ~4 kDa) photolabeled peptides, respectively. Resolution of total tryptic digest of IAARh123-labeled MRP1 by hplc showed three radiolabeled peaks consistent with the three *Staphylococcus aureus V8* cleaved peptides from the Cleveland maps. Together, the results of this study show direct binding of IAARh123 to MRP1 at physiologically relevant sites. Moreover, IAARh123 photolabels three small peptides which localize to the N- and C-domains of MRP1. Finally, IAARh123 provides a sensitive and specific probe to study MRP1-drug interactions.

INTRODUCTION

Selection of tumor cell lines resistant to natural product toxins has been shown to result in the overexpression of either or both transmembrane proteins, P-glycoprotein (Pgp1) and the Multidrug Resistance Protein (MRP1) (1,2). Gene transfer studies using MRP1 cDNA into drug sensitive tumor cells have been shown to confer resistance to structurally dissimilar anti-cancer drugs and to other natural product toxins (3). The human MRP gene family consists of seven orthologs (MRP1-7; (4)), with MRP1, 2 and 3 shown to confer drug resistance when transfected into drug sensitive cells (5,6). The functions of the other members of the MRP gene family are less defined. MRP1 is a member of a large family of membrane trafficking proteins (7) that couple ATP hydrolysis to the transport of diverse molecules across the cell membrane. Sequence analysis of MRP1 has predicted the presence of three membrane spanning domains (MSD_0, MSD_1 and MSD_2) and two nucleotide-binding domains (NBD_1 and NBD_2). The linear arrangement of MRP begins with a hydrophobic domain (MSD_0) which is connected by a linker domain (L_0) to an MDR1-like core (MSD_1-NBD_1-L_1-MSD_2-NBD_2) (7-10). Unlike MSD_1 and MSD_2, which encode six transmembrane sequences each, MSD_0 encodes five transmembrane sequences with an extracytoplasmic N-terminal (9,10). The role of MSD_0 domain in MRP1 functions is not yet clear, however deletion of the linker domain (L_0) between MSD_0 and MSD_1 inhibits Leukotreine C_4 (LTC_4) transport (11,12).

MRP1 is expressed to varying levels in normal human tissues and is thought to mediate the transport of modified cell metabolites (13). Disruption of the *mrp1* gene in mice caused a significant increase in the accumulation of natural product toxins and glutathione in MRP1-expressing tissues (14-16). Although the normal functions of MRP1 are not entirely clear, Wijnholds et al. (16) have recently shown MRP1 to function in mediating inflammatory responses possibly through its transport of the glutathione conjugated eicosanoid, LTC_4. In addition to LTC_4, other molecules that are conjugated to glutathione, glucuronate and sulfate are also substrates for MRP1 (17-20). Interestingly, certain non-conjugated natural product drugs are co-transported with glutathione (21,22). Furthermore, glutathione transport into MRP1-enriched membrane vesicles was stimulated by vincristine (21,22).

Earlier reports have demonstrated direct interaction between LTC_4 and MRP1 (17). More recently, we have shown direct binding between MRP1 and two structurally different quinoline-

based photoreactive drugs (23,24). In the latter study (24), MRP1 was photoaffinity labeled at multiple sites in the N- and C-terminal domains. The photoaffinity labeling of MRP1 with both photoreactive drugs was inhibited by LTC_4 or MK571 and to a lesser extent by other unmodified natural product drugs (23,24). Thus while the findings of those studies suggest the same or overlapping sites in MRP1 for LTC_4 and unmodified quinoline-based drugs, it is not clear if previously identified photoaffinity labeled sites (24) are specific to quinoline-based drugs or represent sites in MRP1 that interact with structurally diverse drugs. To address this possibility, it was of interest to study the interactions between MRP1 and Rhodamine 123, a xanthone containing drug. Rhodamine 123 (Rh123) is an excellent substrate for Pgp1 and its transport occurs by direct binding to Pgp1 (25-27). Several studies have suggested that Rh123 is a substrate for MRP1 (20,28). In this study, we report on the characterization of MRP1 interactions with a photoactive drug analogue of Rh123 (IAARh123). Our results show that IAARh123 photolabels three tryptic peptides in the N- and C-domains of MRP1. Of interest is the observed cross-resistance of MRP1-transfected HeLa cells (~5-fold) to Rh123 without a large decrease in intracellular accumulation.

MATERIALS AND METHODS

Materials - Iodine-125 (100.7 mCi/ml) was purchased from Amersham Biochemical Inc., (Mississauga, Ontario, Canada). Protein A-couples Sepharose was purchased from Pharmacia Inc. (Montreal, Quebec, Canada). Calcein AM was purchased from Molecular Probes Inc., (Eugene Oregon USA). The LTD_4 receptor antagonist MK 571 was kindly provided by Dr. A.W. Ford-Hutchinson, (Merck-Frost Centre for Therapeutic Research, Quebec, Canada; (29)). Leukotreine C_4 (LTC_4) was purchased from Cayman Chemical Co., (Ann Arbor, MI). All other chemicals were of the highest commercial grade available.

Cell Culture and Plasma Membrane preparations - HeLa and HeLa-MRP1 transfectant cells were grown in α-MEM media containing 10% fetal calf serum (Hyclone) (10). HeLa-MRP1 cells were cultured continuously in the presence of 250 ng/ml Etoposide (VP-16); however, cells used for drug transport studies were grown in drug-free media for several days prior to the date of the experiment. Plasma membranes from HeLa and HeLa-MRP1 cells were prepared as previously described (10). The resultant plasma membranes were further purified by sucrose gradient (10) and washed with 5 mM Tris-HCl, pH 7.4. Plasma membrane enriched fractions were resuspended in the same buffer with 250 mM sucrose. Membranes were stored at -80°C if not immediately used. Protein concentrations were determined by the Lowry method (30).

Synthesis and Radio-iodination of photoreactive Rhodamine 123 - A photoactive analogue of Rh123 (aryl azido-Rh123 or AARh123) was synthesized by reacting Rh123 with NHS-ASA in DMF essentially as previously described (25). Briefly, 5 mg (16.5 μmol) of Rh123 were dissolved in DMF containing triethylamine. The mixture was added to 27 μmol of NHS-ASA in an equal volume of DMF. The reaction was allowed to proceed for 48 hr at room temperature with constant agitation. The solvent was removed by vacuum drying and the oily residue was dissolved in 250 μl of methanol. The photoactive derivative of Rh123, AARh123, was purified by high performance liquid chromatography (hplc) using a Vydac 201HS54 C_{18} reverse phase column (4.6mm x 25 cm) with a gradient of 20-100% acetonitrile in 0.025 M ammonium acetate buffer, pH 5.5. The reaction products were monitored at 505 nm or 290 nm.

The hplc peak corresponding to AARh123 was further purified on the same column in the absence of UV detection to avoid photo-destruction. AARh123 was radio-iodinated as previously described (25) to produce IAARh123 (figure 1).

Photoaffinity labeling and Immunoprecipitation - Total membranes were incubated with 1 μM of IAARh123 in 20 μl of labeling solution (5 mM Tris pH 7.4, 250 mM sucrose) for 30 min in the dark. Membranes were then incubated for an additional 10 min on ice followed by UV irradiation at 254 nm for 10 min while on ice (Stratgene 1800 UV crosslinker, Stratagene, La Jolla, CA). Following photoaffinity labeling, samples were mixed with 80 μl of buffer A (1% SDS, 0.05 M Tris pH 7.4) and 320 μl of buffer B (1.25% Triton X-100, 190 mM NaCl, 0.05 M Tris pH 7.4). Immunoprecipitation was carried as previously described (31) using MRP1-specific monoclonal antibodies (QCRL-1, MRPm6 and MRPr1; (32)). Immunoprecipitated proteins were resolved on SDS-PAGE using the Fairbanks system (33). Gels were dried and exposed to Kodak X-AR film at -80°C. Alternatively, proteins were visualized by silver staining, using the NOVEX SilverXpress™ Silver Staining Kit.

Proteolytic Digestions and HPLC - The mild digestion of IAARh123-photolabeled MRP1 to obtain the 111 kDa and 85 kDa polypeptides was accomplished as follows. IAARh123-photolabeled MRP1 was immunoprecipitated with the QCRL-1 Mab. MRP1/QCRL-1complex bound to Protein A-Sepharose was then digested at 37°C for 5 min with low concentrations of trypsin (8 or 16 ng per sample). Digestion was stopped with the addition of protease inhibitors (10 μg/ml leupeptin, pepstatin A, aprotinin and 1 mM PMSF). The digested products of IAARh123- photolabeled MRP1 were eluted from Protein A-Sepharose at 65°C in SDS-PAGE sample buffer. MRP1 fragments were resolved on Fairbanks gels, transferred to a nitrocellulose membrane and probed with MRPm6 and MRPr1 Mabs. The same membrane was also exposed to x-ray film to determine the relative positions of the IAARh123-photolabeled tryptic fragments. Alternatively, the two resulting polypeptides (the 85 kDa and 111 kDa fragments) were immunoprecipitated separately with MRPr1 and MRPm6 Mabs. Briefly, HeLa-MRP1 membranes were photolabeled with IAARh123 and subjected to mild trypsin digestion (1:125 w/w) for 40 min. The digestion was stopped with a solubilization buffer (1% SDS, 0.05 M Tris pH 7.4) containing an excess of protease inhibitors. The trypsin treated sample was divided in

two halves and mixed separately with either MRPr1 or MRPm6 for immunoprecipitation as previously described (24). Following an overnight immunoprecipitation, MRP halves, bound to protein-A sepharose beads, were washed and incubated with 40 μg of V8 protease in 50 mM Na_2HPO_4, pH 7.4. Digestion was allowed for 16 hrs at 37°C and samples were then resolved on Fairbanks gels. For in-gel maps of MRP1, immuno-purified IAARh123-photolabeled MRP1 were resolved on Fairbanks gels and the resulting bands were exhaustively digested with increasing concentrations of *Staphylococcus aureus* V8 protease (0-20 μg/gel slice) in the wells of a 15% Laemmli gel according to the method of Cleveland *et al.* (34). For a complete tryptic digestion, IAARh123-photolabeled and immuno-purified MRP1 was incubated with trypsin (1:5 w/w) for 14 hours at 37°C. Digested samples were vacuum dried and resuspended with 250 μl of 1% trifluoroacetic acid/water and resolved by reverse phase hplc (Vydac 201HS54 C_4 reverse phase column). The chromatographic procedure consisted of an 80 min gradient of 0-100% acetonitrile with 1% trifluoroacetic acid at a flow rate of 1 ml/min. Fractions were collected and checked for radioactivity.

Accumulation of Calcein AM and Rh123 - HeLa and HeLa-MRP1 cells, seeded in 60 mm dishes (1 $X10^5$ cells/dish), were washed 3x with Dulbecco's phosphate buffered salt solution and incubated (45 min, 37°C) with 10 mM Glucose or 10 mM Deoxy-glucose and 100 nM NaN_3. Rh123 or Calcein AM were added to a final concentration of 1 μM and cells were incubated for an additional 30 min. Cells were washed with ice cold Dulbecco's phosphate buffered salt solution and observed using a Nikon TE200 inverted microscope equipped with a B-2A fluorescence filter.

Modification of Rh123 in cells - To determine if Rh123 is metabolically modified in HeLa or Hela-MRP1 transfectants, cells were incubated with or without 1 μM of Rh123 for 1 hour at 37°C. Cells were washed with ice cold phosphate buffer, harvested and lysed in 100 μL of 50mM Tris-HCl (pH 7.4), containing 2 mM $MgCl_2$ and 1% NP40. The cell extracts were centrifuged at 14000xg for 5 min prior to the addition of 900 μL of cold methanol to the supernatant. The mixture was left overnight at -20°C and then cleared by centrifugation at 14000xg for 30 min. The supernatant was carefully removed and analyzed by hplc using a Vydac 201HS54 C_{18} reverse phase column. The chromatographic separation consisted of an

aqueous phase of 0.025 mM ammonium acetate, pH 5.5 and 0-100% acetonitrile gradient. Elution was monitored at 505 nm.

Cytotoxicity assays - HeLa and Hela-MRP1 transfectants were seeded at 2000 cells per well in 24-well plates. Cells were allowed to grow overnight prior to the addition of drugs at increasing concentrations. Cells were grown for 6-7 days and then stained with 0.1% methylene blue in 50% ethanol as previously described (35). The staining of adherent cells was extracted by incubating stained cells with 250 µL of 0.1% SDS in phosphate-buffered saline solution at 37°C for 1 hour with shaking. The released stain from each well was quantified at 570 nm using a Dynatech MR5000 plate reader.

RESULTS

The multidrug resistance protein (MRP1) has been shown to mediate resistance to natural product toxins and other anti-cancer drugs (36). However, the molecular mechanism by which MRP1 mediates resistance to natural product toxins is not well understood. Of interest is the overlap between MRP1 and Pgp1 substrate specificity, which includes drugs from the *vinca* alkaloids (vincristine), the anthracycline antibiotics (doxorubicin), the etoposides (VP16) and certain dyes (Rh123) (2,37). Recent reports have demonstrated MRP1-mediated accumulation of unmodified *vinca* alkaloids into membrane vesicles in the presence of high levels of GSH (38,39). Based on these results, we speculate that the drug binding site(s) in MRP1 are likely to be similar to those of Pgp1; while the mechanism of drug efflux may be different and could require the catalytic presence and/or the co-transport of other molecules such as GSH. To address the question of whether MRP1 interacts directly with unmodified drugs in a similar fashion to Pgp1, we examined the binding of Rh123 to MRP1 in HeLa-MRP1 transfectant cells. Rh123 is among the best substrates for Pgp1-mediated drug efflux and was shown to interact directly with Pgp1 (25). More recently (20,28), Rh123 was also shown to be a substrate for MRP1, however it is not known if Rh123 interacts directly with MRP1. To demonstrate a direct binding of MRP1 to Rh123, a photoactive radio-iodinated analogue of Rh123 (IAARh123) was synthesized (see figure 1) and used to photolabel MRP1 in membrane-enriched fractions from HeLa and HeLa-MRP1 cells. The results in figure 2a (lanes 3 and 4) show the labeling of

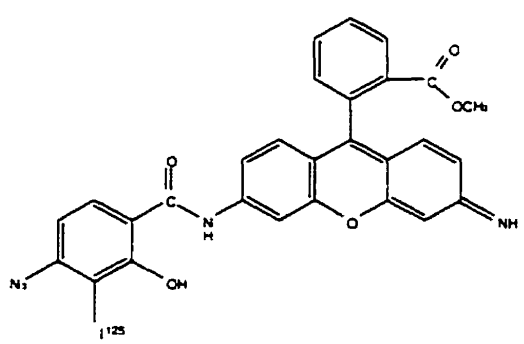

Figure 1. The organic structure of IAARH123, the photoactive analogue of Rh123.

membranes from HeLa and HeLa-MRP1 cells by IAARh123, respectively. A 190 kDa protein in HeLa-MRP1 but not in HeLa cells was photoaffinity labeled by IAARh123. Lanes 1 and 2 of

figure 2a show total membrane proteins resolved on SDS-PAGE and stained by Silver staining, indicating that the 190 kDa photolabeled protein is not the most abundant protein. Moreover, other abundantly expressed proteins (circa 28 kDa to 60 kDa) are barely photoaffinity labeled with IAARh123. Thus the labeling of the 190 kDa by IAARh123 is not due to its high expression and therefore it is selectively labeled. To confirm the identity of the 190 kDa protein as MRP1, HeLa and HeLa-MRP1 membranes were photoaffinity labeled with IAARh123 and then immunoprecipitated with an MRP1-specific monoclonal antibody, QCRL-1 (figure 2b, lanes 3 and 4). Lane 4 shows a single polypeptide with an apparent molecular mass of 190 kDa

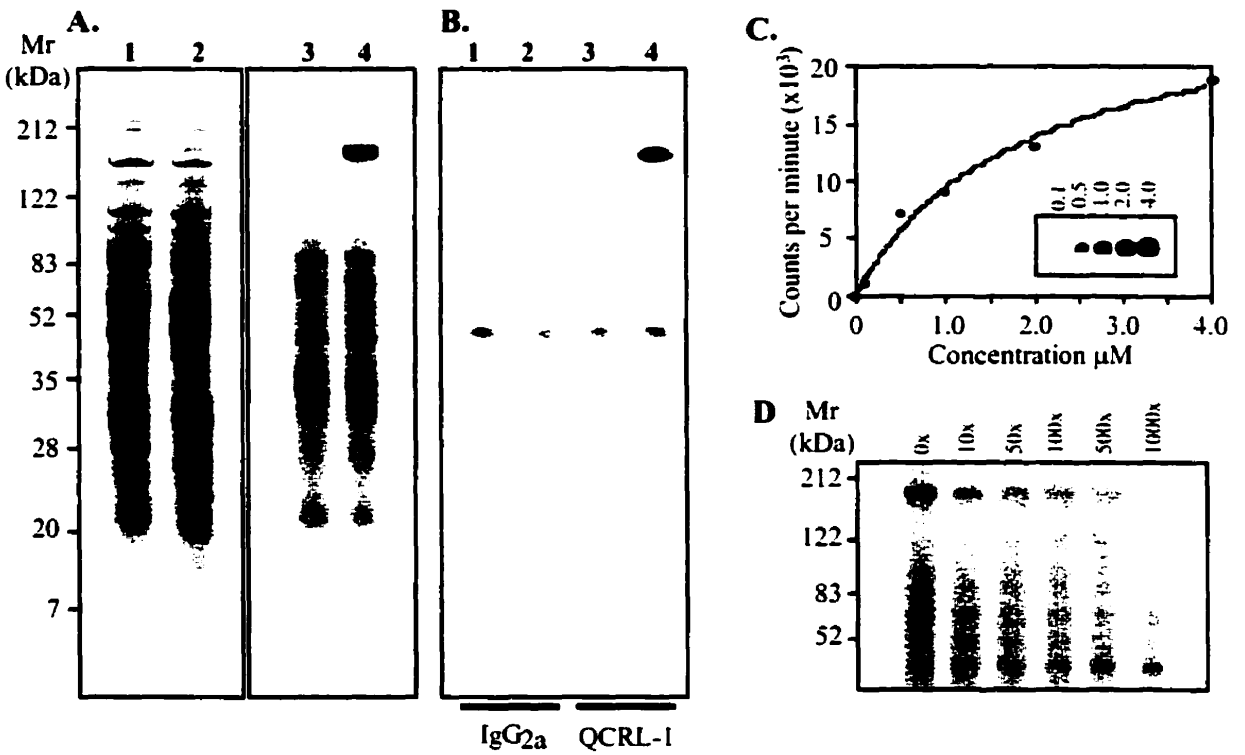

Figure 2. Photoaffinity labeling of MRP by IAARh123. Plasma membranes from drug sensitive (HeLa) and resistant (HeLa-MRP1) cells were photoaffinity labeled with IAARh123 and resolved on SDS-PAGE (lanes 3 and 4, figure 2A). In parallel, lanes 1 and 2 in figure 2A show total membranes from HeLa and HeLa-MRP1 cells stained by Silver stain. Figure 2B shows IAARh123-photoaffinity labeled membranes from HeLa and HeLa-MRP1 cells immunoprecipitated with MRP-specific Mab (QCRL-1) or an irrelevant IgG_{2a}. Figure 2C shows the photoaffinity labeling of HeLa-MRP1 membranes with increasing concentrations of IAARh123 (0.1-4.0 µM). The inset in figure 2C shows the increase in the intensity of a 190 kDa photolabeled protein, which was excised, and the radioactivity quantified. Figure 2D shows the photoaffinity labeled proteins from HeLa and HeLa-MRP1 cells incubated in the absence or presence of molar excess (0.1 - 100 µM) of the uniodinated analogue, AARh123.

that is not immunoprecipitated from HeLa membranes. Similar immunoprecipitations of IAARh123-photolabeled membranes with an irrelevant IgG_{2a} did not precipitate a 190 kDa protein (lanes 1 and 2 of figure 2B). To determine the photolabeling specificity of MRP1 by

IAARh123, membranes from HeLa-MRP1 cells were photolabeled with increasing concentrations of IAARh123 (0.1 μM to 4 μM) or in the presence of increasing molar excess of the uniodinated analogue, AARh123 (figures 2C and 2D, respectively). The results in figure 2C show saturable photolabeling of MRP1 at higher concentrations of IAARh123. Similarly, photolabeling of MRP1-enriched membranes with IAARh123 in the presence of an increasing molar excess of AARh123 showed a dramatic decrease in the photolabeling of MRP1 (figure 2D). Taken together, the results in figure 2 show a direct and specific photoaffinity labeling of MRP1 by IAARh123. Similar labeling results were obtained using membranes from other MRP1 expressing cells, such as H69/AR or HL60/AR (data not shown).

To determine if IAARh123 binds to physiologically relevant site(s) in MRP1, membranes from HeLa-MRP1 cells were photolabeled with IAARh123 in the presence of molar excess of

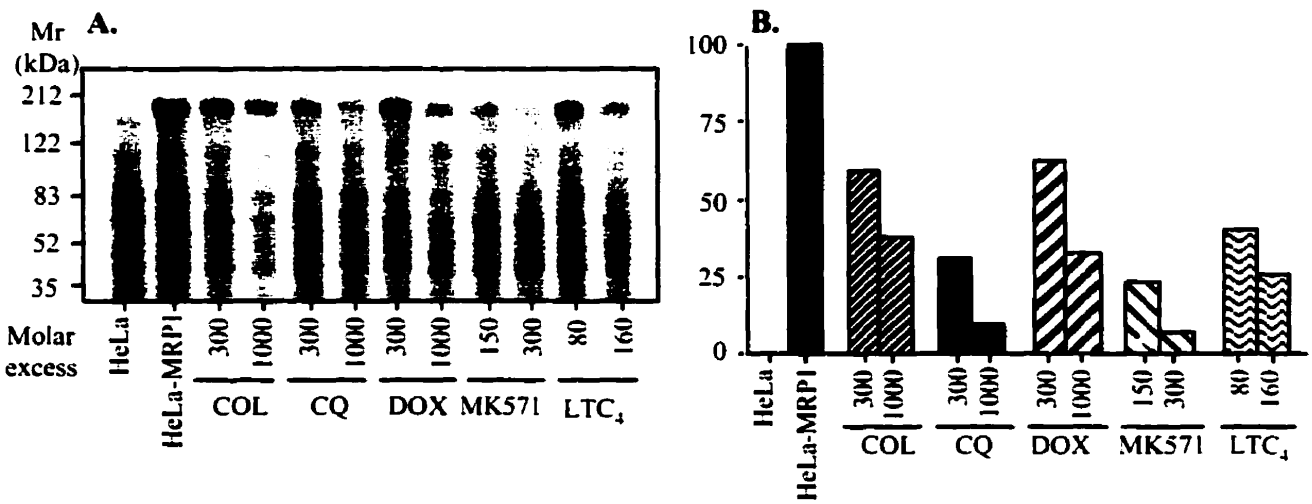

Figure 3. Effects of diverse drugs on the photoaffinity labeling of MRP1 by IAARh123. HeLa or HeLa-MRP1 cells were photoaffinity labeled with IAARh123 in the absence or presence of molar excess (300 -1000-fold) of Colchicine (COL), Chloroquine (CQ), Doxorubicin (DOX), MK571 (150-300) and LTC_4 (80-160). Figure 3B shows a plot of the relative decrease in the photolabeling of MRP with IAARh123 in the presence of the above drugs.

colchicine, chloroquine, doxorubicin, MK571 and LTC_4. The photoaffinity labeling of MRP1 was inhibited at 160-fold molar excess of LTC_4 (Figure 3). Similarly, molar excess (150-fold) of MK 571 caused a significant decrease in the photolabeling of MRP1 by IAARh123. Colchicine, chloroquine and doxorubicin at 1000-fold molar excess were less effective than LTC_4 or MK571 (figure 3). Together, these results confirm the specificity of IAARh123 towards MRP1 and

suggest that IAARh123 binding to MRP1 occurs at the same or overlapping site(s) as MK 571 and LTC_4.

Studies of MRP1 topology using epitope insertion are consistent with the predicted model of MRP1 (figure 4B) which suggests the presence of an extracytoplasmic N-terminal with MSD_0-L_0 plus an MDR1-like core of MSD_1-NBD_1-L_1-MSD_2-NBD_2 (8-10). Moreover, biochemical analysis of MRP1 have demonstrated the presence of two trypsin sensitive sites within the two linker domains (L_0 and L_1; figure 4B) that connect MSD_0 to MSD_1+MSD_2 and MSD_0 +MSD_1 to MSD_2 sequences (24,40). Treatment of MRP1 enriched membranes with

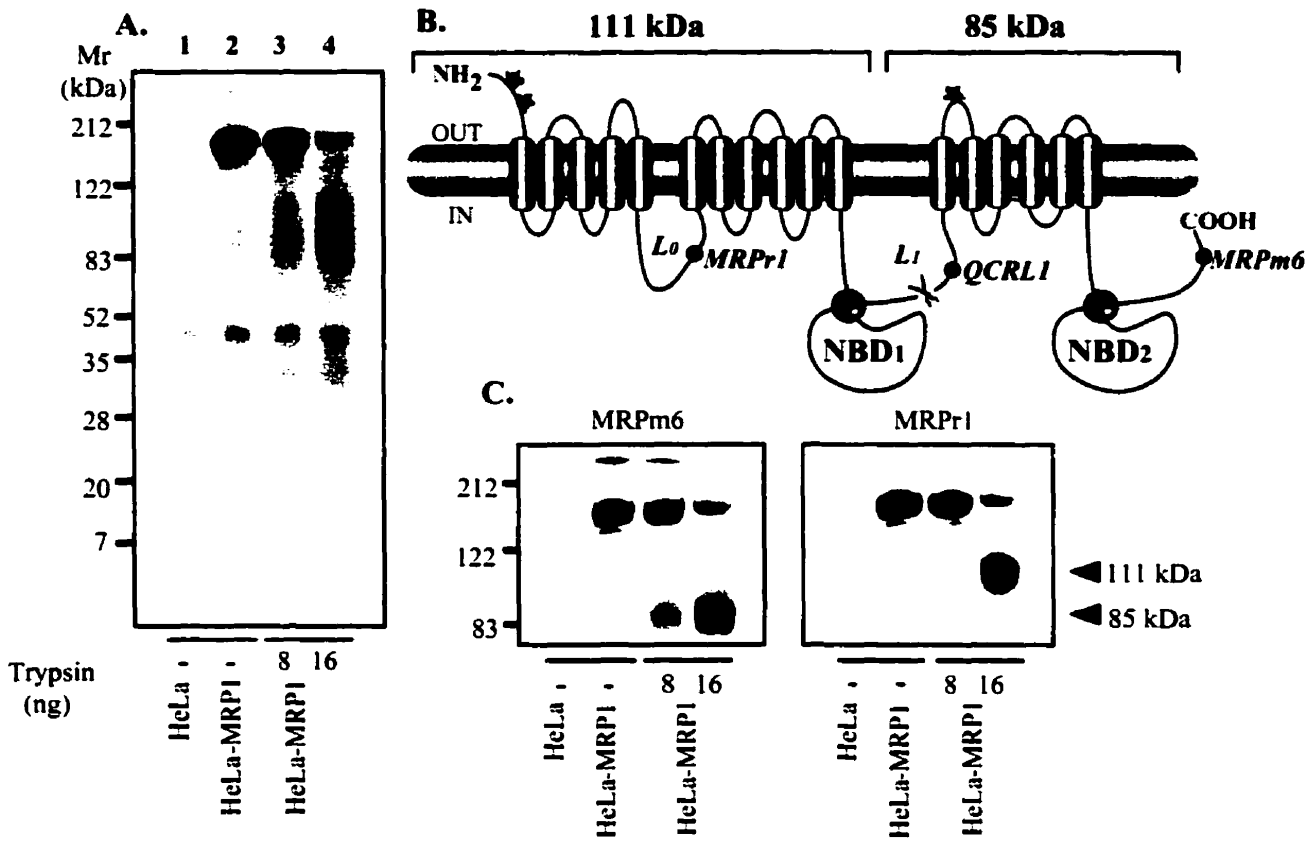

Figure 4. Photoaffinity labeling of two large polypeptides of MRP1 by IAARh123. MRP1 photoaffinity labeled by IAARh123 was purified from HeLa-MRP1 membranes and subjected to mild tryptic digestion. The photolabeled-radiolabeled products were resolved by SDS-PAGE, transferred to nitrocellulose membrane and exposed to x-ray film (figure 4A). Subsequently, the membrane was processed for Western blotting (Figure 4C). Lanes 2-4 of figure 4A show the signal from purified IAARh123-photolabeled MRP1 incubated in the absence and in the presence of 8 and 16 ng of trypsin, respectively. Figure 4C shows the Western blot of the IAARh123-photolabeled MRP1 tryptic digest probed separately with MRPr1 and MRPm6 Mabs. Figure 4B shows a schematic of MRP1 predicted topology indicating the above two Mabs epitopes in MRP1 relative to the protease hypersensitive sites in L_0 and L_1. The nucleotide binding domains (NBD_1 and NBD_2) and the extracellular glycosylation sites (**) are indicated on the schematic of MRP1 secondary structure (Fig. 4B).

limiting amounts of trypsin results in the cleavage at L_1 sequence followed by a second cleavage at L_0 (40). Earlier studies have shown that cleavage at L_1 generates two fragments of 120 kDa and 75-80 kDa polypeptides containing MSD_0-MSD_1-NBD_1 and MSD_2-NBD_2, respectively (8-10,24). Figure 4A shows such a limited trypsin digestion of IAARh123-photolabeled MRP1. Lanes 3 and 4 of figure 4A show two photoaffinity labeled polypeptides (111 kDa and 85 kDa), consistent with the predicted trypsin cut at L_1. To confirm the identity of MRP1 tryptic fragments, tryptic digest of IAARh123-photolabeled MRP1 was analyzed by western blotting with two epitope-specific monoclonal antibodies MRPr1 and MRPm6, which bind to sequences in the 111 kDa and the 85 kDa fragments, respectively. Figure 4C shows Western blots of the same samples as in figure 4A probed with MRPm6 and MRPr1 Mabs, respectively. The results in figure 4C show clearly that MRPm6 recognizes the 85 kDa polypeptide, while MRPr1 recognizes the 111 kDa fragment. Accordingly, the limited trypsin digestion of MRP1 is consistent with earlier proteolysis profiles of MRP1 confirming the identity of the 85 kDa and the 111 kDa polypeptides as MRP1 sequences containing MSD_0-MSD_1-NBD_1 and MSD_2-NBD_2, respectively. In addition, photoaffinity labeling of both polypeptides with IAARh123 suggests the presence of at least one photolabeled site in each fragment of MRP1.

Our earlier report using a quinoline-based photoreactive drug (IACI) demonstrated the photoaffinity labeling of three tryptic peptides in MRP1 (24). To determine the number of IAARh123-photolabeled sites in MRP1, photoaffinity labeled MRP1 was purified with QCRL-1 Mab and subjected to in-gel digestion with increasing concentrations of *Staphylococcus aureus* V8 protease (1-20 μg/gel slice). Figure 5A shows the resultant proteolytic fragments migrating with apparent molecular masses of 6 kDa and 4 kDa. Similarly, purified IAARh123-photolabeled MRP1 was subjected to exhaustive trypsin digestion and the resulting digest was resolved by hplc using reverse phase chromatography. Figure 5B shows the IAARh123-photolabeled tryptic peptides resolved on a C_{18} reverse phase column with 0-100% acetonitrile gradient. The results in figure 5B show three peaks eluting between 30-50% acetonitrile. Hence, by contrast to the exhaustive V8 digestion, the tryptic digestion suggests the presence of three photolabeled peptides. To identify the origin of the 4 kDa and 6 kDa labeled peptides relative to the two large photolabeled domains of MRP1, IAARh123-photolabeled 111 kDa and 85 kDa polypeptides were purified and digested exhaustively with *Staphylococcus aureus* V8 protease. Lanes 1 and 2 of figure 6 show IAARh123-photolabeled 111 kDa and 85 kDa

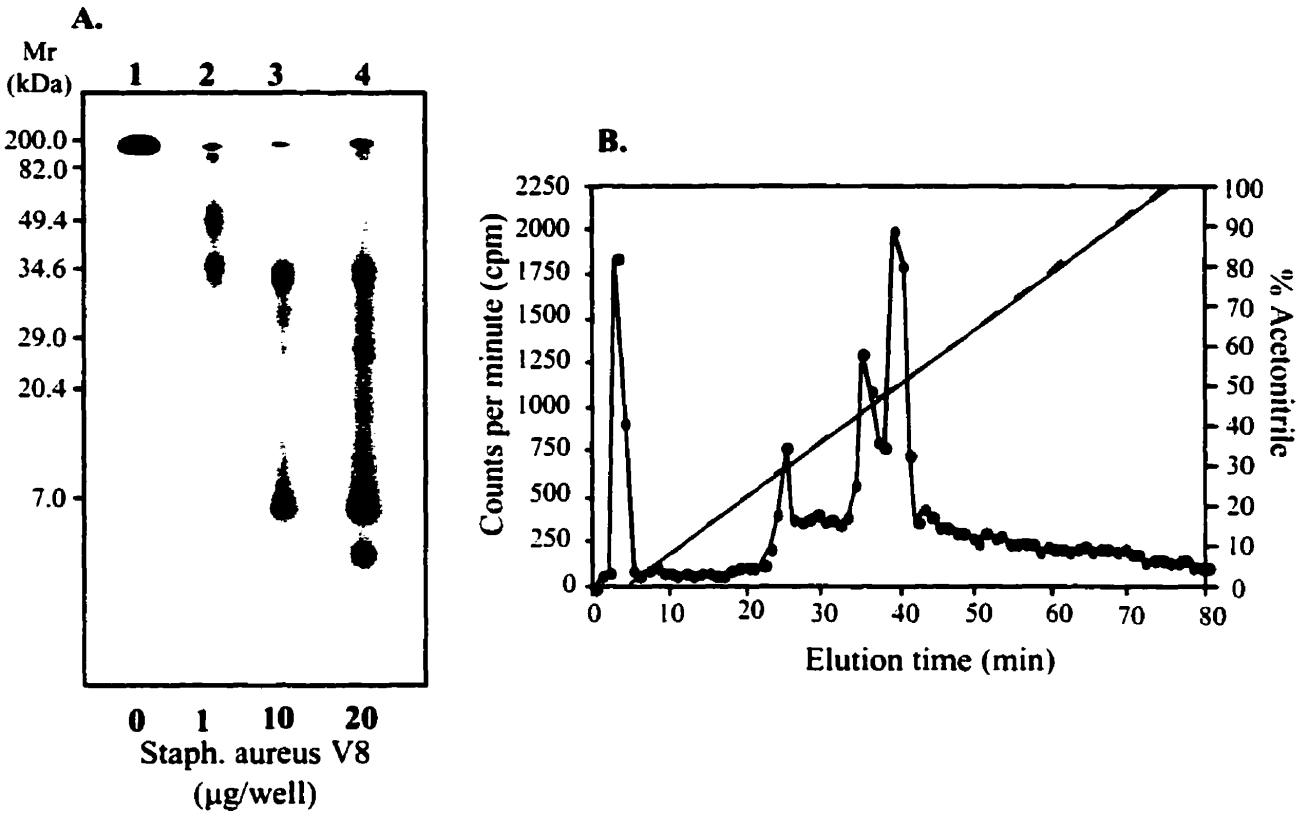

Figure 5. Complete proteolytic digestion of IAARh123-photoaffinity labeled MRP1. Purified MRP photoaffinity labeled with IAARh123 was subjected to complete in-gel or solution digestions. The in-gel digested MRP products were resolved on 15% acrylamide SDS-PAGE, while the solution digested products were resolved by reverse-phase chromatography. Lanes 1-3 of figure 5A show an in-gel digestion of IAARh123-photolabeled MRP with increasing concentrations of *Staphylococcus aureus V8* (1 – 20 μg/well). Figure 5B shows the separation of MRP photoaffinity labeled tryptic peptides on a C_{18} reverse phase column using 0-100% gradient of acetonitrile. The amount of radiolabel in each eluted fraction was plotted versus time. The peak signal at the beginning of the gradient represents the void volume.

polypeptides following immunoprecipitation with MRPr1 and MRPm6 Mabs, respectively. Exhaustive digestion of the 111 kDa polypeptide, which corresponds to MSD_0-MSD_1-NBD_1 of MRP1, resulted in two photolabeled peptides migrating with apparent molecular masses of 4 kDa and 6 kDa (lane 5). Similarly, digestion of the 85 kDa polypeptide, which corresponds to MSD_2-NBD_2 of MRP1, resulted in only one photolabeled peptide of 6 kDa (lane 4). Lane 3 shows the digestion of full length MRP1. Collectively, the results in figure 6 suggest the presence of three IAARh123-labeled peptides in MRP1.

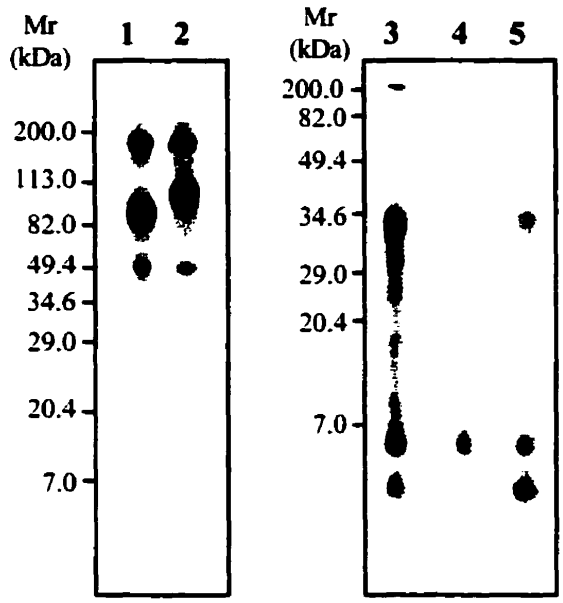

Figure 6. Proteolytic cleavage of immunopurified IAARh123-photolabeled N- and C-halves of MRP. MRP enriched membranes were photolabeled with IAARh123 and subjected to mild tryptic cleavage. Lanes 1 and 2 show the immunoprecipitation of trypsin-digested MRP1 with MRPm6 and MRPr1 Mabs, respectively. Lanes 3, 4, and 5 show complete digestion of immuno-purified IAARh123-photolabeled MRP1, 111 kDa and 85 kDa polypeptides with *Staphylococcus aureus V8*, respectively. The digested fragments were resolved on Fairbanks gel system. The apparent molecular masses of standard proteins are indicated to the left of the gels.

Given the above results, it was of interest to determine if IAARh123 and/or Rh123 are substrates for MRP1 in HeLa-MRP1 cells. Figure 7 shows the growth of HeLa and HeLa-

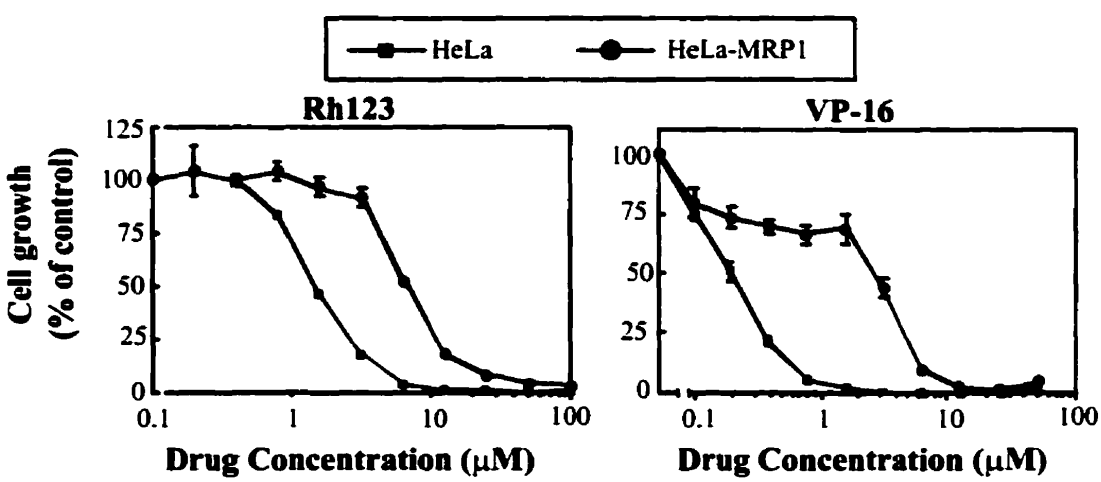

Figure 7. Effects of drugs on the growth of HeLa and HeLa-MRP1 cells. HeLa and HeLa-MRP1cells were grown in the presence of increasing concentrations of Etoposide (VP-16)and Rhodamine 123. Cell growth is expressed as percent of control cells grown in the absence of drug.

MRP1 cells in the presence of increasing concentrations of Rh123 or the etoposide VP16. The results of figure 7 show the IC_{50} of HeLa-MRP1 cells to be 14-fold and 5-fold higher than HeLa cells for VP16 and Rh123, respectively. These results are consistent with earlier observations by Zaman et al, (20) which demonstrated cross-resistance to Rh123 in MRP1 transfected SW1573 cells. However, the latter study did not determine if Rh123 is transported by MRP1 (20). Figure 8 shows the accumulation of Rh123 and Calcein AM in HeLa and HeLa-MRP1 with and without ATP. The results in figure 8 (panels A and B) show a large decrease in Calcein AM accumulation in HeLa-MRP1 versus HeLa cells. Moreover, inhibition of ATP levels resulted in increased Calcein AM fluorescence in HeLa-MRP1 cells to the same level as HeLa cells (panel C versus D). A less dramatic decrease was observed for Rh123 accumulation in HeLa-MRP1 versus HeLa cells (Figure 8, panels E and F). Thus while both cell lines (HeLa-MRP1 and HeLa) showed intracellular Rh123 fluorescence, HeLa-MRP1 cells showed less diffused fluorescence in the cytoplasm as compared to HeLa cells (figure 8, panel E and F). Interestingly, this difference in Rh123 fluorescence between HeLa-MRP1 and HeLa cells ceased when ATP synthesis in both cell lines was inhibited with 100 nM Sodium Azide and 10 mM Deoxy-glucose (figure 8, panel G and H). Although these results are consistent with previous observations suggesting that Rh123 is transported less efficiently by MRP1, it was of interest to know if Rh123 and Calcein AM bind to the same or overlapping site(s). Figure 9 shows the photoaffinity labeling of MRP1 in membranes from HeLa and HeLa-MRP1 in the absence (lanes 1 and 2) and in the presence of increasing concentrations of Calcein AM (lanes 3-6). Calcein AM at 125-fold molar excess inhibited more than 50% of MRP1 photolabeling by [AARh123. Further increase in Calcein AM concentrations resulted in greater inhibition of MRP1 photolabeling (figure 9, lanes 3-6). Consequently, these results demonstrate that HeLa-MRP1 transfectants are able to mediate an energy-dependent transport of Calcein AM, while Rh123 is less efficiently transported. In addition, Rh123 interacts with MRP1 at the same or overlapping site(s) as Calcein AM.

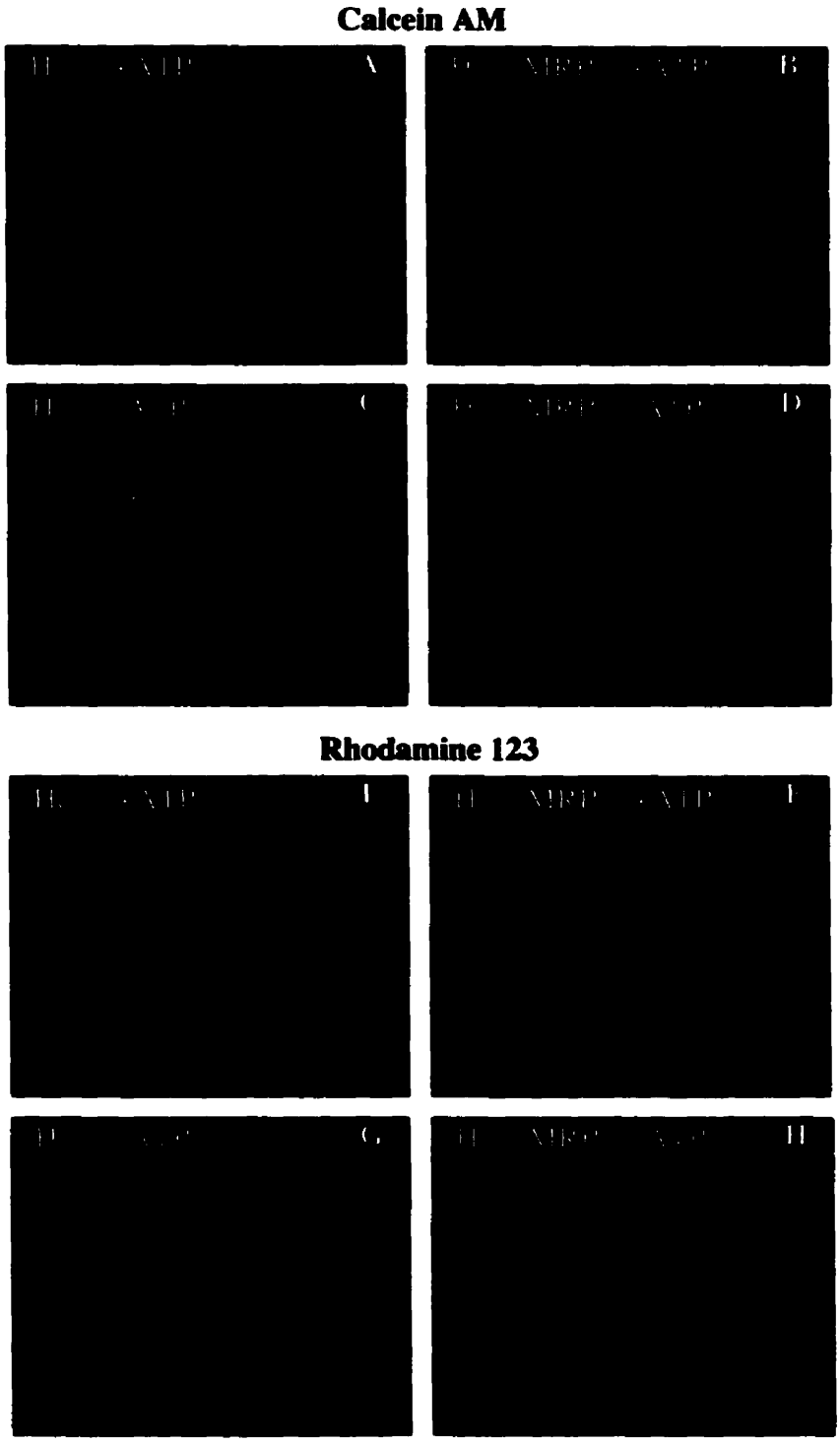

Figure 8. Rhodamine 123 and Calcein AM accumulation in HeLa and HeLa-AM cells. Cells were incubated with Rh123 or Calcein AM at 37°C in the presence of D-glucose or Deoxy-glucose and sodium azide. Panels A and B or E and F show the fluorescence of Calcein AM or Rh123 in HeLa-MRP1 and HeLa cells in the absence or in the presence of ATP synthesis inhibitors (Panels C and D or G and H). Cells were viewed at 400X magnification using Nikon TE200 inverted microscope equipped with a fluorescence filter.

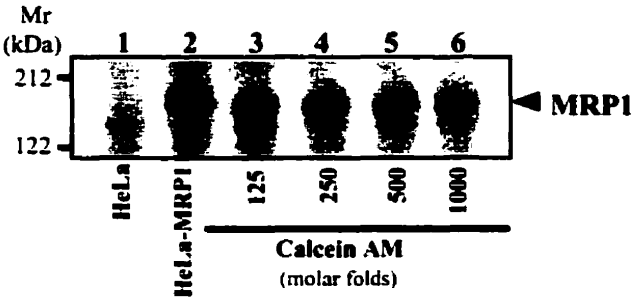

Figure 9. Effects of Calcein AM on MRP1 photoaffinity labeling with IAARh123. Plasma membranes from HeLa and HeLa-MRP1 cells were photolabeled by IAARh123 in the absence (lanes 1 and 2) and in the presence of increasing molar concentrations (125–1000) of Calcein AM (lanes 3-6). The molecular weight markers are indicated to the left of the figure. The migration of MRP1 is indicated to the right of the figure.

The molecular mechanism of MRP1 drug transport is not understood. However, it is presently believed that conjugation of certain compounds to anionic moieties (glutathione, sulfate, and glucuronic acid) may be required for their transport by MRP1. To determine if modification of Rh123 is required for its interaction with MRP1and transport from intact cells, HeLa and HeLa-MRP1 cells were incubated with 1 μM of Rh123 for one hour at 37°C and then

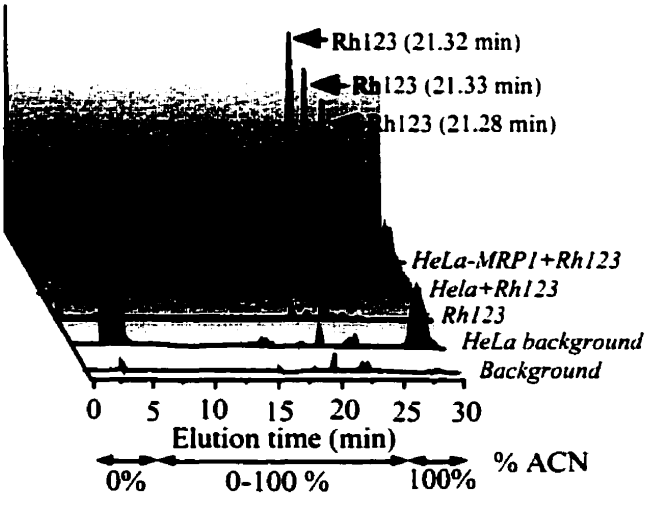

Figure 10. HPLC profile of Rh123 following incubation with HeLa and HeLa-MRP1 cells. Rh123 was extracted from HeLa and HeLa-MRP1 cell lysates (see Methods) following an incubation of cells at 37°C. The mobility of Rh123 extracted from cells was compared to unmodified Rh123. Other controls included extracts from HeLa cells without exogenously added Rh123 and solvent controls. All samples were analyzed by hplc using a Vydac column with C_{18} resin. A gradient of 0 – 100% acetonitrile (ACN) was used to elute Rh123.

the status of Rh123 was compared to unmodified Rh123 by hplc. The chromatograms in figure 10 show the elution time of Rh123 incubated with HeLa or HeLa-MRP1 to be identical to that of control unmodified Rh123. Taken together, these results demonstrate that Rh123 is not modified in HeLa or HeLa-MRP1 cells. Moreover, modification of Rh123 is not required for its binding to and transport by MRP1.

DISCUSSION

We have previously used two photoreactive quinoline derivatives to demonstrate direct and specific binding to MRP1 (23,24). Using these photoreactive drugs we demonstrated that modification with glutathione, glucuronate, or sulfate was not required for binding to MRP1 (23,24). In this report, we have synthesized a photoreactive drug analogue of Rh123 (IAARh123) and demonstrated the photoaffinity labeling of a 190 kDa protein, or MRP1. The identity of the 190 kDa photolabeled protein as MRP1 was confirmed by its reactivity with three MRP1-specific Mabs, (QCRL-1, MRPr1 and MRPm6; (32)). The binding specificity of IAARh123 to physiologically relevant site(s) in MRP1 was confirmed by competitive inhibition studies whereby molar excess of LTC_4 and MK571, known MRP1 substrates, inhibited the photoaffinity labeling of MRP1 by IAARh123. By contrast, higher concentrations of natural product toxins (colchicine, chloroquine and doxorubicin) were required to inhibit MRP1 photolabeling by IAARh123. It is not entirely clear why differences in the capacity of these drugs to inhibit MRP1 photoaffinity labeling exist, especially since both chloroquine and MK571 are quinoline-derivatives. Using two different epitope-specific Mabs (MRPr1 and MRPm6), we demonstrated that mild proteolysis of membranes containing MRP1 produced a 111 kDa and 85 kDa polypeptides corresponding to MSD_0-MSD_1-NBD_1 and MSD_2-NBD_2, respectively. Similar proteolysis of IAARh123-photolabeled MRP1 showed both fragments (111 kDa and 85 kDa) to be photolabeled. However, exhaustive digestion of IAARh123 with trypsin revealed three radiolabeled peptides by hplc. Analysis of the three IAARh123 photolabeled peptides showed the 111 kDa fragment to contain 4 kDa and 6 kDa photolabeled peptides while the 85 kDa fragment contained only the 6 kDa photolabeled peptide. These results are interesting since similar peptides were photoaffinity labeled with IACI, a structurally dissimilar photoreactive quinoline-derived drug (24); suggesting the presence of common drug binding domain(s) that include the three photolabeled peptides of MRP1. It is interesting that photolabeling of Pgp1 with several Pgp1-specific photoreactive drugs produced two photolabeled peptides circa 4 kDa and 7 kDa peptides (25,41,42). Indeed photoaffinity labeling of Pgp1 with IAARh123 led to the photolabeling of the latter two peptides in Pgp1 (25). Further analysis of Pgp1 photolabeled peptides localized the two peptides to transmembrane 5-6 and 11-12 in MSD_1 and MSD_2, respectively (43,44). Later mutational analyses of Pgp1 transmembranes 5-6 and 11-12

confirmed the relevance of these two domains in Pgp1-mediated drug binding and resistance (45,46). Although it is presently not clear what sequences in MRP1 encode for the photolabeled peptides, it is tempting to speculate that each of the photolabeled peptides corresponds to sequences found in one of the three multiple spanning domains (i.e. MSD_0, MSD_1 and MSD_2). In support of the latter speculations, mutations of charged amino acid (Glu^{1208} – Gln and Arg^{1206} – Met) in TM16 of rat MRP2 resulted in the loss of transport activity for glutathione, glucuronate and sulfate conjugates (47). Thus, the 6 kDa IAARh123-photolabeled peptide that is found in the C-terminal half of MRP1 could represent any of the transmembrane sequences in MSD_2. Analysis of MSD_2 sequences for all possible V8 peptides showed a ~6 kDa stretch that includes the sequences of transmembrane 16 and 17. In another study (48), substitution of the carboxyl third of human MRP1 with that of mouse *mrp*1 modulated MRP1 specificity to doxorubicin, whereas the LTC_4 transport was unaffected. Hence, these results support our findings with regards to the notion of more than one drug binding site in MRP1 and that at least one site is localized in the C-terminal portion of MRP1.

The ability of IAARh123 to photolabel MRP1 and Pgp1 specifically and at physiologically relevant sites is interesting since the two proteins share only 15% sequence identity (49). Comparison between Rh123 binding sequences from MRP1 and Pgp1 should increase our understanding of the molecular mechanism of interactions between MRP1 or Pgp1 and drugs. Efforts are ongoing to obtain higher resolution mapping of IAARh123-photolabeled peptides.

Membrane transport systems containing MRP1 have been shown to mediate the accumulation of several normal cell metabolites and other cytostatic compounds that are derivatized with GSH, sulfate or glucuronate (19,38,50). However, it has not been possible to show clear transport of non-derivatized natural product toxins in membranes containing MRP1 in the absence of GSH (19,38,50). Thus, energy dependent transport of vincristine was shown only in the presence of GSH (21). Interestingly, we have previously demonstrated reduced accumulation of unmodified chloroquine and other quinoline-derived drugs in two different MRP1 expressing tumor cells (51). The results in figure 8 of this study show clearly that HeLa-MRP1 cells are capable of energy-dependent accumulation of Calcein AM while Rh123 is less efficiently transported. These findings are surprising since both Calcein AM and Rh123 are structurally similar, sharing the xanthone backbone. Indeed the results in figure 9 show that both

Rh123 and Calcein AM compete for the same or overlapping drug binding site(s). However, unlike Calcein AM, Rh123 carries two cationic charges at physiological pH that may influence its ability to be transported but not its interactions with MRP1. In an earlier study by Twentyman et al (52), MRP1-expressing cells showed 70% clearance of Rh123 following a 2 hr incubation. Alternatively, the inefficient transport of Rh123 by MRP1, in contrast to Pgp1, may be due to the requirement of other rate-limiting ligands. This speculation is consistent with the conclusion from an earlier study (53) whereby depolarization of the membrane potential by genistein accelerated the efflux of Rh123 in MRP expressing cells. Regardless of the mechanism of Rh123 transport, the findings of this study demonstrate clearly that Rh123 interacts directly and specifically with MRP1. Moreover, IAARh123 represents an excellent photoreactive compound that can be used to probe MRP1 and Pgp1 interactions with drugs. In addition, unlike other Pgp1 and MRP1 photoreactive probes, IAARh123 can be iodinated and is fluorescent.

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CONNECTING STATEMENT II

Results from the previous two studies using photosensitive IACI and IAARh123 clearly showed more than one peptide being labeled in MRP1 (results in Chapters 2 and 3). Labeled peptides were localized to both the N- and C-terminals of the protein. More importantly, the same peptides were photoaffinity labeled by two structurally dissimilar compounds, IACI and IAARh123. In addition, photoaffinity labeling of MRP1 with IACI or IAARh123 was inhibited by molar excess of LTC_4 and other MRP1-specific substrates; hence, the photoreactive drugs interact with physiologically relevant site(s) in MRP1. Consequently, it became a priority to achieve a higher resolution mapping of MRP1 sequences that are photoaffinity labeled by IAARh123 and IACI. Knowledge of the amino acids that interact with MRP1 could shed the light on MRP1-drug interactions and its mechanism of drug transport. In Chapter 4 of this Thesis, we describe a higher resolution mapping of MRP1 drug binding sequences.

CHAPTER IV

Major photoaffinity drug binding sites in MRP1 are within TM10-11 and TM16-17

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ABBREVIATIONS

MDR, multidrug resistance; Pgp, P-glycoprotein; MRP, multidrug resistance protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ABC, ATP-binding cassette; Rh123, Rhodamine 123; IAARh123, Iodoaryl azido-Rhodamine 123; Hemagglutinin A, HA; Monoclonal antibody, Mab.

ABSTRACT

MRP1 is an ABC membrane transport protein shown to confer resistance to structurally dissimilar drugs. Studies of MRP1 topology suggested the presence of a hydrophobic N-domain with five potential membrane spanning domains (MSD_0) linked to an MDR1-like core (MSD_1-NBD_1-L_1-MSD_2-NBD_2) by an intracellular linker domain (L_0). MRP1-mediated multidrug resistance is thought to be due to enhanced drug efflux. However, little is know about MRP1-drug interaction and its drug binding site(s). We have previously used two structurally different photoreactive drugs (IACI, Daoud, et al., Biochemistry 2000; and IAARh123 Daoud, et al., Biochemistry 2000 [in press]) to show direct and specific photolabeling of three small peptides in MRP1. In this report, we have used eight MRP1-HA variants that were modified to have hemagglutinin A (HA) epitopes inserted at different sites in the MRP1 sequence. Exhaustive in-gel digestion of all IAARh123-photoaffinity labeled MRP1-HA variants revealed the same profile of photolabeled peptides as seen for wild type MRP1. Photolabeling of the different MRP1-HA variants followed by digestion with increasing concentrations of trypsin or V8 protease (1:800 to 1:5 w/w) and immunoprecipitation with anti-HA Mab, identified two small photolabeled peptides (~ 6-7 kDa) from MRP1-HA(574) and MRP1-HA(1222). Based on the location of the HA epitopes in the latter variants together with molecular masses of the two peptides, the photolabeled amino acid residues were localized to MRP1 sequences encoding transmembranes 10 and 11 of MSD_1 (Ser^{542}-Arg^{593}) and transmembranes 16 and 17 of MSD_2 (Cys^{1205}-Glu^{1253}). Interestingly, the same sequences in MRP1 were also photolabeled with a structurally different photoreactive drug, IACI, confirming the significance of transmembranes 10, 11, 16 and 17 in MRP1 drug binding. Taken together, the results in this study provide the first direct evidence about the domains in MRP1 implicated in drug binding. Furthermore, our findings suggest the presence of a common drug binding site(s) for structurally dissimilar drugs.

INTRODUCTION

Increased expression of P-glycoprotein (Pgp1) or the multidrug resistance protein (MRP1) have been associated with the rise of drug resistance in numerous tumor cell lines, *in vitro* (1,2). Gene transfer studies have also provided direct correlation between Pgp1- or MRP1-expression and resistance to dissimilar anti-cancer drugs, hence the multidrug resistance (MDR) phenotype. In addition, gene disruption studies (3,4) in mice have demonstrated an increased drug accumulation in MRP1 or Pgp1 expressing tissues and organs. Interestingly and despite the low level of sequence identity between MRP1 and Pgp1 (<20%;(5)), the two proteins confer resistance to similar natural product anti-cancer drugs (6). However in contrast to Pgp1, a distinguishing feature of MRP1 endogenous and exogenous substrates is their derivatization by glutathione, glucuronate or sulfate (7-11). Notably, the cysteinyl Leukotriene LTC_4 is a high affinity substrate and an important modulator of inflammatory responses (9). Other endogenous substrates include glucuronate- and sulfate-conjugated bile salts, glutathione-conjugated prostaglandin A_2, as well as oxidized glutathione (GSSG) (8,12,13).

Pgp1 and MRP1 are members of the ABC (or ATP-binding cassette) family of membrane transport proteins (14) that couple the hydrolysis of ATP to drug transport. Members of this family contain one or more blocks of six membrane spanning domains (MSD) linked to a nucleotide binding domain (NBD) (14). In contrast to Pgp1, MRP1 encodes for an additional hydrophobic domain with five potential transmembrane sequences (MSD_0) and an extracytoplasmic N-terminal (15-18). Consequently, the linear organization of MRP1 consists of MSD_0 linked to an MDR1-like core (MSD_1-NBD_1-L_1-MSD_2-NBD_2) through an intracytoplasmic linker domain (L_0). Functional analyses of truncated MRP1 have demonstrated that deletion of the L_0 domain abolished MRP1-mediated LTC_4 transport, whereas deletion of the MSD_0 while maintaining L_0 behaved as the full-length protein (19,20).

The mechanism of MRP1-mediated transport is not well understood, and appears to differ from that of Pgp1. Drug transport studies using Pgp1-expressing plasma membranes have clearly demonstrated an energy-dependent transport through direct binding to Pgp1 (21). Likewise, MRP1-mediated transport of LTC_4 has been shown to be energy-dependent possibly through direct binding to MRP1 (22). However, transport of

unmodified natural product drugs by MRP1 has been shown to occur in association with glutathione (23-26). Using several MRP1-specific photoreactive drugs, we have demonstrated direct binding between MRP1 and unmodified natural product drugs (27-31). Moreover, glutathione was not required for MRP1-drug interaction. In addition, proteolysis of IACI- or IAARh123-photolabeled MRP1 produced three small labeled peptides (27,28). In this report, we have extended the previous findings by mapping the photoaffinity binding sites of the two photoreactive drugs, IACI and IAARh123, in MRP1 sequence. To achieve this, we made use of eight MRP1 variants containing hemagglutinin A (HA) epitope repeats inserted at different positions in MRP1 (17,32). Our results show for the first time the presence of two major drug binding domains, confined to ~50 amino acids each and encoding TMs 10-11 and TMs 16-17.

MATERIALS AND METHODS

Materials - Iodine-125 (100.7 mCi/ml) was purchased from Amersham Biochemical Inc., (Mississauga, Ontario). Protein A-coupled Sepharose was purchased from Pharmacia Inc., (Montreal, Quebec). The monoclonal anti-Hemagglutinin A antibody 16B12 (anti-HA) was from Babco Labs, (Richmond, California). All chemicals were of the highest commercial grade available.

Cell culture and plasma membrane preparations - HeLa cells transfected with vector alone or MRP1-HA cDNA variants (17,32) were used in this study. HeLa-MRP1-HA variants describe cells expressing MRP1 containing one or more copies of hemagglutinin A epitope (YPYDVPDYAS) inserted at amino acids 4, 163, 271, 574, 653, 938, 1001, or 1222 from the N-terminal of MRP1 (see Figure 1). Briefly, cells were grown in alpha MEM medium containing 10% fetal bovine serum (Hyclone). Cells were detached with trypsin-EDTA and washed in phosphate-buffered saline solution, pH 7.4 (PBS). The cell pellets were resuspended in hypotonic Tris-Mg^{++} buffer (1mM $MgCl_2$ and 10 mM Tris-HCl, pH 7.0) containing protease inhibitors (2μg/ml leupeptin, 2μg/ml aprotinin, 1μg/ml pepstatin A) and homogenized in a glass Dounce homogenizer. The resulting cell homogenate was spun for 10 min at 400xg and the resulting supernatant was spun further for 60min at 100,000xg. Plasma membrane enriched pellets were resuspended in 5 mM Tris buffer, pH 7.4 and washed once to remove residual protease inhibitors. The final membrane pellets were resuspended in Tris-sucrose buffer (5 mM Tris, 250 mM sucrose, pH 7.4) using a 27-gauge needle and aliquoted for storage at -70°C. Proteins were quantified using the Lowry assay (33).

Photoaffinity labeling and Protease **digestion** - Plasma membranes from HeLa or HeLa-MRP1-HA variants were photoaffinity labeled with IAARh123 or IACI photoreactive drugs as previously described (27,28). Photolabeled MRP1-HA variants were excised from SDS-PAGE and processed for in-gel digestion with *Staphylococcus aureus V8* protease (20 μg/gel slice) according to the method of Cleveland et al. (34). Alternatively, 100 μg aliquots of photolabeled plasma membranes were digested with

increasing concentrations of trypsin (sequencing grade and TPKC treated, from Roche Diagnostics, Laval, Quebec) at 37°C for 40 min. Following digestion, samples were transferred on ice and the digestion was stopped by the addition of 80 µl buffer A (1% SDS, 0.05 M Tris pH 7.4) containing protease inhibitors (10µg/ml leupeptin, pepstatin A, aprotinin, and 1mM PMSF). Samples were left on ice for 15 min and then diluted with 320 µl of buffer B (1.25% Triton X-100, 190 mM NaCl, 0.05 M Tris pH 7.4). IAARh123- or IACI-photolabeled MRP1-HA was immunoprecipited using anti-HA Mab as previously described (35).

SDS-PAGE and Western blotting - Protein samples were resolved on an SDS-PAGE using the Fairbanks system (36). Radiolabeled proteins were visualized on dried gels following exposure to Kodak X-AR film at -80°C. For immuno-detection of recombinant MRP1-HA variants, 20 µg samples of enriched plasma membranes were resolved on SDS-PAGE and transferred to nitrocellulose membrane by using wet electro-blotting technique essentially as outlined by Towbin et al. (37). Nitrocellulose membranes were blocked in 5% skim milk in PBS and incubated with anti-HA Mab overnight at 4°C. Membranes were washed and incubated with 1:3000 (v/v) of goat anti-mouse antibody conjugated to horseradish peroxidase. Immunoreactive proteins were visualized by chemiluminescence using Pierce SuperSignal Substrate.

RESULTS

Tumor cells over-expressing MRP1 show reduced sensitivity to natural product toxins and certain anti-cancer drugs likely due to enhanced drug clearance (2). However, little is known about MRP1-drug interaction and binding site(s). Using photoreactive drug analogues, we have recently demonstrated the photoaffinity labeling of MRP1 at multiple sites (27,28). In this study, eight different MRP1-HA variants (see Figure 1) were used to identify the sequences of MRP1 drug binding site(s). The expression and characterization of all eight MRP1-HA variants have been previously described (17,32) and their function was determined to be similar to wild type or unmodified MRP1.

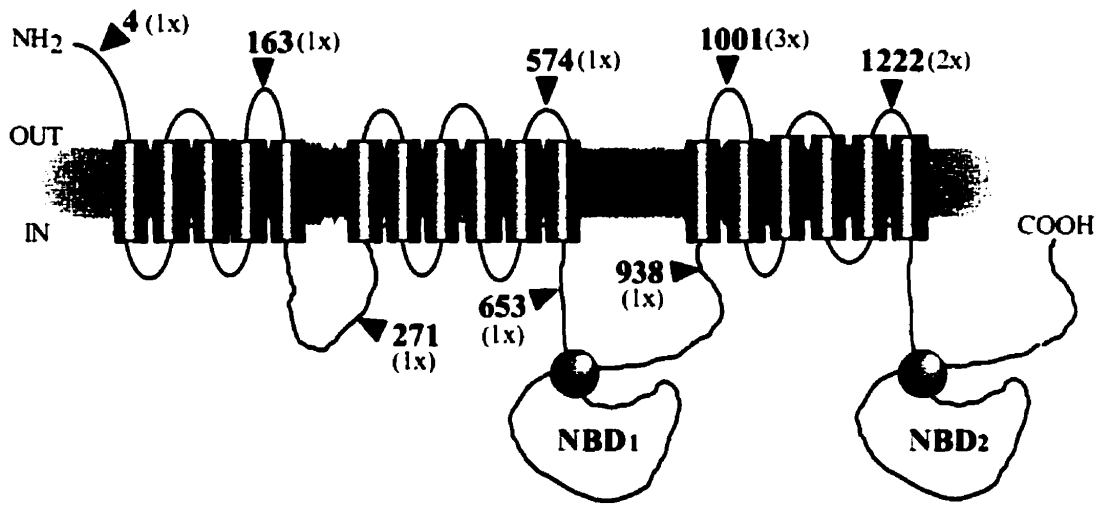

Figure 1. Schematic representation of MRP1 topology. MRP1 is thought to encode for 17 putative transmembrane domains (TM) organized as MSD_0 plus an MDR1-like core (MSD_1+NBD_1+MSD_2+NBD_2). The rectangular bars represent the transmembrane domains of MRP1. The arrows with numbers show the positions of the hemagglutinin A (HA) epitope insertions. The numbers within brackets refer to the number of repeats of the HA epitope. The nucleotide binding domains are indicated as NBD_1 and NBD_2. The extracellular (OUT) and the intracellular (IN) sides of the membrane are also indicated.

Figure 2A shows a Western blot analysis of membranes from untransfected and MRP1-HA-transfected HeLa cells probed with anti-HA Mab. With the exception of the MRP1-HA(574), all other MRP1-HA variants showed high levels of MRP1 (figure 2A). To determine if IAARh123 could photolabel the MRP1-HA variants, plasma membranes were labeled with IAARh123 and proteins were either resolved on SDS-PAGE (figure 2B) or used for immunoprecipitation with anti-HA Mab (figure 2C). Figure 2B shows an IAARh123-photolabeled 190 kDa polypeptide in all eight membrane samples. Moreover,

figure 2C confirms the identity of the labeled protein as MRP1. The lower photoaffinity labeling signal associated with MRP1-HA(574) is consistent with the low level of expression seen in plasma membranes from cells expressing this variant (figure 2A). To determine if the insertion of HA epitopes at different positions in MRP1 has affected the binding domains relative to unmodified MRP1, immunopurified IAARh123-photolabeled MRP1-HA variants were excised from SDS-PAGE gels and digested with *Staphylococcus aureus V8* protease according to the method of Cleveland et al. (34). The results in figure 2D show the same digestion profile for the labeled MRP1-HA variants; hence the insertion of HA epitopes in MRP1 at the specified positions (figure 1) did not affect its drug-interaction. Similar results were also obtained using the quinoline-derived photoreactive drug, IACI (results not shown).

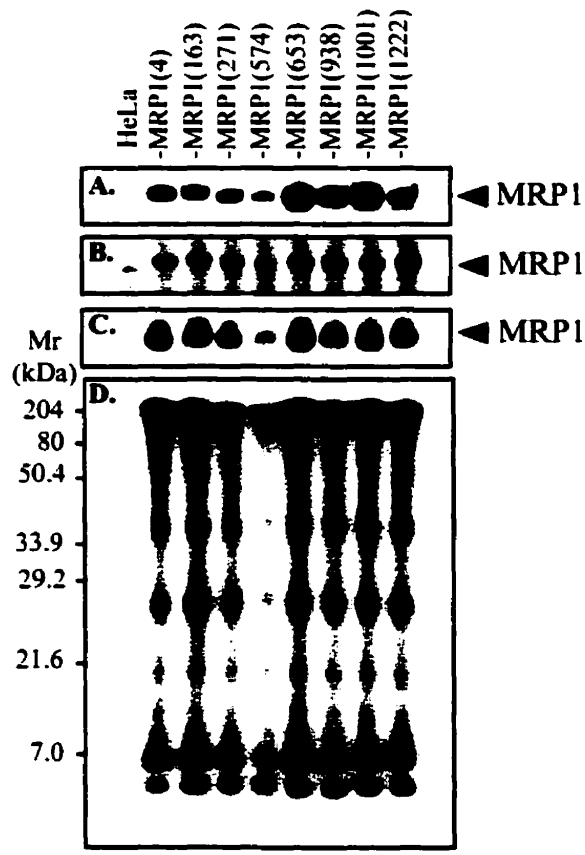

Figure 2. Biochemical characterization of the eight MRP1-HA variants. Plasma membranes from the various MRP1-HA-transfected HeLa cells were resolved on SDS PAGE and probed with anti-HA Mab (Panel A), photoaffinity labeled with IAARh123 (Panel B), immunopurified with anti-HA Mab following photoaffinity labeling by IAARh123 (Panel C) and subjected to in-gel digestion with *Staphylococcus aureus* V8 protease (Panel D). The migration of the molecular weight marker proteins is indicated to the left of the figure.

We have recently shown that MRP1 is photoaffinity labeled at multiple sites with two structurally dissimilar drugs (27,28). Mild proteolytic cleavage of MRP1 with trypsin generated 111 kDa and 85 kDa photoaffinity labeled polypeptides, which respectively produced two and one small photolabeled peptides, upon further digestion (27,28). To achieve a higher resolution map of MRP1 photoaffinity labeled sites, plasma membranes from each of the eight MRP1-HA variants were photoaffinity labeled, digested with increasing concentrations of trypsin (1:800 – 1:5 w/w) and then immunoprecipitated with anti-HA Mab. Only those polypeptides having an HA epitope and a crosslinked IAARh123 were utilized in the analyses of MRP1 binding domains. Figure 3 shows the results of three MRP1-HA variants, in which HA epitopes are inserted at positions 4, 163 and 271 from the N-terminal of MRP1. Resolution of anti-HA

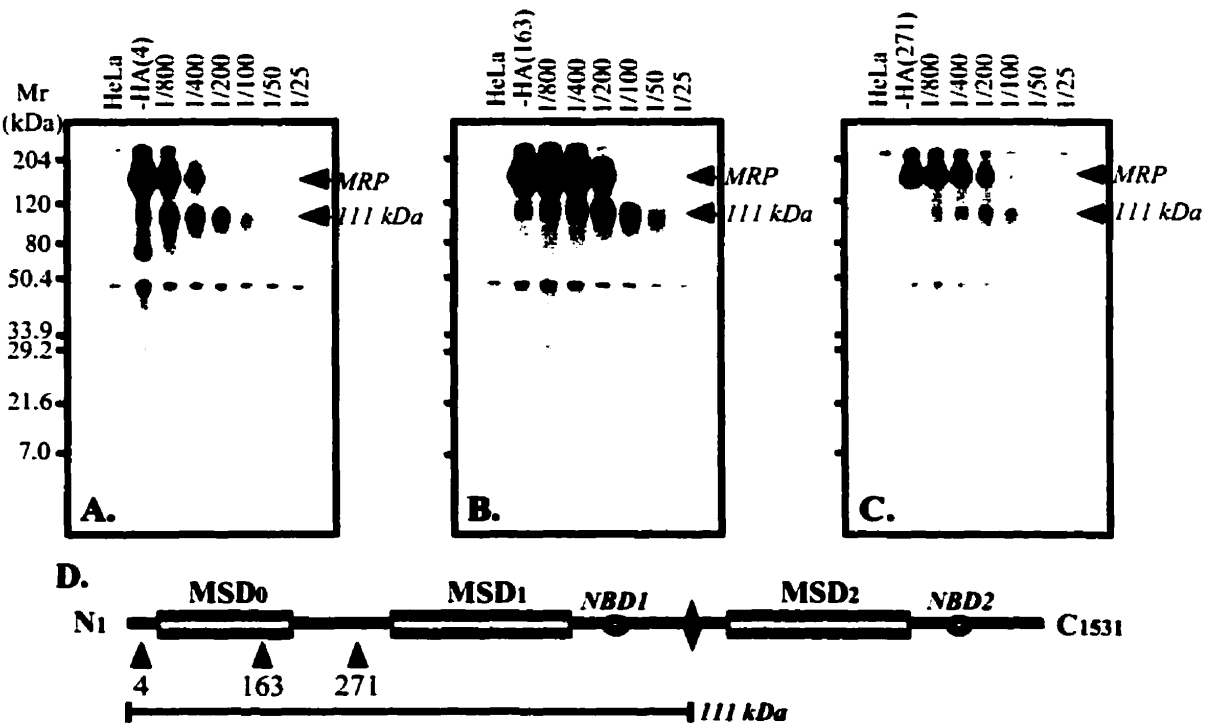

Figure 3. Immunoprecipitation of the tryptic digest of IAARh123-labeled MRP1-HA(4, 163, 271) with anti-HA Mab. Plasma membranes purified from MRP1-HA(4, 163, 271) transfectants were photolabeled with IAARh123 and digested with increasing concentrations of trypsin (1/800-1/25). Figure 3 shows the resulting photolabeled and immunopurified peptides derived from A) MRP1-HA(4), B) MRP1-HA(163), and C) MRP1-HA(271) variants. As the concentration of trypsin was increased, the full-length MRP1 and its resulting 111 kDa fragment disappeared gradually. The bands running at ~50 kDa, appear throughout and are non-specifically immunoprecipitated. The schematic representation shown in figure 3D, indicates the positions of the inserted HA epitopes relative to MRP1, the three multiple spanning domains (rectangles), and the two nucleotide binding domains (circles). The diamond at L1 represents the trypsin sensitive site. The resulting 111 kDa polypeptide is represented under the schematic of MRP1 with a black line.

immunoprecipitated tryptic digest showed one polypeptide with a molecular mass of 111 kDa from MRP1-HA(4), -HA(163) and -HA(271) (figures 3A, 3B, 3C). Hence only the 111 kDa fragment contained the IAARh123-labeled amino acid residue(s) and either one of the three HA epitopes. The schematic in figure 3D shows the three HA epitopes relative to the trypsin sensitive site in the linker domain (L_1). Further digestion of the 111 kDa polypeptide with trypsin led to the loss of the IAARh123-photolabeled residue(s) or the HA-epitopes.

Likewise, analysis of MRP1-HA variants that have HA epitopes inserted at positions 574 or 653 from the N-terminal is shown in figure 4. Proteolytic digestion of IAARh123-photolabeled membranes with increasing concentrations of trypsin generated five and three photoaffinity labeled peptides with MRP1-HA(574) and -HA(653), respectively (figures 4A, 4B). For MRP1-HA(574), five polypeptides with approximate molecular masses of 111 kDa, 44 kDa, 31 kDa, 23 kDa and 6.5 kDa contained both IAARh123 photolabel and the HA epitope. Again, relative to the trypsin sensitive site in L_1 linker domain, the 111 kDa photolabeled polypeptide encodes sequences that include MSD_0, L_0, MSD_1 and NBD_1 as indicated on the schematic in figure 4C. This polypeptide is rapidly degraded to four smaller peptides with apparent molecular masses of 44 kDa, 31 kDa, 23 kDa, and 6.5 kDa, representing sequences between the two trypsin sensitive sites in L_0 and L_1 linker domains (figure 4C). The 44 kDa and 31 kDa IAARh123-photolabeled peptides were immunoprecipitated from the digestion products of both MRP1-HA(574) and -HA(653) variants. Thus, a maximum region of ~280 amino acid residues contains both HA(574) and HA(653) epitopes as well as the IAARh123-labeled site(s). Interestingly, further digestion of MRP1-HA(574) but not MRP1-HA(653), revealed the presence of two smaller photoaffinity labeled peptides (23 kDa and 6.5 kDa; Figure 4A). The immunoprecipitation of a ~6.5 kDa photolabeled peptide from MRP1-HA(574), but not from MRP1-HA(653), positions the peptide between Ser^{542} and Arg^{593}, before the HA epitope at amino acid 653. Consequently, the first IAARh123-photolabeled polypeptide encodes 52 amino acids of MRP1 (Ser^{542}-Arg^{593}) plus 10 amino acid residues as a result of the 1xHA insertion at position 574 of MRP1 (see figure 1).

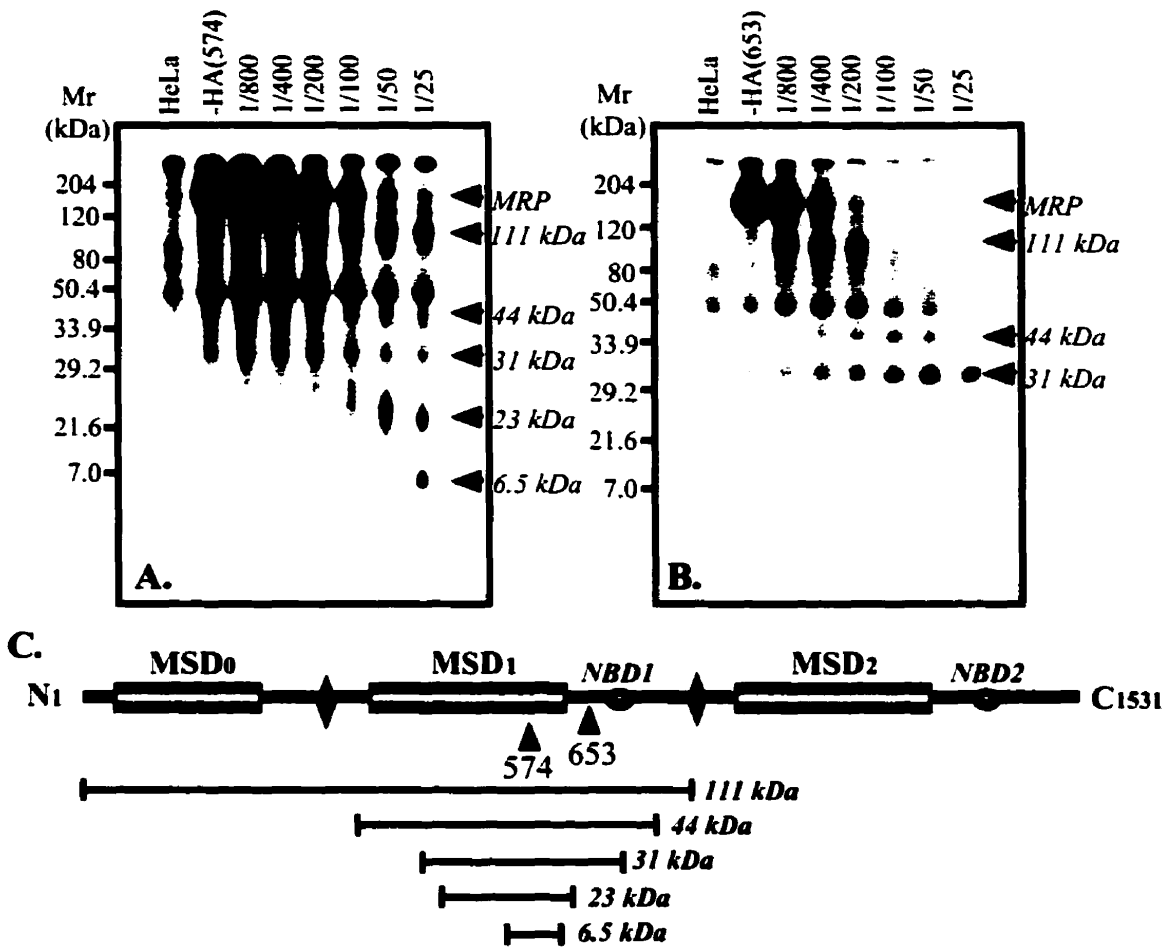

Figure 4. Immunoprecipitation of the tryptic digest of IAARh123-photolabeled MRP1-HA(574, 653) with anti-HA Mab. As in figure 3, IAARh123-photolabeled membranes from MRP1-HA(574) and -HA(653) were digested with trypsin. Figure 4 shows the immunoprecipitation of full-length MRP1 and its resulting 111 kDa fragment which are gradually degraded to the 44 kDa and 31 kDa peptides for MRP1-HA(653) (4B) and to 44 kDa, 31 kDa, 23 kDa, and 6.5 kDa peptides for MRP1-HA(574) (4A). The estimated molecular masses of the resulting IAARh123-photolabeled peptides are indicated to the right of Figure 4A. The relative positions of the HA epitopes and the approximate sizes of the resulting tryptic peptides are indicated in the schematic drawing in figure 4C.

Using three other MRP1-HA variants (938, 1001, and 1222), in which the HA epitopes are localized between the trypsin sensitive site in L1 and the C-terminal of MRP1, plasma membranes were photoaffinity labeled with IAARh123, treated with trypsin and immunoprecipitated with anti-HA Mab. The results in figure 5A, 5B, and 5C show two IAARh123-labeled polypeptides immunoprecipitated from MRP1-HA(938), -HA(1001), and –HA(1222) tryptic digests migrating with apparent molecular masses of approximately 130 kDa and 85 kDa. The 85 kDa IAARh123-photolabeled polypeptide has been previously identified by using epitope-specific Mabs (QRCL-1 and MRPm6

(27,28)) and encodes MRP1 sequences immediately after the trypsin sensitive site in L_1 (see schematic in figure 5D). The 130 kDa polypeptide represents a cleavage of MRP1 at L_0, the second trypsin sensitive site (38), and encodes the MDR1-like core (MSD_1-NBD_1-L_1-MSD_2-NBD_2; see schematic in figure 5D). It should be mentioned that the same 130 kDa IAARh123-photolabeled polypeptide is observed following immunoprecipitation of tryptic digests from MRP1-HA(574) and -HA(653) but not from MRP1-HA(4), -HA(163) and -HA(271) variants (figures 3 and 4). In contrast to MRP1-HA(938) and -HA(1001), immunoprecipitation of MRP1-HA(1222) digest showed four IAARh123-photoaffinity labeled peptides (figure 5); the 130 kDa and 85 kDa

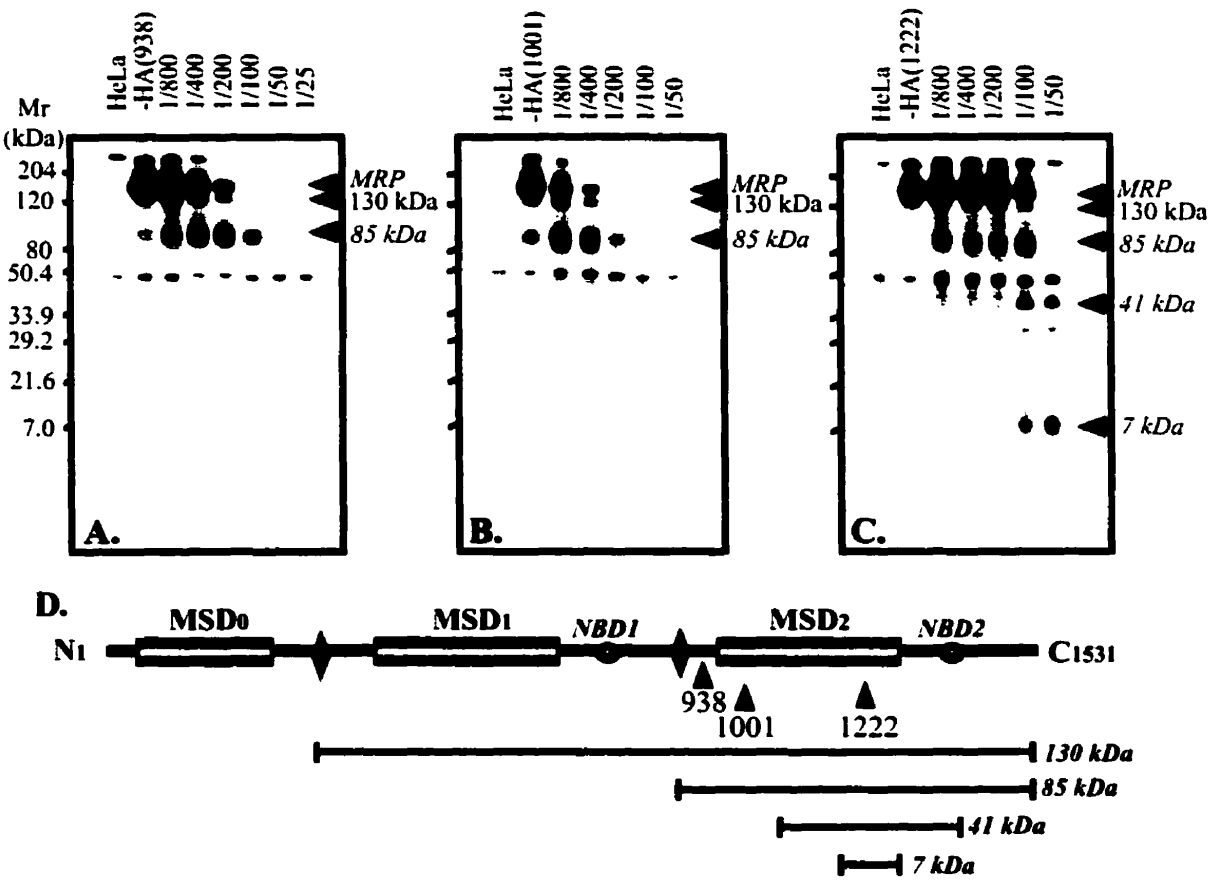

Figure 5. Immunoprecipitation of the tryptic digest of IAARh123-photolabeled MRP1-HA(938, 1001, 1222) with anti-HA Mab. Immunoprecipitation of IAARh123-photolabeled and trypsin digested membranes from cells expressing MRP1-HA(938, 1001, 1222) revealed an 85 kDa photolabeled polypeptide. MRP1-HA(1222) produced two smaller fragments with estimated molecular masses of 41 kDa and 7 kDa, as indicated in Figure 5C. The 130 kDa polypeptide indicated in figures 5A, 5B, and 5C results from the digestion of MRP1 at the trypsin sensitive site in L_0. The latter fragment is clearly seen with MRP1-HA(1001) but less obvious with MRP1-HA(574, 653, 938, 1222) . The schematic in figure 5D indicates the positions of the HA epitopes on MRP1 sequence together with the approximate sizes of the resulting tryptic peptides.

polypeptides plus two additional peptides migrating with molecular masses of 41 kDa and 7 kDa (figure 5C). The 41 kDa photoaffinity labeled polypeptide encodes MRP1 sequences beyond the 1001 epitope insertion site and is equivalent to ~370 amino acids towards the C-terminal end of MRP1 (see schematic representation in figure 5D). The 7 kDa polypeptide represents the smallest photolabeled peptide that can be immunoprecipitated from the tryptic digest of IAARh123-photoaffinity labeled MRP1-HA(1222) (figure 5C). Consequently, the second IAARh123-photolabeled polypeptide contains sequences between Leu^{1203} and Arg^{1249} of MRP1 (plus 20 amino acids residues of the 2xHA insertion at position 1222).

To ascertain the positions of the IAARh123-photolabeled peptides of MRP1-HA(574) and -HA(1222) more closely, photolabeled membrane samples were digested with higher concentrations of trypsin or *Staphylococcus aureus* V8 protease (up to 1:5 w/w) and the total digest was subjected to immunoprecipitation with anti-HA Mab. Figure 6A shows a photolabeled peptide, derived from the exhaustive digestion of MRP1-HA(574) with trypsin, migrating with an apparent molecular mass of a ~6.5 kDa. Careful examination of MRP1 amino acid sequence for the smallest possible tryptic peptide that include the HA epitope at position 574, revealed the following peptide sequence ^{542}SAYLSAVGTFTWVCTPFLVALCTFAVYVTIDEN[HA]NILDAQTAFVSLALF NILR^{593}, where the underlined sequences represent the predicted TM10 and TM11. The calculated molecular mass of the photoaffinity labeled peptide, including the amino acid sequence of the three HA epitopes, is 6.8 kDa. Exhaustive digestion of IAARh123-photolabeled MRP1-HA(574) variants with V8 protease followed by immunoprecipitation with anti-HA Mab did not show any other photolabeled small peptides (data not shown). Similarly, exhaustive digestion of IAARh123-photolabeled membranes of MRP1-HA(1222) with V8 protease revealed the presence of a ~7 kDa photolabeled peptide (figure 6B). Analysis of MRP1 sequence for all possible V8 peptides that include the HA epitope at position 1222, revealed the following amino acid sequence ^{1205}CVGNCIVLFAALFAVISR[HA-HA]HSLSAGLVGLSVSYSLQVTTYLNWLVRMSSE^{1233}, where the underlined sequences represent the predicted TM16 and TM17. The calculated molecular mass of this peptide including the amino acid sequence of the two HA epitopes is ~7.6 kDa.

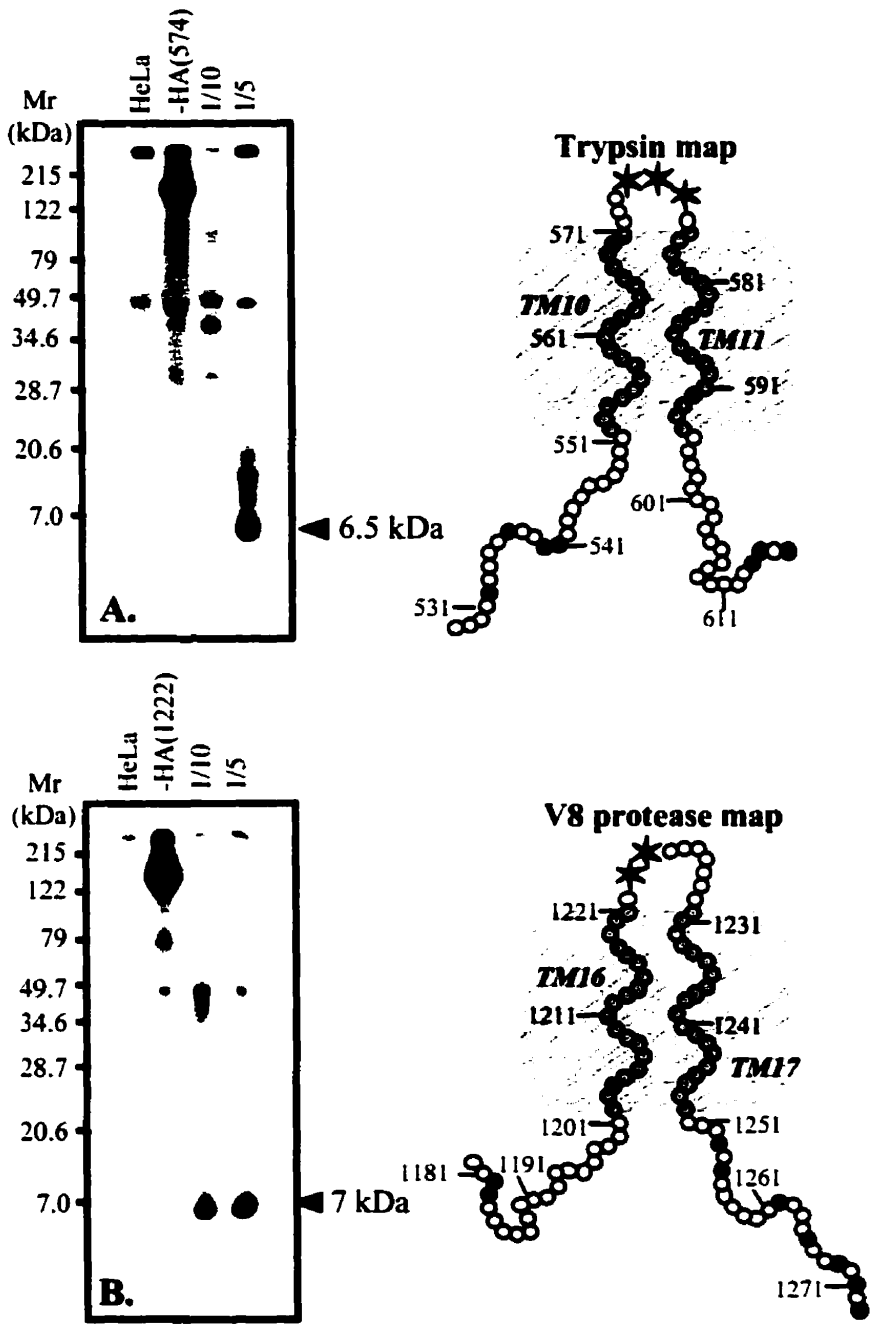

Figure 6. Exhaustive digestion of IAARh123-photoabeled MRP1-HA(574) and MRP1-HA(1222) variants. Figures 6A and 6B show an exhaustive trypsin and V8 protease digestions of IAARh123-photolabeled membranes form MRP1-HA(574) and MRP1-HA(1222), respectively. The smallest immunoprecipited IAARh123-photolabeled peptides, following trypsin digestion of MRP1-HA(574) or V8 protease digestion of MRP1-HA(1222), were 6.5 kDa and 7 kDa, respectively. The schematics to the right of figures 6A and 6B represent the equivalent domains in MRP1. Each circle represents one amino acid residue, with the white circles for those outside the membrane and gray circles for those that form TM domains. Blackened circles are the trypsin sensitive arginines and lysines (6A) or V8 sensitive aspartic and glutamic acids (6B).

As indicated earlier, we have recently demonstrated that both IAARh123 and IACI, two structurally dissimilar photoreactive drugs, labeled similar tryptic peptides which migrate with apparent molecular masses of 4-6 kDa on SDS-PAGE (27,28). Given the localization of IAARh23 drug binding sites in MRP1, it was of interest to determine the IACI-photolabeled sequences using the MRP1-HA variants. Figure 7 shows immuno-purified peptides from IACI-labeled MRP1-HA(574) and -HA(1222), after digestion with trypsin and V8 protease, respectively. Interestingly, figure 7A shows a ~6.5 kDa IACI-photolabeled peptide immunoprecipitated from MRP1-HA(574) tryptic digest. Likewise, MRP1-HA(1222) showed a ~7 kDa IACI-photolabeled peptide (figure 7B). Digestion of IACI-photolabeled plasma membranes, prepared from the other MRP1-HA variants, led to identical photolabeled peptides as IAARh123 (data not shown).

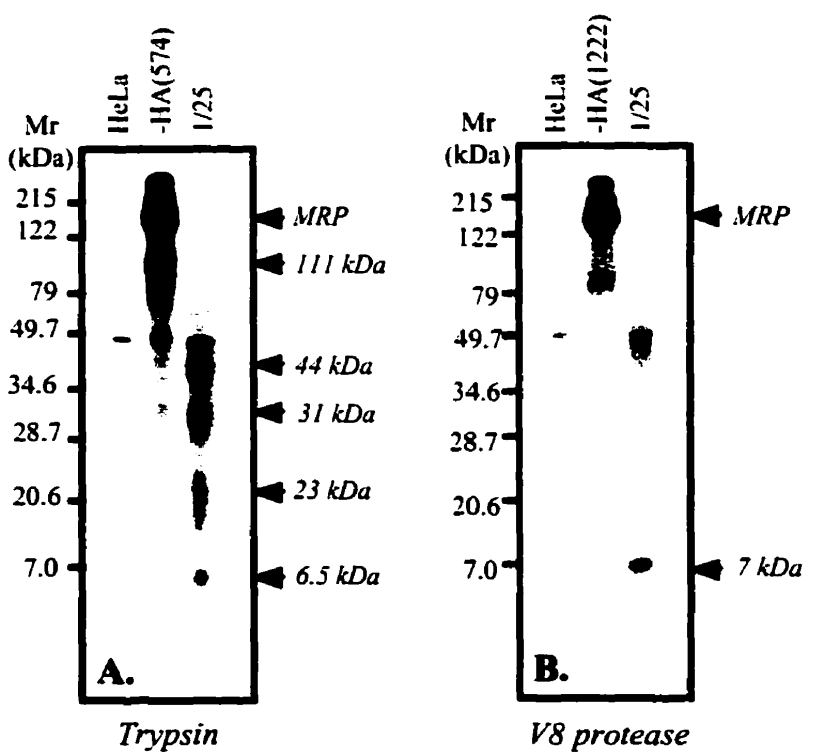

Figure 7. Localization of IACI-photoaffinity labeled peptides in MRP1-HA(574) and MRP1-HA(1222). Plasma membranes from MRP1-HA(574) and -HA(1222) variants were photolabeled with IACI, digested with trypsin (7A) or V8 protease (7B) and immunoprecipitated with anti-HA Mab. Five IACI-photolabeled peptides with estimated molecular masses of 111 kDa, 44 kDa, 31, kDa, 23 kDa, and 6.5 kDa were seen with MRP1-HA(574). Immunoprecipitation of MRP1-HA(1222) V8 protease digest yielded a 7 kDa IACI-photolabeled peptide (7B).

DISCUSSION

Similar to Pgp1, MRP1 is believed to cause resistance to anti-cancer drugs through an enhanced drug clearance mechanism (39). Indeed, there is considerable overlap in substrate specificity to anti-cancer drugs between MRP1 and Pgp1 (39). However, MRP1 also mediates the transport of other normal cell metabolites such as glutathione-, sulfate- and glucuronate-modified ligands. Furthermore, given the structural differences between MRP1 and Pgp1 and the low sequence identity (<20%; (5)), it has been difficult to speculate about the mechanism of MRP1 based on current understanding of Pgp1-mediated drug transport. For example, unlike Pgp1, it has not been possible to demonstrate direct binding between MRP1 and unmodified natural product drugs, likely due to low binding affinities. Moreover, it remains unclear how reduced glutathione mediates MRP1 drug transport. Using a quinoline-based photoreactive drug, we have previously shown direct interaction between MRP1 and natural product drugs (29-31). Using two additional structurally dissimilar photoreactive drugs, we have also shown the photolabeling of three small peptides in MRP1 (27,28). Consistent with our findings, Leier et al. (9) have also shown direct photolabeling of MRP1 by [^{3}H]-LTC_4. Though, it has not been possible to determine which domain(s) in MRP1 interact directly with LTC_4.

Using MRP1-HA variants with HA epitopes inserted at eight positions throughout MRP1 sequence (see figure 1), we identified two short sequences in MSD_1 and MSD_2 that are photolabeled by IAARh123 and IACI. Our approach was based on the ability to immunoprecipitate the smallest photolabeled peptides of MRP1 that contained the HA-epitope. Analyses of the first three MRP1-HA(4, 163 and 271) variants using IACI and IAARh123, revealed the presence of only one photolabeled polypeptide with an apparent molecular mass of 111 kDa. Further digestion of the 111 kDa fragment, which contained three HA epitopes at positions 4, 163 or 271 from the N-terminal did not produce smaller photolabeled peptides. Therefore, MRP1 sequence within the first 274 amino acid residues (relative to the position of the first lysine located right after the HA epitope insertion at position 271) are not photolabeled with IAARh123 or IACI. Alternatively, it is possible that the first 274 amino acid residues of MRP1 are photoaffinity labeled; however following the addition of higher trypsin concentrations the photolabeled site was

separated from the HA-epitope. While this latter possibility is conceivable with one HA-epitope, it is unlikely to occur with all HA epitopes inserted at three different positions in MRP1 (at amino acids 4, 163 and 271 from the N-terminal). Moreover, we and others have previously demonstrated the presence of two trypsin hypersensitive sites, one in each of the linker domains (L_1 and L_0) of MRP1, with cleavage at L_1 proceeding that at L_0 (27,28,38). Consequently, cleavage at L_1 would generate the 111 kDa and 85 kDa polypeptides, followed by a cleavage of the 111 kDa at L_0 producing two polypeptides with apparent molecular masses of 40-60 kDa and 60 kDa (16). The 40-60 kDa fragment would include MSD_0, while the 60 kDa fragment would include MSD_1 (16). The absence of photolabeled 40-60 kDa polypeptides with either one of the three MRP1-HA(4, 163, 271) variants is consistent with the lack of drug interactions within the first 274 N-terminal amino acids. These results are consistent with earlier findings whereby deletion of the first N-terminal 203 amino acids behaved like wild-type MRP1 in vesicle uptake of LTC_4 (19). Having previously demonstrated that IACI and IAARh123 interacts with MRP1 at the same or an overlapping site(s) as that of LTC_4, we speculate that MSD_0 does not interact with either drugs.

Similar analyses of the other five MRP1-HA(574, 653, 938, 1001, 1222) variants revealed two photolabeled peptides with apparent molecular masses of 6.5 kDa and 7 kDa derived from MRP1-HA(574) and -HA(1222), respectively. Proteolytic cleavage at L_0 of MRP1-HA(574, 653, 938, 1001, 1222) variants produced a common polypeptide with a molecular mass of 130 kDa, consisting of MSD_1-NBD_1-L_1-MSD_2-NBD_2 (figure 1). Cleavage at L_1 produced the 111 kDa and 85 kDa fragments form MRP1-HA(574, 653) and MRP1-HA(938, 1001, 1222), respectively. In addition, further proteolytic digestion of MRP1-HA(653) produced two smaller photolabeled peptides (44 kDa and 31 kDa) which encode for MSD_1 and some parts of NBD_1 (figure 4B). In contrast to MRP1-HA(653), similar proteolytic cleavage of MRP1-HA(574) produced two additional photolabeled peptides (23 kDa and 6.5 kDa) which encode TMs 8-11 and TM10-11, respectively. Digestion of MRP1-HA(574) with higher concentrations of trypsin did not result in further cleavage of the 6.5 kDa peptide. Analysis of MRP1 sequence for all potential trypsin cleavage sites near the HA epitope inserted at position 574 of MRP1 revealed the presence of Lys^{541} and Arg^{593}. Thus cleavage at these positions would

produce a 62 amino acid peptide taking into consideration the 10 residues from the HA insertion (see schematic diagram in figure 5C).

Earlier in-gel digestion studies of IACI- or IAARh123-photolabeled 111 kDa fragments, which encodes MSD_0-L_0-MSD_1-NBD_1 and a part of L_1, showed two photolabeled peptides with apparent molecular masses of ~6 kDa and ~4 kDa (27,28). Based on the observed results with MRP1-HA(4, 163, 271, 574, 653), which yielded only one photolabeled peptide containing the HA epitope at position 574, the previously observed photolabeled peptides (4 kDa and 6 kDa) may represent incomplete digestion of the photolabeled fragment (Leu^{536}-Glu^{623}) at internal V8 protease cuts at Asp^{572} or Asp^{578}. Thus, V8 protease cuts at Asp^{572} or Asp^{578} would produce a fragment with estimated molecular mass of ~4 kDa (Leu^{536} to Asp^{572}/Asp^{578} or Asp^{572}/Asp^{578} to Glu^{623}) in addition to a ~6 kDa fragment representing Leu^{536}-Glu^{623}.

Tryptic digestion of MRP1-HA(1222), by contrast to MRP1-HA(938 and 1001), produced two photolabeled peptides (41 kDa and 7 kDa) in addition to the 85 kDa polypeptide (figure 5). The 41 kDa photolabeled peptide did not contain the first 1013 N-terminal amino acid residues of MRP1 (relative to the position of the first lysine located right after the HA epitope insertion at position 1001) and is likely to encode part of the MSD_2 and NBD_2. The second smallest photolabeled peptide produced from the total cleavage of the MRP1-HA(1001) is likely to encode the last two transmembrane domains of MSD_2. Analysis of MRP1 amino acid sequence for potential trypsin cuts near the 1222 HA epitope showed three potential sites, Arg^{1202}, Arg^{1222} and Arg^{1249}. Cleavage at Arg^{1202} and Arg^{1249} would generate a 67 amino acid peptide, including the two 10-residue repeats for the HA epitope, with a calculated molecular mass of 7.6 kDa versus an apparent molecular mass of 7 kDa. Interestingly however, exhaustive trypsin digestion of MRP1-HA (1222) variant did not produce a smaller peptide that could represent cleavage at Arg^{1222}. One possibility is that the Arg^{1222} site was not accessible to trypsin. Alternatively, cleavage at Arg^{1222} did occur but the peptide generated from this cleavage was not photolabeled. Consequently, the photolabel is crosslinked to sequences between Arg^{1202} and Arg^{1222} or within TM16. The localization of the 7 kDa photolabeled peptide in MRP1-HA (1222) was further confirmed following exhaustive proteolytic digestion with V8 protease which produced a 7 kDa peptide. Scanning MRP1 sequences for potential

V8 sites revealed two glutamate residues (Glu^{1204} and Glu^{1253}) near HA(1222). Hence the resulting polypeptide of 69 amino acid residues has a calculated molecular mass of 7.5 kDa versus an apparent mass of 7 kDa on SDS-PAGE. The localization of the 7 kDa IAARh123-photolabeled peptide to MRP1 sequences, which include TMs 16 and 17, is consistent with proteolytic cleavage of MRP1 with two different proteases. The latter findings are in agreement with an earlier study suggesting a possible role of the C-terminal domain (MSD_2 and NBD_2; or residues 959-1531) in determining MRP1 resistance to and transport of anthracylclines (40).

The photolabeling of the same peptides of MRP1 by two structurally dissimilar photoreactive drugs (IAARh123 and IACI) suggests common binding domains. However, given the size of the photolabeled peptides with a combined sequence of 100 amino acid residues, it is conceivable that the photolabeled peptides can encode several binding sites for different groups of compounds. Alternatively, the photolabeled peptides in MRP1 represent domains that are distant from the drug transport site(s). Although the latter possibility is plausible, earlier mapping studies of Pgp1 drug binding site(s) using photoreactive drugs have shown that photoaffinity labeled sequences represent functionally important drug binding sites and mutations of amino acids in such domains modulate Pgp1 drug transport (41-43). However, several questions relating to Pgp1 broad substrate specificity and the ability of Pgp1 to accommodate the binding of drugs remain unanswered. The photoaffinity labeled domains of MRP1, which encode TMs 10-11 and TMs 16-17 are separated by ~400 amino acid residues in its linear sequence (5). As such, photolabeling of MSD_1 and MSD_2 sequences in MRP1 suggest one of two possibilities; a) the two photolabeled sequences from MSD1 and MSD2 are brought together by protein folding to form one large binding cavity that can accommodate interactions with many structurally dissimilar drugs; or b) the two photolabeled peptides represent two separate drug binding sites in MSD_1 and MSD_2 that are allosterically regulated. Although, it is not possible to determine which model best fits the observed results in the current study, earlier reports with Pgp1 suggested two or more spatially separate sites linked allosterically through protein conformation (44,45).

Secondary structure predictions of MRP1 have suggested the presence of a hydrophobic domain with five transmembrane domains (MSD_0) and an extracytoplasmic

N-terminal, linked to an MDR1-like core consisting of two MSDs and two NBDs, in tandem (MSD_1-NBD_1-MSD_2-NBD_2). In contrast to MRP1, secondary structure predictions of Pgp1 have suggested two tandemly repeated MSD and NBD or an MDR1 core. Alignment of the MRP1 and Pgp1 predicted secondary structures revealed TMs 5-6 and TMs 11-12 of Pgp1 to correspond to TMs 10-11 and TMs 16-17 of MRP1, respectively. Thus, the drug binding sites of both Pgp1 and MRP1 are localized to parallel domains in both proteins. This finding is unexpected in light of the low sequence identify between the two proteins (<20%; (5)). Comparison of the amino acid sequences of the four major transmembrane domains in MRP1 and Pgp1 showed 14%, 9%, 4%, and 14% sequence identity between TMs 5, 6, 11, 12 of Pgp1 and TMs 10, 11, 16, 17 of MRP1, respectively. In addition, helical wheel presentation of the transmembrane domains did not reveal any obvious similarities between MRP1 and Pgp1 transmembrane sequences.

The findings that the photolabeled sequences of MRP1 encode transmembrane domains is interesting, since similar results have been obtained with Pgp1, whereby TMs 5-6 and 11-12 were also shown to mediate Pgp1-drug interactions (42-45). However, unlike Pgp1 which mediates the transport of hydrophobic drugs mainly, from within the lipid bilayer, MRP1 appears to interact with and mediate the transport of drugs from the cytoplasm and the hydrophobic environment of the lipid bilayer (46-48). Consequently, Pgp1-drug interaction model may be an oversimplified version of MRP1-drug interactions.

In summary, the results of this study identified two domains in MRP1, to a resolution of ~50 amino acid residues, that are photoaffinity labeled with two structurally dissimilar drugs. Moreover, based on positions of the photolabeled peptides, the results of this study show that the drug binding domains are localized to TMs 10-11 of MSD1 and TMs 16-17 of MSD2. Efforts to identify the precise amino acid residues that are crosslinked to IAARh123 and IACI are ongoing. In addition, it will be of interest to know if the photolabeled sites identified in this study are the same or different from the binding site(s) of normal MRP1 substrates (eg. LTC_4 and other cell metabolites).

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CHAPTER V

Summary and Conclusions

Photoaffinity labeling refers to a technique developed over the past 30 years to probe both structural and functional properties of biological targets, in particular membrane proteins (for reviews, Brunner, 1989, 1993). The widespread use of this technique can easily be traced to two key advantages it offers over more conventional approaches. The first arises from the reasonable chemical stability of photochemical reagents in the absence of light, which permits them to be targeted prior to activation to their specific receptor molecules. The second advantage results from the exceptional reactivity of many photolytically generated intermediates and their ability to attack a broad range of functional groups occurring in biomolecules. By far most of the reagents made to date are based on nitrenes or carbenes as intermediates (Brunner, 1993). The photolabile precursors are azides, diazirines, and diazo compounds. The particular interest in nitrenes and carbenes originates from their ability to attack even aliphatic C-H bonds.

For example, the identification of P-glycoprotein as a specific drug binding protein involved the synthesis of a number of photoactive drug analogues (for review, Safa, 1998). Examples include analogues of anti-cancer drugs (vinblastine, colchicine, taxol, and doxorubicin) and of chemosensitizers (verapamil, forskolin, cyclosporin A, prazosin, and azidopine). The use of these photoactive drug analogues was based on the assumption that a reversible complex occurs between the modified drug and Pgp binding site(s), which specifically recognizes the structural characteristics of the parent drug (Safa, 1998). Ultraviolet irradiation then transforms the photoactive analogue into a reactive nitrene intermediate, which then covalently attaches at or near the drug binding site(s). The use of photoaffinity labeling clearly demonstrated a direct and specific interaction between Pgp and a number of drugs. In addition, high resolution immunologic mapping of photolabeled Pgp sites suggested that homologous transmembrane domains of each cassette of Pgp contain drug-binding sites. Sequences that include TM5-6 in MSD_1 and TM11-12 in MSD_2 were found involved in drug binding. The latter two domains were found photolabeled with the photoactive analogue [^{125}I]-IAAP (Greenberger, 1993). This was supported when other Pgp-specific photoactive drug analogues were used, such as [^{3}H]-azidopine (Bruggemann et al., 1992) and [^{125}I]-forskolin (Morris et al., 1994). Whether these two sites are independent binding sites or

two domains of the same site remains to be determined. It must be appreciated that photoactive probes impose certain limitations on mechanistic conclusions of Pgp. First, the addition of the photoactive moiety changes the structure of the drug itself. Therefore, it was important to show that Pgp transported the modified drug equally well to the parent drug. Second, the reactive domain of the molecule may be a distance from the actual site of drug contact. This issue was resolved by using more than one structurally distinct photoactive drug analogues, to examine whether the labeled site(s) were the same or not.

As for MRP, little is known about the molecular mechanisms governing its drug interaction and mediated transport. Accordingly, defining the arrangement of the drug-binding site(s) of MRP will be invaluable not only to understand how drugs interact with this protein, but also to design more useful and specific inhibitors of MRP function. In a parallel approach to Pgp, adopting photoaffinity labeling as a tool to study the interaction of MRP with cytotoxic drugs and MDR reversing agents is a good starting point. Unfortunately, a number of Pgp-specific photoactive drugs were not successful in labeling MRP. This observation is not surprising if we consider the number of differences between MRP and Pgp. In particular, the necessity of a pre-modification of some transported drugs by small molecules (e.g., glutathione, glucuronate, sulfate) is crucial. Alternatively, the presence of GSH during transport is required, since it facilitates the transport of some drugs through a co-transport mechanism. Furthermore, both MRP and Pgp share a low 15% amino acid identity; not to mention the major topological difference introduced by the additional five transmembrane domains at the N-terminal of MRP. Therefore, it was of particular interest to develop at least one photoactive compound that interacts specifically with MRP. Fortunately, over the past six years research in our laboratory lead to four new photoactive drugs. The first was a quinoline-based photoactive compound (IAAQ), the first photoactive compound known to interact specifically with MRP1 (Vezmar et al., 1997). The second and third, IACI (Daoud et al., 2000) and IAARh123 (Daoud et al., (2000) submitted to JBC), are discussed in chapters 2 and 3 of this thesis. Recently, a fourth photoactive compound, an analogue of glutathione (^{125}I-ASA-GSH), has been synthesized and purified. Its characterization is currently ongoing.

In brief, in chapter 2 we presented a photoreactive quinoline-based drug *N*-(hydrocinchonidin-8′-yl)-4-azido-2-hydroxybenzamide (IACI), which showed a specific labeling of MRP. Molar excess of leukotriene C_4, doxorubicin, colchicine and other quinoline-based drugs, including MK571, inhibited the photoaffinity labeling of MRP. Drug transport studies in H69/AR cells showed lower IACI accumulation, which was reversed by depleting ATP levels. Initial mapping of IACI-photolabeled site(s) in MRP demonstrated the presence of labeled residue(s) on both sides of a trypsin sensitive site in the linker domain L_1. Using low trypsin concentrations, MRP was cleaved into two labeled polypeptides: an ~85 kDa fragment which includes the MSD_2 and the NBD_2; and a ~111 kDa which includes the MSD_0 and MSD_1 plus NBD_1. Cleveland maps of purified IACI-labeled 85 kDa and 111 kDa polypeptides revealed a 6 kDa and 6 kDa plus 4 kDa photolabeled peptides, respectively. In addition, resolution of exhaustively digested IACI-photolabeled MRP by hplc showed two major and one minor radiolabeled peaks.

In chapter 3, we described the synthesis and binding characteristics of the photoactive analogue of Rhodamine 123 (IAARh123). Parallel to IACI, photolabeling of MRP1 with increasing concentrations of IAARh123 approached saturation levels, and was inhibited with excess of the non-radiolabeled analogue (AARh123). Furthermore, the photoaffinity labeling of MRP1 with IAARh123 was greatly reduced in the presence of excess Leukotreine C_4 or MK571. Cell growth assays using Rhodamine 123 showed 5-fold increase in the IC_{50} of MRP1-transfected HeLa cells relative to HeLa cells. Analysis of Rhodamine 123 fluorescence in HeLa and HeLa-MRP1 cells, in the absence or presence of ATP, suggested that cross-resistance to Rhodamine 123 is in part due to reduced drug accumulation in the cytosol of HeLa-MRP1 cells. Interestingly, labeled residues in MRP were again traced to both sides of the trypsin sensitive site in L_1. Exhaustive proteolysis of purified IAARh123-labeled 85 kDa and 111 kDa polypeptides revealed one (6 kDa) and two (6 kDa plus 4 kDa) photolabeled peptides, respectively. Resolution of total tryptic digest of IAARh123-labeled MRP1 by hplc showed three radiolabeled peaks consistent with the three *Staphylococcus aureus V8* cleaved peptides from the Cleveland maps.

Finally, in chapter 4 we described our findings for the high resolution mapping of IACI- and IAARh123-photolabeled sites. Briefly, we have used eight MRP1-HA variants

that were manipulated to have hemagglutinin A (HA) epitopes inserted at different sites in MRP1 sequence. Photolabeling of the different MRP1-HA variants with IAARh123 followed by digestion with increasing concentrations of trypsin and immunoprecipitation with anti-HA Mab, identified two small photolabeled peptides (~ 6-7 kDa) from MRP1-HA(574) and MRP1-HA(1222). Based on the location of the HA epitopes in the latter variants, together with the molecular masses of the two peptides, the photolabeled residues were localized within sequences that include transmembranes 10 and 11 of MSD_1 and transmembranes 16 and 17 of MSD_2. The same sequences in MRP1 were also photolabeled with IACI, confirming the importance of transmembranes 10, 11,16, and 17 in MRP1 drug binding. Taken together, the results in this study provided the first direct evidence for MRP1 drug binding sites.

The quinoline moiety (see appendix 1) at the base of the MRP-specific photoreactive analogues IAAQ (Vezmar et al., 1997) and IACI (chapter 2) is found in many therapeutically important drugs that include several anti-malarial and anti-inflammatory drugs. A well known drug that contains a quinoline moiety is the antimalarial chloroquine. Interestingly, we have previously demonstrated direct binding and transport of chloroquine by MRP (Vezmar and Georges, 1998). The study showed that chloroquine, similar to leukotriene C_4 and MK571, inhibits the photoaffinity labeling of MRP by IAAQ. Moreover, MK571, reversed the resistance of H69/AR cells to chloroquine (Vezmar and Georges, 1998). The specific interaction of quinolines with MRP was further demonstrated when chloroquine, quinine, quinidine and primaquine, four quinoline-based drugs, potentiated the toxicity of doxorubicin in a concentration dependent manner (Vezmar and Georges, 2000). Another compound of high reactivity with MRP1 is the quinoline-based, LTD_4 receptor antagonist, MK571 (Gekeler et al., 1995). Research in our laboratory has supported MK571 specificity to MRP (Vezmar et al., 1997; Vezmar and Georges, 1998; Daoud et al., 2000; Vezmar and Georges, 2000). In fact, competition experiments using IAAQ, IACI and IAARh123 suggested that MK571 is as good a substrate for MRP as LTC_4. In parallel to MRP1, quinoline-based drugs were also shown to reverse Pgp-mediated MDR (Takeshita et al., 1998; Wattel et al., 1998). Therefore, we speculate an important role in binding to MRP1 for the quinoline moiety in

quinoline-containing drugs. In addition, quinoline-based drugs are clinically well tolerated, and their use as MDR reversing agents should be considered.

Given the specificity of IACI to MRP1 and the possibility that MRP1 may encode more than one binding site, it was of interest to map the site(s) of interaction in the protein. As a result, low resolution mapping of IACI-photolabeled domains identified three major sites, one derived from the C-terminal half (MSD_2+NBD_2) and two others from the N-terminal half (MSD_0+MSD_1+NBD_1). While these sites are of interest, it was not clear whether they are specific to quinoline-based drugs only or represent sites in MRP1 that interact with structurally diverse drugs. To address this possibility, we examined the interactions between MRP1 and Rhodamine 123, a xanthone-containing drug (see appendix 1). Rhodamine 123 is an excellent substrate for Pgp1 and its transport occurs by direct binding to Pgp1 (Nare et al., 1994). In addition, several studies have suggested that Rhodamine 123 is a substrate for MRP1, although MRP1 transports Rhodamine 123 less efficiently (Twentyman et al., 1994; Zaman et al., 1994). Indeed, while the results in chapter 3 demonstrated that HeLa-MRP1 cells are capable of energy-dependent accumulation of Calcein AM, Rh123 was less efficiently transported. Even though Calcein AM and Rh123 have the same xanthone backbone, Rh123 carries two cationic charges at physiological pH which may influence its ability to be transported. Alternatively, the inefficient transport of Rh123 by MRP1, in contrast to Pgp1, may be due to the requirement of other rate-limiting ligands. Regardless of the mechanism of Rh123 transport, the findings of this study demonstrate clearly that Rh123 interacts directly and specifically with MRP1. It is interesting that a number of Rh123 family members have shown remarkable potential in binding to MRP. In particular, Rh6G has shown excellent competition with IAARh123 (results not shown). Furthermore, cell growth analysis using Rh6G revealed better transport than Rh123 (results not shown). Further characterization of Rhodamine family members should identify a Rh123 analogue that could bind as efficiently to MRP1 as Rh123, but with a better transport profile. Again, IAARh123-photolabeled sites in MRP1 were located on both sides of the trypsin sensitive site at L_1 and the peptides that resulted from the V8 protease Cleveland maps showed a particular resemblance in number and size to the peptides that were labeled by IACI.

With both IACI and IAARh123 showing very similar binding profiles, while possessing considerable differences in structure, it is tempting to suggest that different MRP substrates share a common binding site(s). In order to map these sites, we made use of the MRP1-HA variants (described in chapter 4) to identify the smallest photolabeled peptides of MRP1 that contained the HA-epitope. As a result, exhaustive digestion of MRP1-HA(574) and -HA(1222) produced two small peptides: a 6.5 kDa derived from MRP-HA(574), and a 7 kDa from MRP-HA(1222). Analysis of MRP sequence in the vicinity of the HA insertions, revealed the presence of TM 10-11 in the 6.5 kDa fragment and TM 16-17 in the 7 kDa fragment. Based on the secondary structure of MRP1, TM 5-6 and TM 11-12 of Pgp1 would correspond to TM 10-11 and TM 16-17 of MRP1, respectively. Therefore, the drug binding sites of both Pgp1 and MRP1 are localized to parallel domains in both proteins. While the gaps of sequence identity and structural differences between MRP and Pgp are large, it is difficult to extrapolate from current knowledge of Pgp on the mode of action of MRP. Therefore, based on the knowledge generated in chapter 4, two scenarios are equally plausible. Either the two domains in MSD_1 and MSD_2 would fold together forming one binding site and accommodate interactions with many structurally dissimilar drugs, or the two photolabeled domains would represent two separate drug binding sites that are allosterically regulated.

The role of GSH in drug binding in comparison to its role in drug transport has been addressed in both chapters 2 and 3. Wijnholds et al. (Wijnholds et al., 1997) have shown MRP1 to function in mediating inflammatory responses possibly through its transport of the glutathione conjugated eicosanoid, LTC_4. In addition to LTC_4, other molecules that are conjugated to glutathione, glucuronate and sulfate are also substrates for MRP1 (Leier et al., 1994b; Zaman et al., 1994; Jedlitschky et al., 1996; Loe et al., 1996b). Interestingly, certain non-conjugated natural product drugs are co-transported with glutathione (Loe et al., 1996b; Loe et al., 1998). Furthermore, glutathione transport into MRP1-enriched membrane vesicles was stimulated by vincristine (Loe et al., 1996c; Loe et al., 1998). While glutathione plays a crucial role in drug transport, it doesn't seem important for drug binding. In fact, IACI and IAARh123 labeled MRP in cells and plasma membranes equally well. The plasma membranes that were used for that purpose were pre-washed several times in 5-10 mM Tris (pH 7.4) before suspension in the assay

buffer. These conditions decreased the levels of GSH to traces levels. In addition, the incubation of IACI and IAARh123 with cells, did not result in a modification to either drugs, as determined by identical retention times during hplc analysis of incubated and pure drugs. Therefore, it is evident that neither a modification by nor the presence of GSH is required for the binding of IACI and IAARh123 to take place. Whether this observation could be extended to other MRP substrates requires further investigation. In an effort to expand our understanding of MRP-drug interactions, we have recently synthesized a photoactive-radioiodinated GSH (^{125}I-ASA-GSH). Mass spectrometry analysis proved that the synthesis product is indeed a GSH analogue, carrying an arylazide group through its sulfhydryl group. Preliminary photolabeling assays showed a 190 kDa protein photolabeled in MRP1-transfected HeLa cells. Immunoprecipitation analysis using MRP1-specific antibodies, determined the identity of the labeled 190 kDa protein as MRP1 (appendix 2). Thus, the availability of a photoactive analogue of glutathione will be of major importance to map its binding site(s) in MRP1, and then compare it to the identified sites of IACI and IAARH123 in MSD_1 and MSD_2.

Consequently, the combined knowledge of the binding sites of natural product toxins and glutathione, will have two major implications. First, it will provide a better understanding of the binding dynamics in MRP and will provide solid basis for answering mechanistic questions. Second, it will bring to light the different domains involved in drug binding, setting by that the stage for a dimensional analysis of the binding site(s), and thus leading to a better design of reversing agents possibly without a major disruption of the physiological role(s) of MRP1.

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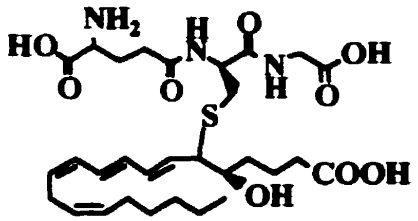
APPENDIX I



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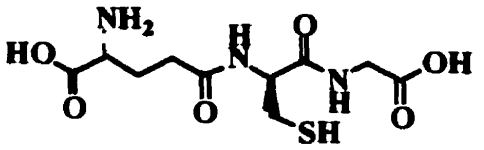

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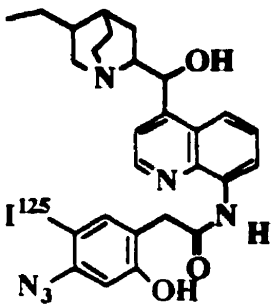

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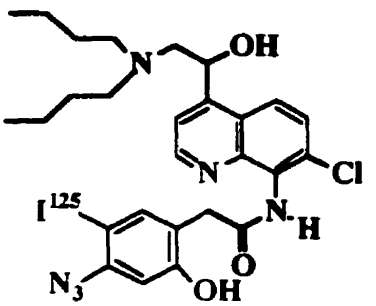

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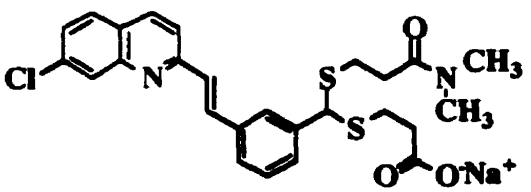

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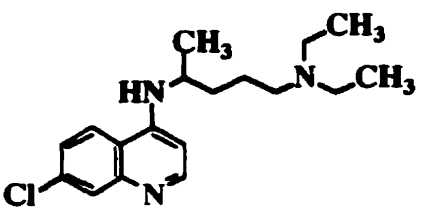

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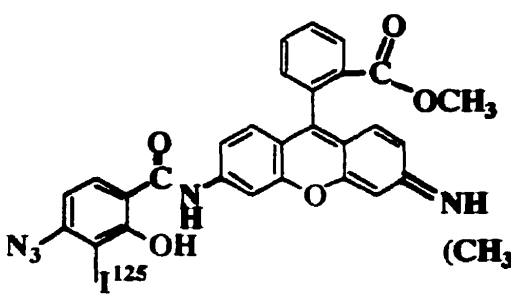

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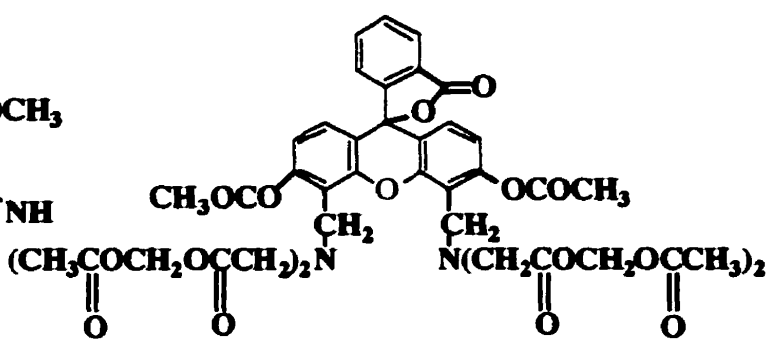
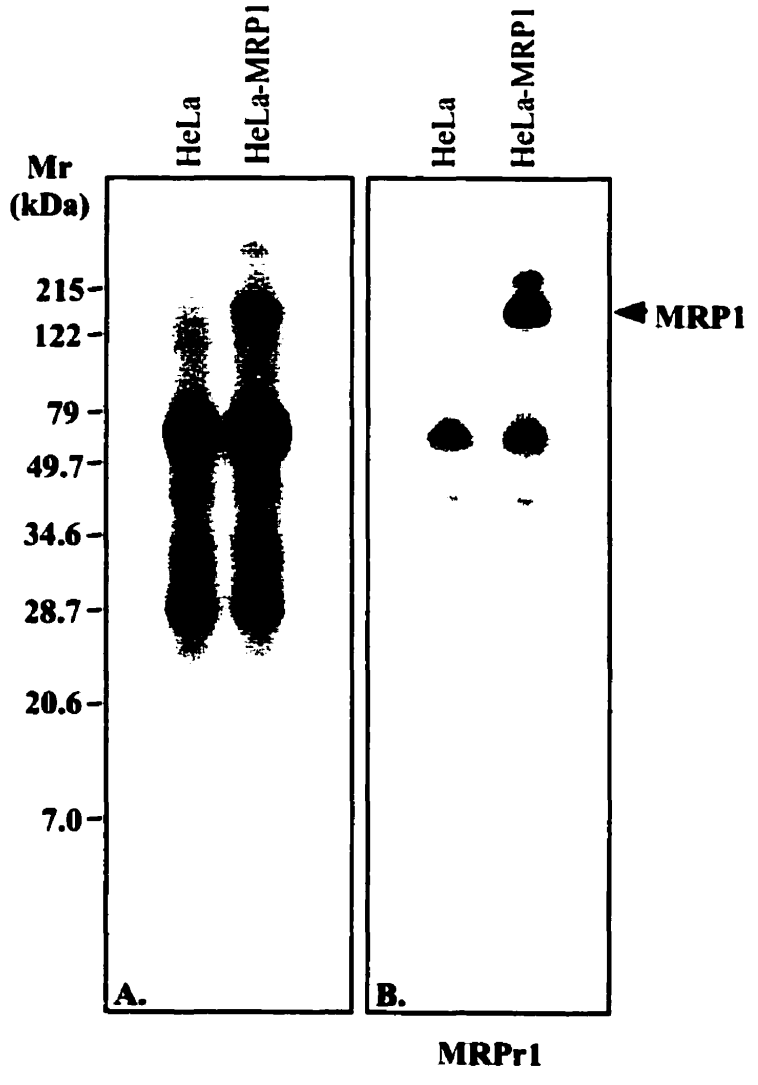


Calcein AM

Glutathione (dark blue); Quinoline (light blue); Xanthone (red); arylazide (green)

APPENDIX II



A. Photoaffinity labeling of HeLa and HeLa-MRP1 plasma membranes with ^{125}I-ASA-GSH.

B. Immunoprecipitation of ^{125}I-ASA-GSH-photolabeled MRP1 using MRPr1 Mab.