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PHARMACOLOGICAL EFFECTS OF QUINOLINE-RELATED COMPOUNDS IN HUMAN TUMOUR CELLS OVEREXPRESSING THE MULTIDRUG RESISTANCE PROTEIN (MRP)

by

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A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

The emergence of multidrug resistant tumours during the course of chemotherapeutic treatment of cancer patients is a major obstacle in cancer chemotherapy. Although several mechanisms may contribute to the appearance of multidrug resistance phenotype (MDR) in tumour cells, reduced drug accumulation and the ability of cells to undergo apoptosis are thought to be very important in expression of MDR. The work in this thesis focuses on the mechanism responsible for the reduced drug accumulation in tumour cells, mainly the multidrug resistance protein (MRP1).

The molecular mechanism underlying the binding and efflux of drugs by the MRP1 is currently not well understood. Several studies have now demonstrated that the cysteinyl leukotriene C_4 (LTC_4) and other glutathione (GSH) S-conjugated anions are substrates for the MRP. To learn more about MRP-drug interactions, we characterized the binding of MRP to a non-glutathione photoactive quinoline compound (abbreviated, ASA-AQ) (Chapter II). Since MRP mediated multi-drug resistance can be modulated by the anionic quinoline LTD_4 cysteinyl leukotriene receptor antagonist (MK571), we speculated that other quinoline-based compounds are likely to interact with MRP. In Chapter III, we show that MDR cells that express MRP1 are more resistant to the antimalarial drug, chloroquine. We also show that chloroquine is a substrate for MRP1 drug efflux.

Taken together, the results of this thesis describe the interactions of MRP1 with a quinoline-based photoactive drug and the antimalarial drug chloroquine.

ABRÉGÉ

L'apparition de tumeurs résistantes à plusieuris composés pharmaceutiques est un obstacle important au traitement des patients atteints de cancer suivant une chimiothérapie. Bien que plusieurs facteurs peuvent contribuer à l'apparition du phénotype de résistance multimédicamenteuse (MDR) dans les cellules cancéreuses, une baisse de l'accumulation des composés chimiques dans les cellules et leur capacité d'induire l'apoptose semblent aussi trés important à l'expression du phénotype MDR. Nos travaux consistaient donc à étudier le mécanisme responable de la diminution de l'afflux de médicaments dans les cellules cancéreuses, plus précisément, la protéine associée à la résistance multimédicamenteuse (MRP).

Le mécanisme moléculaire responsable de la fixation et du flux des substances chimiques au niveau de la protéine MRP est à ce jour trés peu connu. Plusieurs études ont démontré que LTC_4 (leucotriène C_4), ainsi que d'autres anions glutathione (GSH) conjugués au sulfur servent de substrat à MRP. À fin de mieux comprendre l'interaction entre MRP et les substances chimiques, nous avons caractérisé le taux de fixation de MRP à un composé de quinoline photoactif non-couplé à la glutathione (ASA-AQ) (chapitre II). Étant donné que MRP peut être modulée par LTD_4, récepteur antagoniste (MK571), nous présumons que des composés similaires à base de quinoline peuvent agir sur MRP. Au chapitre III, nous démontrons que les cellules à phénotype MDR qui expriment MRP sont plus résistantes à la chloroquine, qui est utilisée contre la malaria. Nous démontrons également que la chloroquine est utilisée comme substrat par MRP au flux des composés chimiques.

Les résultats de ce mémoire décrivent l'interaction de la protéine associée à la résistance multimédicamenteuse avec un composé photoactif à base de quinoline et la chloroquine.

SUGGESTED SHORT TITLE:

Effects of quinoline compounds on multidrug resistance protein.

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ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Elias Georges, who gave me an opportunity to come to McGill University and allowed me to test my knowledge and skills acquired during my undergraduate studies in the United States. His endless ideas, words of wisdom and encouragement, and above all financial support over the past two years are greatly appreciated.

Many thanks to the people in Dr. George's lab who gave me tremendous technical support from the day of my arrival to the lab. Special thanks to Dr. Jean-François Pouliot for teaching me numerous laboratory techniques, Dr. Ying Wang and superb-technician Mrs. Zhi Liu (Margaret) for answering many questions. Thanks for your professionalism and friendly smiles.

Thanks to the professors and students at the Institute of Parasitology who were always around with a friendly smile and words of support when it was mostly needed. Also a big thanks to my friends on the "top" floor of the Institute who made those long hours in the lab more interesting and who had to put up with my loud voice.

A special thanks to Dr. Elodie Ghedin for correcting the chapters II and III, and for all the words of encouragement, unselfish help and friendship. Thanks to Ann Kalita M.Sc., for extraordinary effort and accomplishment to break the speed record while trying to deliver this thesis on time. Of course,

thank you for your friendship Ann. Thanks to Christiane Trudeau for translating the abstract on French, and Shawn Mohammed for proof-reading the chapter I.

Last, but not least, thanks to my family, especially my brother who was always around to listen to my lab stories and who made sure I stayed focused, and to my mother for hundreds of electronic mail letters making sure that I stay in good health. Thanks to my father for all the advices he gave me which meant more than a life of experience. Your everlasting love and support helped me on my path in reaching my goals.

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STATEMENT OF CONTRIBUTION

The experimental work reported herein was done by Marko Vezmar.

This thesis was written by Marko Vezmar.

Dr. E. Georges acted as research/thesis supervisor.

Dr. L. Tilley provided us with essential material for the research as stated in "Acknowledgment" section of chapters II & III of this thesis.

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LIST OF ABBREVIATIONS

ASA-AQ	Aminoquinoline
CQ	Chloroquine
DOX	Doxorubicin
DTT	Dithithreitol
EDTA	Ethylenediamine tetra-acetic acid
GST	Glutathione S-transferase
LTC_4	Leukotriene C_4
MDR	Multidrug resistance
MK571	(L-660,771) Leukotriene D_4 cysteinyl leukotriene receptor (CLT_2) antagonist
MRP	Multidrug-resistance associated protein
MTT	(3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium salt
P-gp	P-glycoprotein
PMSF	Phenylmethyl-sulfonylfluoride
QCRL-1	MRP-specific monoclonal antibody
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SCLC	Small Cell Lung Cancer
VCR	Vincristine
VLB	Vinblastine

GENERAL INTRODUCTION

Resistance to cytotoxic chemotherapy is a common problem in cancer patients and a major obstacle to the successful treatment of many human tumours (reviewed in Lehnert, 1996). To date, multidrug resistance (MDR) is associated with the overexpression of two integral membrane proteins, the 170kDa mdr1/P-glycoprotein (Ueda *et al.*, 1987; Gros *et al.*, 1986; Riordan *et al.*, 1985) and the more recently identified 190kDa multidrug resistance-associated protein (MRP) (Cole *et al.*, 1992). These proteins belong to the ATP-binding cassette (ABC) (Higgins *et al.*, 1992) or traffic ATPase superfamily of proteins (Ames *et al.*, 1992).

MRP is a phosphoglycoprotein (reviewed in Loe *et al*; 1996) shown to mediate the MDR phenotype in some tumour and MRP-transfectant cells by effluxing cytotoxic drugs across the cell membrane in an energy-dependent manner (Cole et al., 1996). However, the molecular mechanism by which MRP mediates the transport of structurally and functionally dissimilar compounds remains unclear. It has been proposed that MRP plays a role in the sequestration of drugs away from their cellular targets (Cole *et al.*, 1992; Slapak *et al.*, 1994). The ability of MRP to transport GSH conjugates (Muller *et al.*, 1994; Leier *et al.*, 1994; Jedlitschky *et al.*, 1994) and oxidised glutathione (GSSG) (Leier *et al.*, 1996) has lead to the possibility that MRP may be a GSH conjugate transporter. MRP has also been described as a multispecific organic anion transporter (MOAT) (Oude Elferink *et al.*, 1994; Mayer *et al.*,

1995), while others suggested that it may also be involved in the regulation of endogenous channels and other transporters (Jirsch *et al.*, 1994). To date, a limited number of compounds have been identified that can be transported by MRP, therefore it is of interest to search for new substrates in order to better understand the mechanism of drug efflux by MRP.

The cross-resistance of MRP expressing adriamycin-selected human leukaemic cells HL60/AR to antimalarial chloroquine has been known for some time (Cole *et al.*, 1989), but no studies have been undertaken to determine the interaction between quinoline-containing drugs and MRP. Furthermore, the leukotriene LTD_4 receptor antagonist MK571, that shows structural similarity to a photoactive analogue of aminoquinoline (ASA-AQ) due to the presence of a quinoline group (Figure 2), has been shown to specifically modulate MRP-associated multidrug resistance (Gekeler *et al.*, 1995). Based on these observations, I have examined the biochemical interaction of ASA-AQ to MRP, with respect to direct binding, energy-dependent transport and the effects of ASA-AQ on the growth of H69 and H69/AR cells (CHAPTER II).

The availability of photoactive radioiodinated drugs that bind specifically to MRP could facilitate future analyses of MRP drug interactions. Due to the structural similarity between the ASA-AQ and quinoline-like compounds and due to direct binding of ASA-AQ to MRP, it was of interest to examine chloroquine effect in tumour cells expressing MRP (CHAPTER

III). The availability of photoactive radioiodinated drugs that bind specifically to MRP could facilitate future analysis of MRP drug interactions. The importance attached to chloroquine as an antimalarial drug and its direct binding to MRP could suggest possible role of MRP in malaria drug resistance to chloroquine.

CHAPTER I

LITERATURE REVIEW

I.1 Introduction

There are approximately 1,000,000 new patients with cancer in the United States and Canada each year. About 50% of the patients with cancer can be cured by surgery and radiation therapy. Of the remaining 50%, about 10% are curable with systemic chemotherapy, including children with leukaemia and sarcomas and adults with testicular cancer. However, the majority of metastatic cancers are not curable by chemotherapy or any other therapies. These fall into two categories: cancers that are intrinsically resistant to chemotherapy (i.e., there is no significant response to chemotherapy); and those cancers which respond initially to chemotherapy but then acquire resistance during the course of therapy. Tumours from patients with intrinsic or acquired drug-resistant phenotypes are commonly resistant to different chemotherapeutic drugs that share no structural or functional properties. Hence, such tumours are said to be multidrug resistant.

Interestingly, a similar phenomenon has also been observed *in vitro* using tumour cells lines that are selected for resistance to a single cytotoxic agent. Upon exposure to a single chemotherapeutic agent, these drug-resistant cells display a broad and unpredictable cross-resistance to unrelated cytotoxic drugs, many of which are used in cancer treatment (Gottesman *et al.*, 1993). The spectrum of drugs encompassed by this form of multidrug resistance most commonly includes several classes of natural products

amongst which are alkaloids or antibiotics, *Vinca* alkaloids (vincristine and vinblastine), the anthracyclines (doxorubicin and daunorubicin), the epipodophyllotoxins (etoposide and teniposide), mitoxiantrone, VP-16, taxol, and topotecin, but not drugs such as bleomycin, methotrexate, cis-platinum, or alkylating agents (Ford *et al.*, 1995). The only commonality that has been observed in this diverse group of anti-cancer drugs is that they are lipophilic, heterocyclic natural products of fungal, bacterial or plant origin (Georges *et al.*, 1990).

Drugs that are associated with multidrug resistance (MDR) have diverse cellular targets: tubulin assembly (colchicine, colcemid and vinca alkaloids), transcription (actinomycin D), translation (puromycin); or they induce DNA damage (anthracyclins, melphalan and emetine) (Biedler *et al.*, 1970; Riordan and Ling, 1985; Gerlach *et al.*, 1986). However, these drugs have different modes of action and cellular targets, and until now no obvious common functional moieties have been identified (Zamora, *et al.* 1988).

Various molecular mechanisms have been associated with MDR in experimental tumour models. These include increased drug detoxification by the glutathione (GSH) system due to overexpression of glutathione S-transferases (Tsuchida *et al.*, 1992); overexpression of the human major vault protein LRP (Scheper *et al.*, 1993), which may play a role in vesicular sequestration of drugs; failure to activate drugs due to reduced levels of cytochrome P_{450}- associated enzyme, including CYP2B6 (Nebert *et al.*, 1991);

inactivation of topoisomerase II activity (Takano *et al.*, 1991); enhanced repair of drug-induced DNA damage; inability of tumour cells to enter apoptosis due to overexpression of *bcl* 2 oncogene (Lowe *et al.*, 1993) or mutation in tumour suppressor gene p53 (Dittmer *et al.*, 1993); and, finally, enhanced efflux of cytotoxic drugs by transporter proteins such as P-glycoprotein (P-gp) or by newly discovered human multidrug-resistance associated protein (MRP) (reviewed by Lehnert *et al.*, 1996).

The recognition that the emergence of a complex drug resistance phenotype of broad specificity in human tumours could limit successful chemotherapy has provided the impetus to study MDR cells as a model for clinical drug resistance. Multidrug-resistant cell lines that have been studied intensively over the last 15 years include colchicine-selected chinese hamster ovary lines (CHO) (Ling *et al.*, 1974), vinblastine-selected human carcinoma KB lines (Akiyama *et al.*, 1985), vincristine-selected Chinese hamster lung DC-3F lines (Meyers *et al.*, 1985), vinblastine-selected human ovarian carcinoma SKOV3 lines (Bradley *et al.*, 1989), a variety of vinblastine-selected leukaemic CEM lines (Beck *et al.*, 1983), and non P-gp MDR cell lines such as doxorubicin-selected human small cell lung cancer (H69/AR) (Cole *et al.*, 1992) and human leukaemic cell lines (HL60/AR) (Krishnamachary *et al.*, 1993). These cell lines express human multidrug resistance associated protein (MRP).

I.2 Characteristics of the multidrug resistance phenotype associated with P-glycoprotein and multidrug resistance-associated protein (MRP)

Ling and co-workers first reported that multidrug resistance in Chinese hamster ovary cells is linked to an increase in the levels of a 170 kDa membrane glycoprotein (Juliano and Ling, 1976). Further, transfection of P-gp cDNA into drug sensitive cells from different sources indicates that the increased expression of P-glycoprotein alone is sufficient to produce a multidrug resistant phenotype (Gros *et al.*, 1986; Ueda *et al.*, 1987). However, there are now many examples of multidrug-resistant cell lines and tumours where P-gp is not involved, but rather newly found MRP. MRP has been indentified in non-P-gp multidrug resistant cell lines from a variety of tumour types, including leukaemias, fibrosarcoma, and non-small cell lung, other small cell lung, breast, cervix, prostate and bladder carcinomas. Small cell lung carcinoma, non-P-gp multidrug resistant cell line (H69/AR), which was derived from the drug sensitive parental cell line H69 by selection in doxorubicin, is nowadays the most widely characterized. Overexpression of MRP in H69/AR due to amplification of its gene causes multidrug resistance (Cole *et al.*, 1992).

Transport studies revealed that MDR cells expressing P-gp are able to maintain a lowered intracellular drug concentration to a large degree via the increased activity of an energy-dependent drug efflux mechanism (Ling *et al.*, 1983) or due to decreased cell permeability (Ling *et al.*, 1974). Drugs

involved in the multidrug resistance spectrum are believed to enter the cell by simple diffusion, since influx is generally nonsaturable. Several groups have shown that multidrug-resistant cells have an enhanced ability to expel or pump drugs out and that this efflux is energy-dependent, since it can be blocked by metabolic inhibitors such as sodium azide and potassium cyanide (Riordan and Ling, 1985).

MRP confers drug resistance pattern similar to that of the P-gp, but with some differences (Cole *et al.*, 1996; McGrath and Center, 1988). For example, recent studies indicate that MRP confers only low levels of resistance to paclitaxel and colchicine (Zaman *et al.*, 1994; Cole *et al.*, 1994), while these compounds have been reported to be among the best substrates for P-gp (Kirschner *et al.*, 1992). Furthermore, another notable difference is the ability of MRP to confer low levels of resistance to arsenic and antimony-centered oxyanions (Cole *et al.*, 1994).

Reduced drug accumulation and increased drug efflux are also characteristics of MRP expressing drug-selected cell lines (Cole *et al.*, 1996; McGrath and Center, 1988), as it has been shown in altered distribution of anthracyclines. Many groups (Breuninger *et al.*, 1995; Cole *et al.*, 1996; Zaman *et al.*, 1994) have shown ATP-dependent reduced drug accumulation and enhanced drug efflux using MRP transfectant cells.

I.3 *MRP* and *P-gp* gene structure and regulation

The gene family that encodes P-glycoprotein has three distinct gene classes (I, II, III) present in rodents and two gene classes (I, II) present in humans (Ng *et al.*, 1989). Only classes I and II have been shown to confer the MDR phenotype when cDNA encoding P-glycoprotein was transfected into drug-sensitive cells (Gros *et al.*, 1988). P-glycoprotein genes map to chromosome 1 in hamsters (Jongsma *et al.*, 1987, and Teeter *et al.*, 1986), to chromosome 5 in mice (Martinsson *et al.*, 1987), and to chromosome 7 in humans (Trent *et al.*, 1987; Fojo and Roninson, 1986). Chromosomal mapping studies of the P-glycoprotein gene in multidrug-resistant cells show that under certain selecting conditions the gene may be translocated to various positions in the genome and undergo extensive amplification. Double minutes (DMs) and homogeneously staining regions (HSRs) are both common karyotypic alterations found in many multidrug-resistant cells (Bradley *et al.*, 1988). Human MDR1 gene consists of more than 100kb of DNA. Human MDR1 downstream promoter contains no TATA element, but does contain a GC-rich region, a CAAT box, a heat-shock consensus element, and an AP-1-like element (Gottesman *et al*, 1993).

The gene family that encodes MRP has two distinct gene classes. The human *MRP1* gene has been discovered in human small cell lung cancer (SCLC), where it has been mapped to chromosome 16 at band p13.13-13.12 (Kuss *et al.*, 1994; Slovak *et al.*, 1993) and it is commonly referred to as MRP.

On the other hand the rat hepatocanalicular isoform (*MRP2*) has been cloned and transiently expressed in COS-7 cells (Taniguchi *et al.*, 1996) and it shows homology to canalicular multispecific organic anion transporter (cMOAT). The expression of MRP mRNA (size 7.8-8.2 kb) was increased 100- to -200 fold in H69/AR (human SCLC selected with doxorubicin) compared to the parental H69 cells (Cole *et al.*, 1992). The *MRP* gene is also amplified in double minutes (DMs) chromosomes (Slovak *et al.*, 1993) that range from 2 to 100 per cell. In H69/AR cell lines, multiple copies of the *MRP* gene are associated with large hsrs (homogenously staining regions) on chromosomes other than 16, while in multidrug resistant fibrosarcoma cell lines, *MRP* is amplified in its normal location (Slovak *et al.*, 1993). Therefore it can be concluded that it is possible that chromosome-specific repetitive sequences near the *MRP* gene may contribute to the frequency with which *MRP* is amplified.

At present very little is known about the molecular processes regulating transcription of the *MRP* gene. The *MRP* promoter is similar to promoters of many so-called housekeeping genes, lacking both TATA and CAAT motifs (Kurz *et al.*, 1995; Zhu *et al.*, 1995), but it is extremely GC-rich. Analysis of the 5′ flanking region of the gene reveals potential binding sites for the Sp1 (*trans* -acting factor) which has led to postulation that the sequences can be sites of modulation of gene activity in response to a variety of stimuli. The region encoding the 5′ untranslated leader sequence of MRP mRNA is also

GC rich and contains GCC triplet repeats (Kurz *et al.*, 1995; Zhu *et al.*, 1995). These repeats are polymorphic as it is manifested in MRP repeats.

Currently, it is not known if there are any human diseases related to the MRP defect, however some of the characteristics described above have been implicated in the formation of fragile sites at several chromosomal loci and in the aetiology of Huntington's disease (Monckton *et al.*, 1995).

I.4 Structure of P-gp and MRP and posttranslational modification

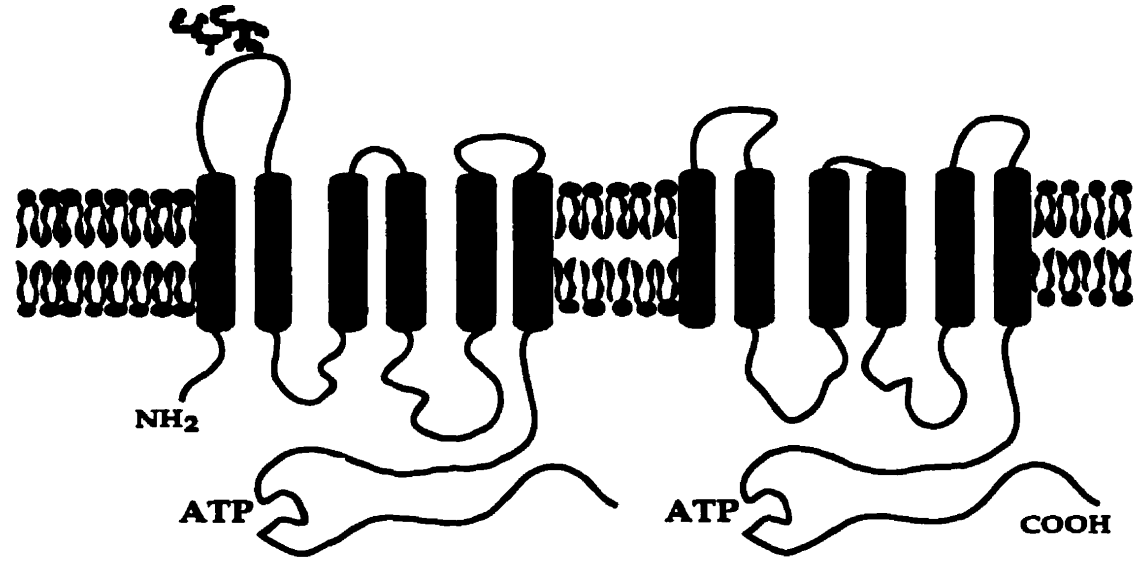
P-gp and MRP both belong to a large superfamily of eukaryotic ABC transporters (ATP Binding Cassette) that includes the CFTR (cystic fibrosis transmembrane conductance regulator), the TAP1/TAP2 (antigenic peptides associated with the endoplasmic reticulum of immune cells) (Kelly *et al.*, 1992; Neefjes *et al.*, 1993), the STE6 (the alpha-mating factor transporter in yeast), the PFMDR (homolog of P-gp in *Plasmodium falciparum*), and pigment uptake proteins in *Drosophila* (Juranka *et al.*, 1989).

Structural characteristics of eukaryotic ABC proteins are a tandemly duplicated molecule with six transmembrane segments and a NBD (Nucleotide binding domain) in each half. The P-glycoprotein sequence consists of a tandem duplication of approximately 590 amino acids, with an additional loop of approximately 60 amino acids which serves as a linker region connecting the two halves of the protein. Each half of P-glycoprotein encodes for a hydrophobic N-terminal domain, and a more hydrophillic C-

terminal region. The hydrophobic domain encodes six putative transmembrane regions, while the hydrophillic domain contains a consensus sequence for an ATP-binding motif (Gros *et al.*, 1986; Gerlach *et al.*, 1986) (Figure 1., *upper panel*). The predicted secondary structure of MRP appears to be distinct from P-gp. Other differences involve the presence of 13 amino acids between Walker A and B motifs of NBD1 of all other members of the ABC family, otherwise absent in the MRP sequence. Another distinguishable feature of MRP in comparison to P-gp, but not to other members of the ABC superfamily, is the greater divergence of primary sequence between the first and second NBDs than between the two NBDs of the P-glycoprotein. Most of the sequence similarity (15%) between MRP and P-gp is found within the NBD. MRP consists of 1531 amino acids with a molecular weight of 190 kDa. The original structure proposed for MRP contains eight transmembrane domains in the NH_2-proximal half of the molecule and four in the COOH-proximal half (Cole *et al.*, 1992). More recently, another predicted secondary structure of MRP has been proposed. In this latter model, MRP is thought to contain a total of 18 putative transmembrane domains (Figure 1., *lower panel*). Eleven or 12 transmembrane domains are located in the N-terminal half- 5 or 6 of which are located in the first 230 amino acids of the molecule- and 6 transmembrane domains are in the C-terminal half of the protein (Stride *et al.*, 1996).

P-glycoprotein is highly glycosylated, with carbohydrates accounting for approximately 20-40 kDa of its apparent molecular mass (Kartner *et al.*, 1985; Greenberger *et al.*, 1987). P-glycoprotein has three glycosylation sites. These N-linked oligosaccharide chains are found on the first extracellular domain from the N-terminus (Asn-X-Ser/Thr). MRP contains 12 Asn-X-Ser/Thr motifs which are the potential sites for N-glycosylation. Three of these occur on the outside of the membrane and lie within the II, V and VI extracellular loops of the protein, therefore suggesting that MRP is highly N-glycosylated (Grant *et al.*, 1994; Krishnamachary *et al.*, 1993; Cole *et al.*, 1993). The functional significance of MRP N-glycosylation is unclear. However, it is possible that the glycosylation influences MRP processing and stability (Almquist *et al.*, 1995).

P-glycoprotein is phosphorylated *in vivo* at serine residues in mouse, human and hamster MDR cells (Hamada *et al.*, 1987; Schurr *et al.*, 1989; Richert *et al.*, 1988; Georges *et al.*, 1996). Early studies suggested that changes in P-glycoprotein phosphorylation can alter its MDR phenotype (Hamada *et al.*, 1987). P-gp is phosphorylated by cAMP-dependent protein kinase A and protein kinase C. Phosphorylation occurs on both serine and threonine residues (Mellado *et al*, 1987). P-glycoprotein appears to be phosphorylated in the basal state in MDR human cells but can be further phosphorylated on different serine residues by activators of protein kinase C (the phorbol esters PMA and OAG). More recently, it was shown that mutations of several



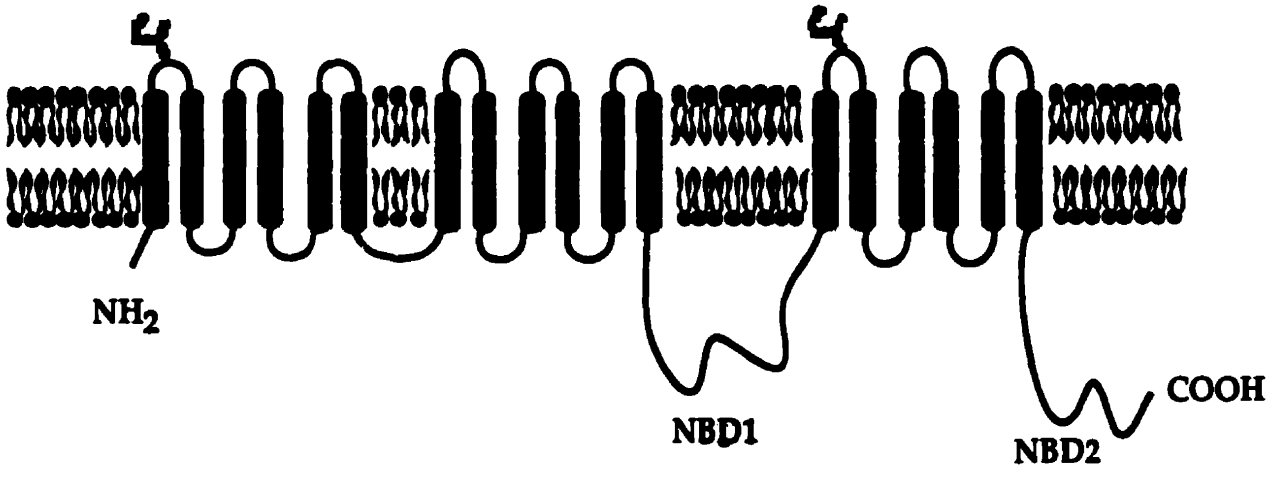


Figure 1. Structure of P-glycoprotein (*upper panel)* and multidrug resistance associated protein (MRP) (*lower panel)*

serine residues to alanine in P-gp did not affect its MDR function (Kajiji *et al.*, 1994). However, further work is required to clarify the role of phosphorylation on P-glycoprotein function, since it has been shown that the presence of drugs can induce phosphorylation of other serine residues which were not identified in previous studies (Georges *et al.*, 1997, manuscript submitted).

MRP has potential sites for phoshorylation by cAMP/cGMP-dependent protein kinases, protein kinase C, protein kinase II, and tyrosine kinases (Cole *et al.*, 1992), however the specific amino acids in MRP that are phosphorylated have not been identified. *In vivo* labelling of MRP, and immunoprecipitation with MRP-specific antibody from H69/AR cells and MRP-transfectants reveal that MRP was phosphorylated *in vivo* in both cell lines (Almquist *et al.*, 1995).

However these results do not exclude the possibility of increased levels of other phophoproteins of a similar size to MRP. It has been reported that when protein kinase C activity is inhibited, the level of MRP phosphorylation is reduced in tandem with increased drug accumulation in human leukaemic cells (HL60/AR) (Ma *et al.*, 1995). However some protein kinase inhibitors can act non-specifically with other cellular components and can also be substrates for transport (Epand *et al.*, 1993).

I.5 Expression of MRP and P-gp in normal tissues and tumours

P-glycoprotein expression has been shown in several normal tissues and organs. Within the organs that express P-glycoprotein, its expression is very localized. In the liver, P-gp is expressed in the biliary canalicular front of hepatocytes and the apical surface of epithelium cells lining the small biliary ductules (Thiebaut *et al.*, 1987). In colon, jejunum, and rectum, expression is similarly localized to the apical surface of the superficial columnar epithelial cells (Thiebaut *et al.*, 1987). P-gp expression in the kidney is greatest in the brush border of the proximal tubule (Thiebaut *et al.*, 1987). It is also expressed in endothelial cells that contribute to the blood brain barrier, and it has been suggested that P-glycoprotein functions to prevent toxic compounds from gaining entry into the cerebrospinal fluid. Disruption of P-gp function in blood-tissue barriers has been shown to result in enhanced central neurotoxicity, nausea and vomiting, or sterility (Hegmann *et al.*, 1992). Similarly, P-glycoprotein is highly expressed in the uterus and may function as a barrier to protect the fetus from toxins (Arceci *et al.*, 1990). These results suggest a possible normal role for P-glycoprotein in transport or secretion. P-glycoprotein may be a common exporter of toxic compounds. The high sequence conservation of P-glycoprotein across species suggests that its function is of fundamental importance.

MRP mRNA is expressed at low levels in many tissues, including haematopoetic cells (Cole *et al.*, 1992; Abbaszadegan *et al.*, 1994; Zaman *et al.*,

1993). The tissue distribution of mRNAs for both human and murine MRP shows the highest levels of expression in the testes, skeletal muscle, heart, kidney and lung (Cole *at al.*, 1992; Stride *et al.*, 1996; Kruh *et al.*, 1995). MRP mRNA is also detectable in the brain and spleen, while the levels are minimal in the liver and intestine (Stride *et al.*, 1996). The physiological function of MRP in these tissues has yet to be determined. MRP overexpression is evident in some tumour types such as human leukaemias (acute myeloid, chronic lymphocytic), lung (NSCLC and SCLC), neuroblastoma and some squamous cell carcinomas (Nooter *et al.*, 1996; Ota *et al.*, 1995).

The question of whether P-glycoprotein expression, or increase in its expression, is prognostic of MDR phenotype in tumour cells is currently under intensive investigation in several laboratories. High levels of P-glycoprotein have been found in many intrinsically drug resistant tumours from the colon, kidney and adrenal gland (Fojo *et al.*, 1987; Kanamaru *et al.*, 1989; Kakehi *et al.*, 1988), as well as in other tumours which had acquired the MDR phenotype after chemotherapy (for example, in acute non-lymphoblastic leukaemia (Ma *et al.*, 1987). In a large survey of tumour biopsies for P-glycoprotein overexpression, it was concluded that high levels of P-glycoprotein mRNA were detected in most tumour types treated with chemotherapeutic drugs (Goldstein *et al.*, 1989). Interestingly, however, high levels of P-glycoprotein mRNA were also detected in some tumour biopsies

prior to chemotherapy. In more recent studies (Chan H.S *et al.*, 1990a, 1991) using immunohistochemical staining for P-glycoprotein in childhood leukaemia, soft tissue sarcomas and neuroblastomas of children, P-glycoprotein expression was found prognostic of MDR and of durable response. Although much more work is needed to confirm the role of P-glycoprotein as a prognostic marker of MDR of tumour cells from patients, the above studies (Chan H.S *et al.* 1990a, 1991) provide convincing evidence that (at least in some cancers) P-glycoprotein levels predict the MDR phenotype of tumours.

Nowadays, besides the acetoxymethylester of calcein which has been shown to undergo energy-dependent efflux from MRP-overexpressing cells (Feller *et al.*, 1995), therefore showing the limitations of the specificity of the assay, there has not been a test developed that is sensitive and specific enough for detection of MRP in tumour cells. Five MRP-specific mAbs have been described. The monoclonal Abs, QCRL-1 (QCRL-2,QCRL-3) recognize intracellular MRP epitopes, therefore this specific and sensitive immunodetection of MRP can be used in facilitating knowledge of the biology and perhaps clinical relevance of this novel protein (Hipfner *et al.*, 1995).

I.6 Aspects of MRP-mediated multidrug resistance mechanism

The mechanism by which MRP confers resistance to a wide range of

drugs is not well understood. Energy dependent reduced drug accumulation and enhanced drug efflux is a characteristic observed in both cells that overexpress MRP (Zaman *et al.*, 1994; Cole *et al.*, 1994; Breuninger *et al.*, 1995) and those that overexpress P-gp.

While there are several proposed models for the mechanism of P-gp drug transport, no model has been described for MRP. Researchers have described P-gp as a membrane "vacuum cleaner", where two halves of protein are folded together and are involved in extruding drugs from the lipid bilayer. Alternatively, P-glycoprotein is thought to function as a "flippase". In the flippase model, P-glycoprotein detects drugs within the inner leaflet of the plasma membrane and "flips" them to the outer leaflet or directly to the extracellular space (Higgins *et al.*, 1992). Another model proposes that ATP hydrolysis is linked to transport of protons into the transporter, with chloride following passively. Once within the transporter, these ions will draw water into the transporter and out of the plasma membrane. Amphipathic drugs within the membrane should follow the water, and drugs will be removed from the membrane (Gill *et al.*, 1992).

Previous experiments showed energy-dependent altered distribution of anthracyclins in drug-selected MRP-overexpressing cell lines which has led the hypothesis that MRP participates in sequestering drugs away from their cellular target (Cole *et al.*, 1992; Slapak *et al.*, 1994). Cole *et al.* (1994) have postulated that MRP may participate directly in the active transport of drugs

into subcellular organelles or influence drug distribution indirectly. Perhaps MRP is involved in ion transport. Therefore due to its overexpression, cytoplasmic or intraorganelle pH can be altered and a relative decrease in pH would result in greater sequestration of the drugs which are protonated under acidic conditions (i.e. Vinca alkaloids, anthracyclins). Although there is no evidence as of now that MRP is directly involved in the vesicular sequestration of drugs, it has been hypothesized that the difference in distribution of MRP between various membrane compartments may contribute to the difference in drug accumulation and efflux kinetics in cell lines overexpressing MRP. The reason for this hypothesis comes from the observation that there are differences in subcellular distribution of MRP. MRP is predominantly expressed in the ER (endoplasmic reticulum) in some cells (Krishnamachary *et al.*, 1993). In transfectant cells (Almquist *et al.*, 1995) and in SW1573 (Zaman *et al.*, 1994), MRP was found predominantly on the plasma membrane and in the post-golgi vesicles (Flens *et al.*, 1994; Barrand *et al.*, 1995). Human leukaemic cell lines, HL60 and HL60/AR that have been selected in stepwise selection with doxorubicin, express a majority of MRP in the endoplasmic reticulum (ER). On the other hand, SCLC cells, H69/AR, express MRP predominantly on the plasma membrane (Marquardt *et al.*, 1992). What exactly controls these differences in subcellular localisation is not yet known, but variations in trafficking of MRP may be involved in the vesicular sequestration of drugs.

The ability of MRP to transport GSH conjugates (Muller *et al.*, 1994; Leier *et al.*, 1994; Jedlitschky *et al.*, 1994), oxidised glutathione (GSSG) (Leier *et al.*, 1996), has lead to the possibility that MRP may be a GSH conjugate transporter. The glutathione S-conjugate export carrier mediates excretion of bivalent anionic conjugates and is believed to play a role in the elimination of conjugated xenobiotics (Muller *et al.*, 1994). Other evidence that MRP is a GSH conjugate transporter is the increased ATP-dependent glutathione S-conjugate carrier activity due to the overexpression of *MRP* gene in human cancer cells. Recently Almquist *et al.* (1996) demonstrated that ATP-dependent uptake of unmodified vincristine in membrane vesicles of MRP-transfectant cells is possible only in the presence of GSH. The mechanism by which GSH-dependent vincristine transport occurs is unknown. However following depletion of GSH, increased drug accumulation in MRP-overexpressing cells is observed. Therefore it is possible that GSH interacts directly with MRP and this interaction is necessary for transport.

It is also believed that MRP transports drugs in a fashion similar to a multispecific organic anion transporter (MOAT) found in liver canalicular membranes, due to the ATP-dependent transport of a variety of hydrophobic anionic compounds (Oude Elfernik *et al.*, 1994; Mayer *et al.*, 1995).

Besides its function as a transporter, MRP may be involved in the regulation of endogenous channels and other transporters. It is known that ABC transporters can modulate the activity of ion channels, including

outwardly rectifying Cl^- channels (CFTR) (Gabriel *et al.*, 1993; Jovov *et al.*, 1995), and volume regulated Cl^- channels (P-gp) (Higgins *et al.*, 1995), and inwardly rectifying K^+ channels (SUR) (Inagaki *et al.*, 1995). In this respect, it is interesting to note that Cl^- and K^+ channel activity is increased in H69/AR cells relative to the drug-sensitive parental cells (Jirsch *et al.*, 1993; Jirsch *et al.*, 1994).

However, the mechanisms by which MRP transports drugs and by which MRP may alter channel activity are as yet unknown.

I.7 Experimental modulation of MRP

It has recently been demonstrated that MRP can transport the cysteinyl leukotriene, LTC_4, and some other GSH conjugates such as *s*-dinitrophenylglutathione, and oxidised glutathione (GSSG) (Leier *et al.*, 1994). A common characteristic of these compounds is anionic charge and hydrophobicity. LTC_4 has been the best characterised substrate for MRP and it has been found to bind to MRP with the highest affinity. LTC_4 is an arachidonic acid derivative which is involved in a receptor-mediated signal transduction pathway controlling smooth muscle contraction and vascular permeability. After synthesis, it is exported from the cell in an energy-dependent manner and then converted to cysteinyl leukotrienes, LTD_4 and LTE_4 (Loe *et al.*, in press). Together, cysteinyl leukotrienes play an important role in the pathogenesis of human bronchial asthma and also make up the

substance of anaphylaxis.

There has not been much research done in analysing the structural features of chemosensitisers that reverse MRP-mediated multidrug resistance. Chemosensitisers are a structurally diverse group of compounds that are able to reverse the MDR phenotype. Due to their potential to overcome the multidrug resistance clinically, there is an increased interest to study them (Ford *et al.*, 1993). Chemosensitisers such as verapamil and trifluoroperazine are among the most effective stimulators of the ATPase activity associated with P-gp (Sharom *et al.*, 1995). However, most of these agents have much less or no effect at reversing resistance in MRP multidrug-resistant cell lines. This conclusion was supported with evidence that verapamil, and to a lesser degree cyclosporin A, has a modest effect as a reversing agent in doxorubicin-selected fibrosarcoma HT1080/DR4 cell lines (Cole *et al.*, 1989). The same observation has been seen in several other MRP-overexpressing cell lines (doxorubicin-selected non-small lung cancer cell lines, COR-L23/R and MOR/R). Furthermore, it has been shown in aforementioned cell lines that SDZ PSC-833, the most effective chemosensitizer of P-gp mediated MDR, had little if any effect as a reversing agent of resistance (Barrand *et al.*, 1993). SDZ PSC-833 commonly exhibits 10-fold increased potency over cyclosporin A in P-glycoprotein-mediated multi-drug resistance cells (Gaveriaux *et al.*, 1991). These differences could be due to the different substrate specificities that are exhibited by the two

transporters.

Compounds that can increase drug accumulation and modulate resistance in MRP-overexpressing cells include dihydropyridines (nicardipine (Cole *et al.*, 1989) and NIK250 (Abe *et al.*, 1995 and Tasaki *et al.* 1995)), the tiapamil analogue DMDP (Cole *et al.*, 1992), the bisindolylmaleimide protein kinase C inhibitor GF109203X (Gekeler *et al.*, 1995), the cyclosporin analog PSC 833 (Barrand *et al.*, 1993), the isoflavanoid tyrosine kinase inhibitor genistein (Versantvoort *et al.*, 1993), the quinolone difloxacin (Gollapudi *et al.*, 1995), and the diiodinated benzofuran amiodarone (van der Graaf *et al.*, 1994). MK571, the anionic quinoline LTD_4 cysteinyl leukotriene receptor (CLT_2) antagonist (see figure 2.) has been shown to suppress the photoaffinity binding of LTC_4 to MRP (Gekeler *et al.*, 1995) therefore specifically modulating MRP-associated multidrug resistance. Finally, buthionine sulphoximine (BSO), a potent inhibitor of glutathione (GSH) synthesis, can partially reverse resistance to daunorubicin and vincristine in cells overexpressing MRP (Versantvoort *et al.*, 1995).

As we can see from these studies, there appears to be no uniform structural features which can predict the ability of a compound to reverse MRP-associated multidrug resistance. Some of these compounds restore accumulation only at concentrations that are highly toxic to the cell, therefore they are not very useful for *in vivo* studies. An important point to address, in regard to modulation of multidrug resistance, is the inability to

attribute the effects of potential reversing agents exclusively to their action on MRP since most of the studies were done in drug selected cell lines, which may have undergone some alterations contributing to resistance.

Having said all this, we are still searching for the ideal substrate and chemosensitizer in order to overcome the obstacle of multidrug-resistance.

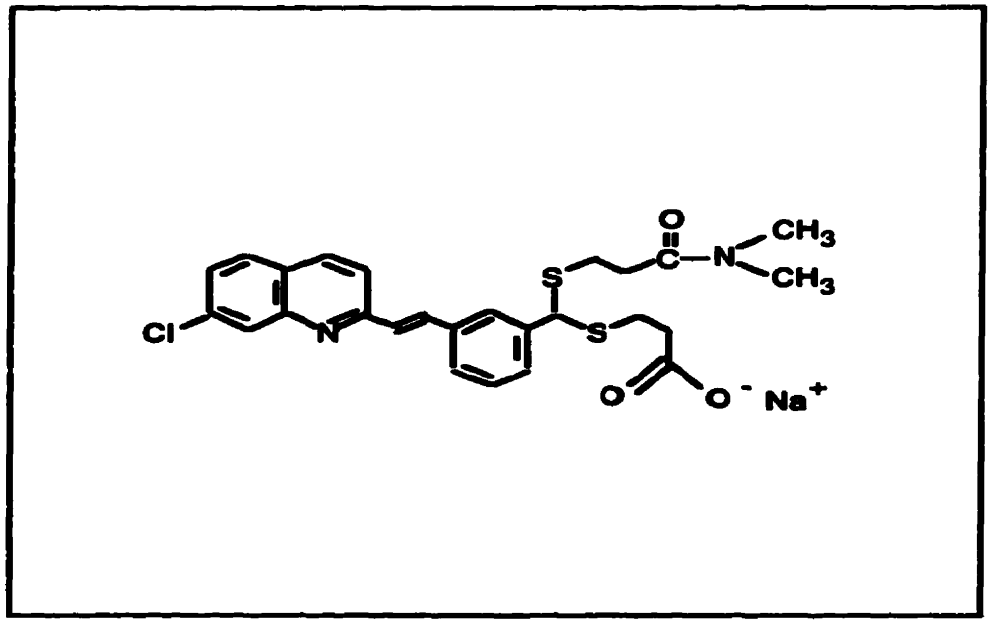


Figure 2. Structure of MK 571

OBJECTIVES

The LTD_4 receptor antagonist MK571, a quinoline-based drug has been previously shown to reverse MRP-mediated MDR and to inhibit MRP-drug interactions. Although it is presently not known if certain moieties, other than the GSH, are recognized by MRP, it was of interest to study the interactions of a photoactive quinoline-based drug ASA-AQ with MRP in plasma membranes and MDR intact cells.

Specifically, in chapter II (MANUSCRIPT I), I have looked at the interaction between aminoquinoline photoactive analogue (abbreviated ASA-AQ) and MRP with respect to:

1) photoaffinity labelling and direct binding of ASA-AQ to MRP;

2) transport of ASA-AQ in cells overexpressing MRP; and

3) cross-resistance of MRP overexpressing cells to ASA-AQ

In Chapter III (MANUSCRIPT II), I have looked at the interaction between antimalarial drug, chloroquine and MRP with respect to:

1) competition of chloroquine for photoaffinity labelling of MRP with its substrate ASA-AQ;

2) cross-resistance of MRP overexpressing cells to chloroquine; and

3) energy-dependent transport of chloroquine in cells expressing MRP

CHAPTER II

A Photoactive Quinoline-based Drug, N-{4-[1-hydroxy-2-(dibutylamino)- ethyl] quinolin-8-yl}-4-azido-2-salicylamide, Binds to a Physiologically Relevant Site in The Multidrug Resistance Protein (MRP1)

(Manuscript I)

Marko Vezmar, Leann Tilley and Elias Georges

(In preparation)

ABSTRACT

Several studies have now demonstrated that the cysteinyl leukotriene C_4 (LTC_4) and other glutathione (GSH) S-conjugated anions are substrates for the multidrug resistance protein (MRP). However, the molecular mechanisms underlying the binding and efflux of drugs by MRP are currently not well understood. To learn more about MRP-drug interactions, it was of interest to characterize the binding of MRP to a photoactive quinoline compound, N-{4-[1-hydroxy-2-(dibutylamino)-ethyl]quinolin-8-yl}-4-azido-2-salicylamide (ASA-AQ). Using [^{125}I] ASA-AQ, we show the photoaffinity labelling of a 190 kDa protein in membranes from resistant Small Cell Lung Cancer cells (H69/AR) but not from the parental (H69) drug sensitive cells. The identity of the 190 kDa protein, as MRP, was confirmed by immunoprecipitation with the monoclonal antibody, QCRL-1. The photolabelling of MRP with [^{125}I] ASA-AQ was saturable and was inhibited with unlabeled ASA-AQ. Interestingly, molar excess of LTC_4 and MK571, but not vinblastine or GSH, inhibited the photoaffinity labelling of MRP with [^{125}I] ASA-AQ. Cell growth and drug transport studies showed H69/AR cells to be less sensitive to, and accumulate less ASA-AQ, as compared to the parental cells H69. Furthermore, the reduced sensitivity to, and drug accumulation of ASA-AQ in H69/AR cells was reversed with MK571 and doxorubicin but not with vinblastine. The results of this study show direct and specific binding between MRP and unmodified quinoline-

based compounds. In addition, this is the first demonstration that a non-glutathione conjugate interacts directly with MRP1.

INTRODUCTION

Selection of tumour cell lines with certain anticancer drugs has led to the isolation of multidrug resistant (MDR) cells that accumulate less drug than the parental drug sensitive cells (Bradley *et al.*, 1988; and Gottesman *et al.*, 1993). Characterization of the MDR cells revealed a correlation between the drug resistance phenotype and the overexpression of two cell surface proteins, the P-glycoprotein (Bradley *et al.*, 1988; Gottesman *et al.*, 1993) and the 190kDa multidrug resistance protein (MRP1) (Cole *et al.*, 1992; Cole *et al.*, 1993; Krishnamachary *et al.*, 1993; and Almquist *et al.*, 1995). The cDNA transfection studies with full length genes of *mdr1 (Pgp1)* or *mrp1* confirmed their role in MDR (Ueda *et al.*, 1987; Gros *et al.*, 1986; and Grant *et al.*, 1993). Further, drug transport studies using *mdr1* or *mrp1* transfectants demonstrated that both P-gp1 and MRP1 are responsible for an energy-dependent drug efflux (Gros *et al.*, 1986; Zaman *et al.*, 1994; Cole *et al.*, 1994 and Breuninger *et al.*, 1995). The normal physiological function of P-gp1 and MRP1 are presently not known. However, tissue distribution studies (Thiebaut *et al.*, 1987; Cole *et al.*, 1992; Kruh *et al.*, 1995; and Stride *et al.*, 1996) suggest the transport of normal cell metabolites and general detoxicification of natural toxins (Chaudhary *et al.*, 1991; Cordon-Cardo *et al.*, 1990; Gottesman *et al.*, 1993). Disruption of *mdr1* gene from the mouse genome was shown to increase the accumulation of drugs in tissues that express high levels of P-gp1 (Schinkel *et al.*, 1994). More recently, P-gp1 was shown to

lipid translocase of a broad specificity in epithelial LLC-PK1 cells (Van Helvort *et al.*, 1996). Similarly, double knock-outs of *mrp1* from embryonic stem cells showed a significant increase in drug toxicity to several lipophilic anticancer drugs and sodium arsenite as compared to wild type embryonic stem cells (Lorico *et al.*, 1996).

P-gp1 and MRP1 are members of a large family of membrane transporters that bind to and hydrolyze ATP (Higgins *et al.*, 1992). Although, P-gp1 and MRP1 share several functional and structural homologies (Cole *et al.*, 1992), important differences exist between these two members of the ATP-dependent transporters (Cole *et al.*, 1992; Szczypka *et al.*, 1994). Among these differences are the membrane topology and substrate specificity (Papadopoulou *et al.*, 1994). For example, unlike the predicted 6+6 membrane topology for P-gp1, MRP1 is thought to contain a hydrophobic N-terminal domain leading to a predicted 12+6 membrane topology (Stride *et al.*, 1996). Furthermore, although, both MRP1 and P-gp1 have broad and overlapping substrate specificities, MRP1 was shown to transport several organic anions, GSH conjugates and heavy metal oxyanions (Leier *et al.*, 1994; Jedlitschy *et al.*, 1994; Loe *et al.*, 1996; and Muller *et al.*, 1994). The ability of MRP1 to mediate the transport of the latter compounds in addition to lipophilic cations (natural *vinca* alkaloids and some antibiotics) suggests differences in the mechanism of drug transport. In addition, unlike P-gp1 which binds to, and transports many structurally dissimilar drugs (Safa *et al.*,

1986, 1988, 1989, 1992; Tanai *et al.*, 1990), the mechanism of MRP1 drug transport remains to be determined. Recent studies (Leier *et al.*, 1994 and 1996; Muller *et al.*, 1994) have demonstrated that both LTC_4 (intrinsically photoreactive compound) and the glutathione conjugate S-(p-azidophenylacyl)-glutathione are transported by, and interact directly with MRP1. However, both compounds contain the glutathione moiety.

In this study, we have used a photoactive quinoline-based drug (ASA-AQ) to demonstrate a direct and specific binding to MRP in intact Small Cell Lung Cancer Cells (H69/AR) and in plasma membranes prepared from these cells. Moreover, we show that the photoaffinity labelling of ASA-AQ to MRP occurs at a physiologically relevant site since is was competed with known substrates of MRP (MK571 and LTC_4).

MATERIALS AND METHODS

Materials - Iodine-125 (100.7 mCi/ml) and [^{125}I]-iodoarylazidoprazosin (2200 Ci/mmol) were purchased from Amersham Biochemical Inc. (Mississauga, Ontario, Canada). Protein-A coupled sepharose was purchased from Pharmacia Inc., Quebec, Canada. The LTD_4 receptor antagonist MK571 was kindly provided by Dr. A.W. Ford-Hutchinson (Merck-Frosst Centre for Therapeutic Research, Point Claire-Dorval, Quebec, Canada). Leukotriene C_4 (LTC_4) was purchased from Cayman Chemical Co. (Ann Arbor, MI). The human Small Cell lung cancer lines H69 and H69/AR, and the MRP-specific QCRL-1 monoclonal antibody were a kind gift from Dr. Susan P. C. Cole (Cancer Research Laboratories, Queen's University, Kingston, Ontario, Canada). The monoclonal antibody C494 was a generous gift from Dr. V. Ling at British Columbia Cancer Center (Vancouver, Canada). All other chemicals were of highest commercial grade available.

Cell Culture and Plasma Membrane preparation - Drug sensitive (H69) and resistant cells (H69/AR) 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 M 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 essentially as described by Lin *et al* (1987). In brief, cells were

collected by low speed centrifugation and washed three times with ice-cold Phosphate buffer saline, pH 7.4 (PBS). Cells were homogenized in 50 mM mannitol, 5mM Hepes and 10mM 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 precipitated by high speed centrifugation at 100,000X g for 1 hour at 4°C in a Beckman SW28 rotor. The enriched plasma membrane pellet was washed with 10 mM Tris-HCl, pH 7.4 and resuspended in the same buffer containing 250 mM sucrose. Membrane fractions were stored at -80°C if not immediately used. Protein concentrations were determined by the Lowry method (Lowry *et.* al., 1951)

Radioiodination of ASA-AQ and photoaffinity labelling - Iodination was carried out in the dark. ASA-AQ (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 1M 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. [^{125}I] ASA-AQ was eluted with 2.5 ml 100% methanol and the solution was vacuum dried in the dark. The dried residue was resuspended in DMSO and the concentration of the radioactive-photoactive drug was determined to be 20 μM.

For photoaffinity labelling of cells, H69 or H69/AR cells were washed with PBS and preincubated for 20 minutes at 37°C in the presence or in the absence of a molar excess of vinblastine, LTC_4 or MK571 before the addition of [^{125}I] ASA-AQ (0.25 μM). Cells were incubated at room temperature in the dark for 30 minutes and then transferred to ice for 10 minutes. Following the latter incubation on ice, cells were irradiated for 10 minutes on ice with a UV source at 254 nm (Stratagene UV crosslinker, Stratagene, La Jolla, CA). Cells were centrifuged for 5 minutes at 2500 rpm in a microcentrifuge, the supernatant containing free radioactive drug was removed and cells were lysed in 20 μl of 50 mM Tris (pH 7.4) containing 1% NP40, 5 mM $MgCl_2$ and protease inhibitors (3 μg/ml of leupeptin and 2 mM PMSF). [^{125}I] ASA-AQ labelled proteins were isolated by brief centrifugation at 4°C and resolved by SDS-PAGE. Photoaffinity labelling of plasma membrane fractions, 15 μg aliquots of membranes prepared from H69 and H69/AR cell lines were photoaffinity labelled as described above.

Immunoprecipitation and SDS Gel electrophoresis- -Intact cells photoaffinity labelled with [^{125}I] ASA-AQ 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 for 10 minutes at 12000X g at 4°C. Equal amounts of cell lysate proteins were separately incubated overnight at 4°C with 10 μg of C494 and QCRL-1 monoclonal antibodies or an irrelevant IgG2a. Protein-A coupled sepharose (Pharmacia Inc.) was added to the latter cell lysate and allowed to incubate for 1 hour at room temperature. The protein-A sepharose beads were then washed four times in the above lysis buffer and incubated in 10 mM Tris-HCl, pH 8.0 containing 2% SDS, 50 mM dithiothreitol (DTT), 1 mM EDTA (Buffer I). Equal volume of buffer II (2X buffer I and 9 M urea) was added to Sepharose beads and the incubation was maintained for 10 minutes before a one minute centrifugation at 12000X g in a microcentrifuge. The eluted proteins were then resolved by SDS-PAGE using the Fairbanks gel system with some modifications (Fairbanks *et al.*, 1971). Gel slabs containing the resolved proteins were fixed in 50% methanol, dried and exposed to XAR Kodak film at -70 °C.

Cytotoxicity assay - Cells were harvested in the exponential growth phase and plated (0.5 - 1.0X10^4 cells/well) into 96-well plates. Cells were allowed to recover for 24 hours before the addition of ASA-AQ in the dark in the

absence or presence of micromolar concentrations of MK571. Cells were allowed to grow in the dark for four days at 37°C before the addition of the MTT dye (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide). The plates were incubated in the dark for 4 hours at 37°C and the coloured crystals formed from the tetrazolium salt were solubilized by the addition of 50 µl of 10% Triton X-100 in 0.01 N HCl followed by vigorous pipetting. The 96-well plates were heated in the microwave oven for 1 minute at the low power setting and 10 µl of ethanol was added to disperse the formed bubbles. Plates were read at 570 nm using an LKB microtiter plate reader.

Drug Transport - For drug accumulation, $1X10^6$ cells were washed three times in PBS containing 5 mM D-glucose or 10 mM 2-deoxyglucose and 100 nM sodium azide and incubated for 45 minutes with 0.25 µM [^{125}I] ASA-AQ in the absence or the presence of a molar excess of vinblastine, MK571 or doxorubicin. The incubation was stopped with the addition of 1 ml of ice-cold PBS and cells were then washed three times with the same solution. The cell pellets were lysed in 100 µl of 1M NaOH followed by neutralization with an equal volume of 1M HCl and the accumulated radiolabel was determined by 1219 Racbeta model counters (LKB, Wallace). It is emphasized that the above studies with [^{125}I] ASA-AQ were done under a safety light to prevent the photodestruction of the photoreactive groups. Furthermore, the integrity of the photoreactive moiety was monitored by the absorbance at 254

nm and controls to check for non-specific photolabelling of proteins were included in each experiment (data not shown).

RESULTS

Photoaffinity labelling of MRP with ASA-AQ- The LTD_4 receptor antagonist MK571, a quinoline-based drug (Figure 1a), has been previously shown to reverse MRP-mediated MDR (Gekeler *et al.*, 1995) and to inhibit MRP-drug interactions (Gekeler *et al.*, 1995). Although it is presently not known if certain moieties, other than the GSH, are recognized by MRP, it was of interest to study the interactions of a photoactive quinoline-based drug (ASA-AQ; Figure 1b) with MRP in plasma membranes and MDR intact cells. To determine if ASA-AQ interacts with MRP, drug sensitive (H69) and resistant (H69/AR) SCLC cells were incubated in the presence of 250nM of [^{125}I] ASA-AQ and were UV irradiated (see experimental procedures). The results in Figure 2a show a 190kDa protein photolabelled with [^{125}I] ASA-AQ in H69/AR, but not in H69 cells. When the same cells were incubated in the presence of 20 nM of IAAP ([^{125}I]-iodoarylazidoprazosin), which was shown to photoaffinity label P-glycoprotein (Safa *et al.*, 1990), no 190 kDa protein was photoaffinity labelled, suggesting that IAAP is not a substrate for MRP (Figure 2a).

To determine the binding specificity of [^{125}I] ASA-AQ towards the 190 kDa protein in H69/AR cells, photoaffinity labelling of cells was carried out in the presence of increasing concentrations of [^{125}I] ASA-AQ (0.25 μM to 2.5 μM). Figure 2b, *inset*, shows the [^{125}I] ASA-AQ photoaffinity labelling of the 190 kDa protein to be saturable in the range 0.1-2.5 μM of the drug. Steady-

state saturation is achieved at 2.0 μM which is evident by the intensity of the labelling (*see inset* , Figure 2b). To confirm the specificity of ASA-AQ binding to the 190 kDa protein, photolabelling of whole cells was carried out in the presence of molar excess (150 - 2000-fold) of non-radiolabelled ASA-AQ. Figure 2c, *lanes a-d,* show a marked decrease in the photoaffinity labelling of the 190kDa protein in the presence of a molar excess of ASA-AQ. The non-specific labelling, which is evident in lower bands (most in the 120 kDa protein), was not significantly affected with excess unlabelled ASA-AQ as that of the 190kDa protein.

To confirm the identity of the 190kDa, [^{125}I] ASA-AQ photoaffinity labelled cell lysates from H69/AR cells were immunoprecipitated with the MRP specific mAb QCRL-1. Figure 3 shows a 190kDa [^{125}I] ASA-AQ photoaffinity labelled protein specifically immunoprecipitated with mAb QCRL-1 from H69/AR, but not from H69 lines. No [^{125}I] ASA-AQ photoaffinity labelled 190kDa protein was immunoprecipitated with the P-glycoprotein specific mAb C494 (Figure 3). These results demonstrate that the 190 kDa protein photoaffinity labelled with [^{125}I] ASA-AQ is indeed MRP as confirmed by its specific binding to mAb QCRL-1 (Hipfner *et al.*, 1994).

Inhibition of photoaffinity labelling of MRP - To demonstrate that the binding of ASA-AQ to MRP occurs at a physiologically relevant site(s), photoaffinity labelling of MRP was examined in the presence of a molar

excess of LTC_4, MK571 and vinblastine (Vlb). The photoaffinity labelling of MRP with [^{125}I] ASA-AQ was decreased in the presence of 50-fold molar excess of LTC_4 and completely inhibited at 75-fold molar excess of LTC_4 (Figure 4a). Furthermore, the photoaffinity labelling of MRP by [^{125}I] ASA-AQ was also decreased at 300-fold and even to a greater degree at 1000-fold molar excess of MK571. In contrast, similar concentrations of Vlb, which is a poor substrate for MRP, did not significantly decrease the photoaffinity labelling of MRP with [^{125}I] ASA-AQ. These results demonstrate that ASA-AQ binds directly to MRP in MDR cells and its binding can be competed with substrates for MRP. Moreover, given that [^{125}I] ASA-AQ photoaffinity labelling of MRP was inhibited in the presence of excess MDR-associated drugs or substrates of MRP and reversing agents suggests a common binding domain on MRP.

It is presently not yet clear if GSH conjugation is required for the binding and/or transport of drugs via MRP (Almquist *et al.*, 1996; Zaman *et al.*, 1996). In the above photoaffinity labelling experiments of MRP with [^{125}I] ASA-AQ, intact cells were used and therefore it cannot be determined if ASA-AQ is modified prior to its interaction with MRP or that GSH binding is required for ASA-AQ binding. To determine if unmodified ASA-AQ binds directly to MRP, plasma membranes prepared from H69/AR cells were labelled with [^{125}I] ASA-AQ in the absence and presence of molar excess of vinblastine and MK571. Figure 4b shows identical results to the experiment

done in intact cells (Figure 4a), concluding that photolabelling with ASA-AQ does not require GSH conjugation. Although, our membrane extraction method is likely to remove most of the cellular GSH from H69/AR cells (which intrinsically contain low levels of GSH (Cole *et al.*, 1994)) and since most glutathione transferases are soluble, it is unlikely that ASA-AQ will be modified during our photoaffinity labelling experiments. However, to rule out the possibility that in intact cells ASA-AQ is modified by GST, H69/AR cells were incubated with [^{125}I] ASA-AQ as described earlier for photoaffinity labelling experiments and the label was extracted and analyzed by HPLC using a C_{18} column before and after incubation. The results from the HPLC runs (data not shown) show identical elution times for [^{125}I] ASA-AQ without and with incubation with cells.

ASA-AQ is a substrate for MRP in H69/AR cells - Given the above results, with regard to the photoaffinity labelling of MRP with [^{125}I] ASA-AQ, it was of interest to determine if ASA-AQ is a substrate for MRP in intact cells. The results in figure 5 show the effects of increasing concentrations of ASA-AQ on the growth of H69 and H69/AR cells. H69/AR appear to be less sensitive to ASA-AQ than the parental H69 drug-sensitive cells with IC_{50} of 20 μM and 38 μM, respectively. Furthermore, MK571 significantly potentiated the toxicity of ASA-AQ in H69/AR (figure 5) but not in H69 (data not shown) cells.

To determine if the decreased sensitivity of H69/AR cells to ASA-AQ cells was mediated by a decrease in drug accumulation, figure 6 show the accumulation of [^{125}I] ASA-AQ in H69 and H69/AR cells in the absence and in the presence of a molar excess of vinblastine, doxorubicin or MK571. H69/AR cells show a lower steady state drug accumulation than H69 cells (figure 6b versus 6a). However, molar excesses (1-25 μM) of MK571 or doxorubicin but not Vlb potentiated the accumulation of [^{125}I] ASA-AQ almost (85+/-3.6% for Dox; and 86.7+/-3.0% for MK 571) to the level as that seen in drug sensitive H69 cells (Figure 6b versus 6a).

DISCUSSION

In this study we demonstrate the photoaffinity labelling of MRP1 in SCLC H69/AR cells using a photoactive quinoline-based drug (ASA-AQ). Furthermore, we show that MRP1 photoaffinity labelling with ASA-AQ is inhibited with molar excess of LTC_4, MK571, but not Vlb. Given that both LTC_4 and MK571 have been previously shown to interact directly with MRP1 and that LTC_4 is a normal cell metabolite with the highest affinity for MRP (Leier *et al.*, 1994), the latter results suggest that ASA-AQ interacts with a physiologically relevant site on MRP1. Although the drug binding site(s) of MRP is not presently known, we speculate that ASA-AQ binds to the same domain as that of MK571 or LTC_4. This speculation is further supported by the structural similarities between MK571 and ASA-AQ. Both compounds contain the quinoline moiety. However, it remains to be determined if other quinoline based compounds also bind to or are substrates for MRP1 drug efflux mechanism.

Alternatively, ASA-AQ and MK571 may bind to another site that is conformationally linked to LTC_4 binding domain(s). Indeed, unlike P-gp1, MRP1 has been shown to interact specifically with GSH-conjugated compounds, GSH-analogues and oxidized glutathione (Muller *et al.*, 1994: Leier *et al.*, 1994 and 1996; Jedlitschky *et al.*, 1994), in addition to other unmodified lipophilic natural products and heavy metal oxyanions (Cole *et al.*, 1994). Therefore, it is likely that ASA-AQ and other quinoline based

drugs interact with a different binding domain in MRP1. Clearly more work with respect to MRP1-drug interactions is required to support the hypothesis of one or multiple drug binding sites in MRP1. Evidence from another broad spectrum transporter (the P-gp1) is consistent with multiple domains forming the drug binding site (Nare *et al.*, 1994; Greenberger *et al.*, 1993; Loo *et al.*, 1995)

Earlier studies (Muller *et al.*, 1994: Leier *et al.*, 1994 and 1996; Jedlitschky *et al.*, 1994) have shown that MRP1 transports GSH S-conjugated drugs and certain natural products and arsenate. Although, the role of GSH in MRP1-mediated drug binding and transport remains unclear (Tew *et al.*, 1994; Versantvort *et al.*, 1995), it has been suggested that GSH binding to MRP1 allows for increased drug binding an ATP dependent transport of vincristine (Loe *et al.*, 1996). In this study, we show that MRP1 photoaffinity labelling with [^{125}I] ASA-AQ does not require GSH conjugation. Furthermore, the presence of exogenously added GSH (up to 1.5 mM) did not modulate the photoaffinity labelling of MRP1 with ASA-AQ. In addition, our results show H69/AR cells to be less sensitive to ASA-AQ than H69 parental cells which is consistent with reduced ASA-AQ drug accumulation. Taken together, these results are interesting and suggest that for certain drugs GSH conjugation is not required for drug binding to, or transport by MRP1.

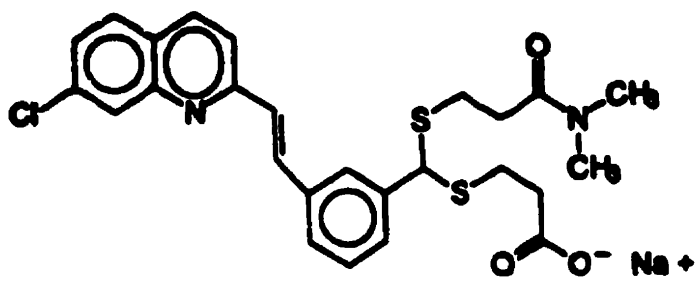
In conclusion, we have used a photoactive drug (ASA-AQ) to

demonstrate a direct and specific binding to MRP1 in intact cells and plasma membranes from H69/AR SCLC cells. We speculate that ASA-AQ binds to the same or conformationally linked domain as that of LTC_4 or MK571 as both drugs inhibit photoaffinity labelling. It would be of interest in future studies to determine if ASA-AQ photoaffinity labels the same or different sequences as LTC_4. Furthermore, the availability of photoactive radioiodinated drugs that binds specifically to MRP1 should facilitate future analysis of MRP drug interactions.

ACKNOWLEDGEMENTS

We thank Dr. Leann Tilley of the Walter and Eliza Hall Institute of Medical Research for providing the photoactive analogue N-{4-[1-hydroxy-2 (dibutylamino)- ethyl] quinolin-8-yl}-4-azido-2-salicylamide (ASA-AQ), and Dr. Susan Cole for providing Small Cell Lung Cancer (SCLC) cell lines (H69 and H69/AR). This research was supported by a grant from the National Cancer Institute of Canada.

A.



B.

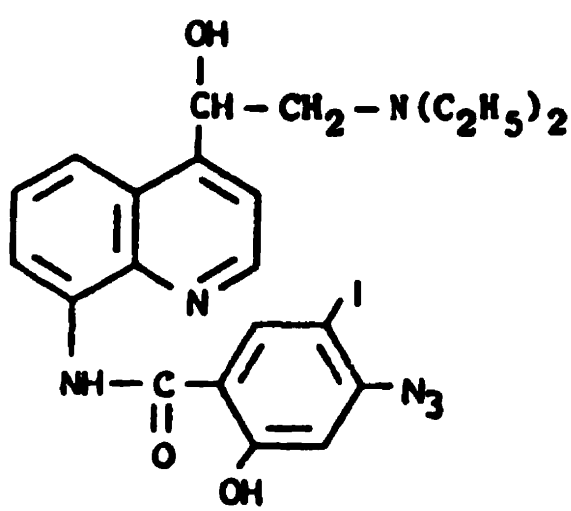


Figure 1. Organic structures of MK571 and ASA-AQ. Leukotriene D_4 receptor antagonist (**A**), and N-{4-[1-hydroxy-2-(dibutylamino)ethyl]quinolin-8-yl}-4-azido-2-salicylamide (**B**). Both compounds contain a quinoline moiety.

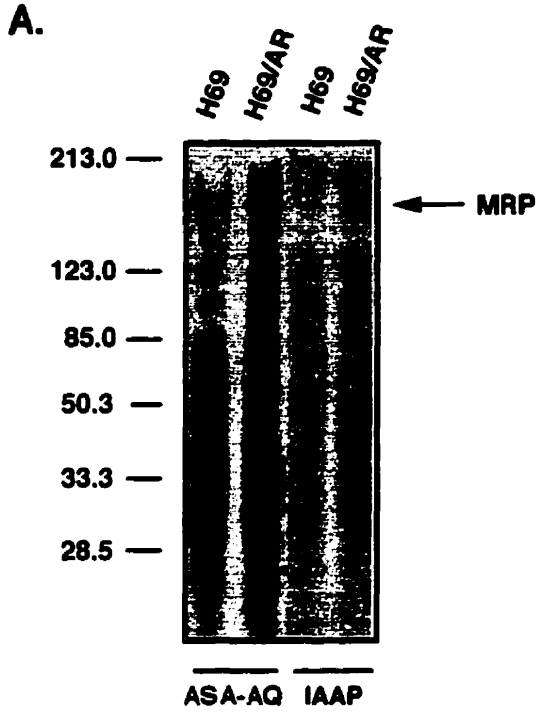


Figure 2a. Photoaffinity labelling in H69 and H69/AR cells with [^{125}I]ASA-AQ. Drug sensitive (H69) and resistant (H69/AR) cells were photoaffinity labelled with either 20 nM IAAP (lane 3 and 4) or 0.25 μM [^{125}I] ASA-AQ (lane 1 and 2). The samples were run on a SDS-polyacrylamide gel (Fairbanks). 190kDa protein is photoaffinity labelled, exclusively, in H69/AR cells with [^{125}I] ASA-AQ.

B.

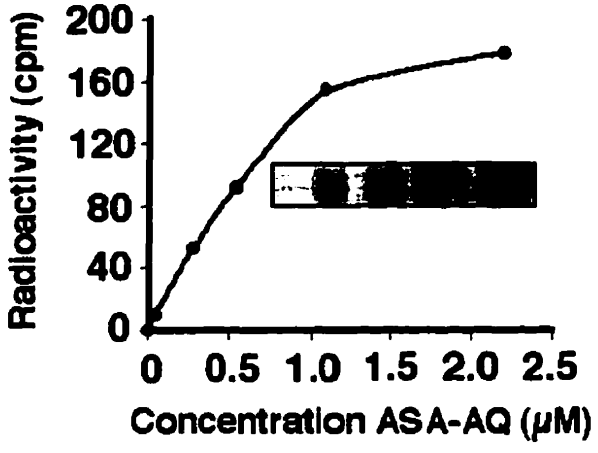


Figure 2b. Saturability of [^{125}I]ASA-AQ labelling in H69/AR cells. Photoaffinity labelling of H69/AR cells is in the presence of increasing concentrations of [^{125}I] ASA-AQ (0-2.5 μM). The inset shows the increase in the intensity of the 190kDa photoaffinity labelled band which was excised and the amount of radiolabel was quantified. The graph is a presentation of the amount of radioactivity (cpm) versus the concentration of the [^{125}I]ASA-AQ (μM).

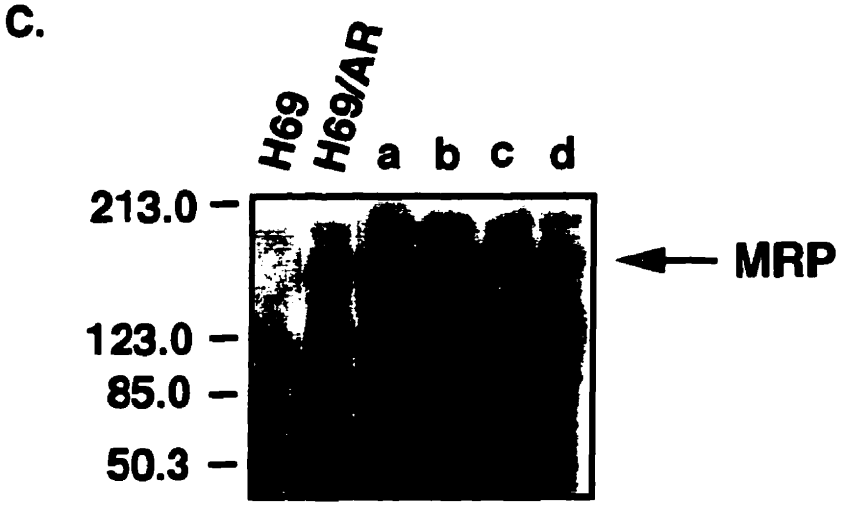


Figure 2c. Specificity of [^{125}I]ASA-AQ labelling in H69/AR cells. Photoaffinity labelled proteins from H69 or H69/AR cells were incubated in the absence or presence of excess (40, 125, 250, 500 μM) non-radiolabeled ASA-AQ (lanes a-d), and the samples were run on a SDS-polyacrylamide gel (Fairbanks). Notice, only protein of a correct size (190 kDa) was labelled in H69/AR cells, while there is an absence of labelling in H69 cells.

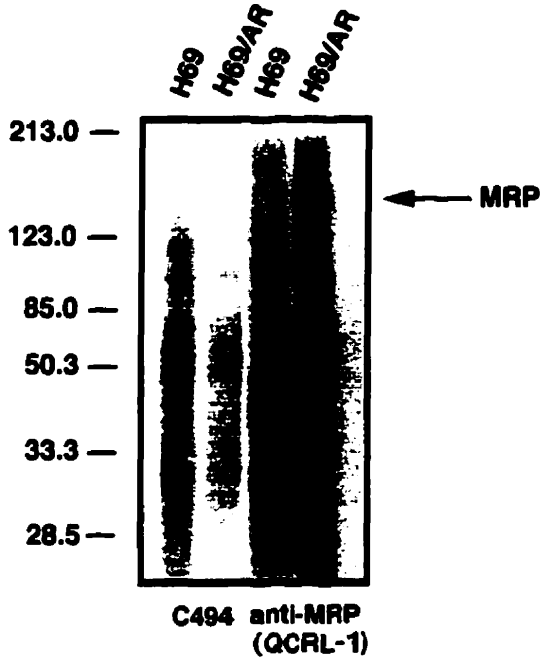


Figure 3. ASA-AQ photoaffinity labells MRP in H69/AR cells. Immunoprecipitation of MRP from the cell lines photoaffinity labelled with [^{125}I] ASA-AQ. 1X10^{6} cells of drug-sensitive (H69) and -resistant (H69/AR) cell lines were photolabelled with 0.25 μM [^{125}I] ASA-AQ and immunoprecipitated with anti-MRP mAb (QCRL-1) (lanes 3 and 4) and C494 mAb (lanes 1 and 2). QCRL-1-MRP complexes were incubated with protein-A sepharose and the protein-antibody complexes were eluted from protein-A beads in SDS sample buffer and resolved on SDS-PAGE. The position of MRP is marked with an arrow head.

A.

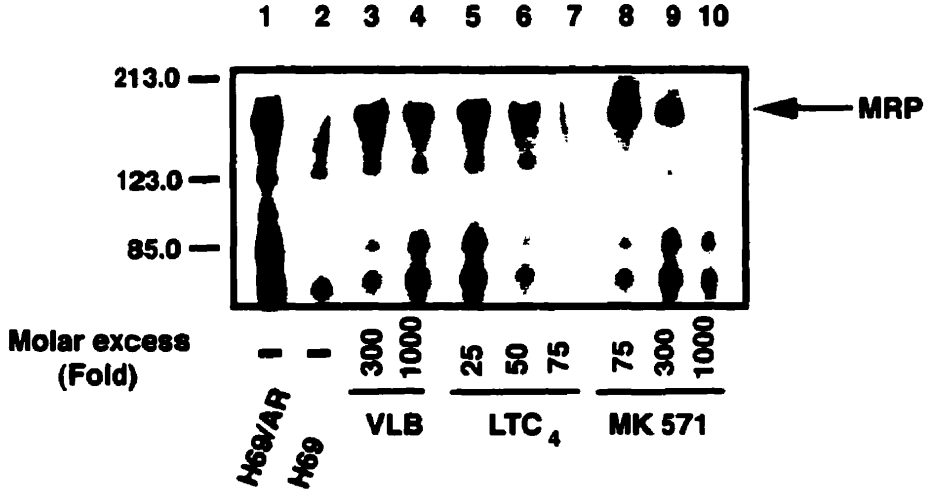


Figure 4a. Effects of Vinblastine, MK 571 and LTC_4 on photoaffinity labelling of MRP with [^{125}I] ASA-AQ in intact cells. H69 or H69/AR cells were photoaffinity labelled with [^{125}I] ASA-AQ in the absence or presence of a molar excess of vinblastine (VLB) (lanes 3 and 4), LTC_4 (lanes 5-7) and MK571 (lanes 8-10). Cells were preincubated for 20 minutes at 37°C in the presence of a molar excess of drugs before addition of [^{125}I] ASA-AQ (0.25 μM). [^{125}I] ASA-AQ (0.25 μM) photoaffinity labelled proteins were isolated by brief centrifugation at 4°C and resolved by SDS-polyacrylamide gel (Fairbanks).

B.

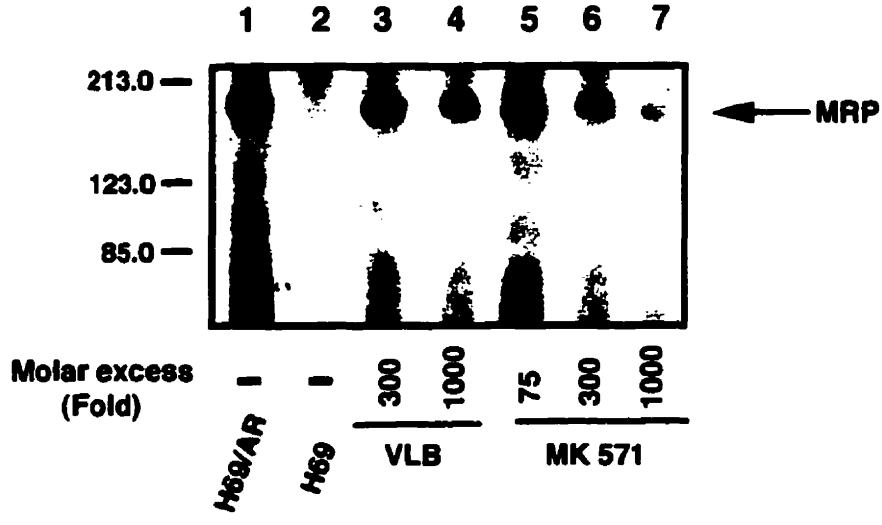


Figure 4b. Effects of Vinblastine and MK 571 on photoaffinity labelling of MRP with [^{125}I] ASA-AQ in plasma membranes. Photoaffinity labelling of plasma membranes of H69 and H69/AR (15μg of protein) was done in the absence or presence of a molar excess of vinblastine (VLB) (lanes 3 - 4) and MK571 (lanes 5 - 7). Notice, only protein of the correct size (190kDa) was labelled in H69/AR plasma membrane preparations.

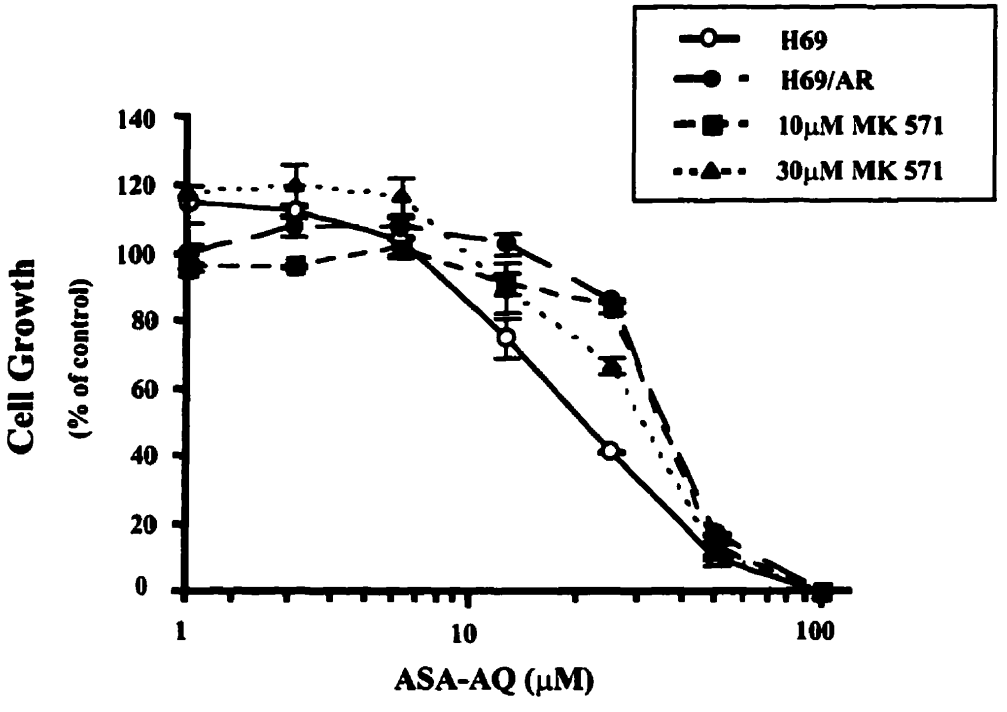


Figure 5. Effects of ASA-AQ on the growth of H69 and H69/AR cells. The effects of ASA-AQ on the growth of H69 and H69/AR cells was determined by incubating cells with increasing concentrations of ASA-AQ alone or together with 10 μM and 30 μM of MK 571. The level of cell growth was determined using MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Each value is mean +/- SD of the two experiments in which triplicates were assayed.

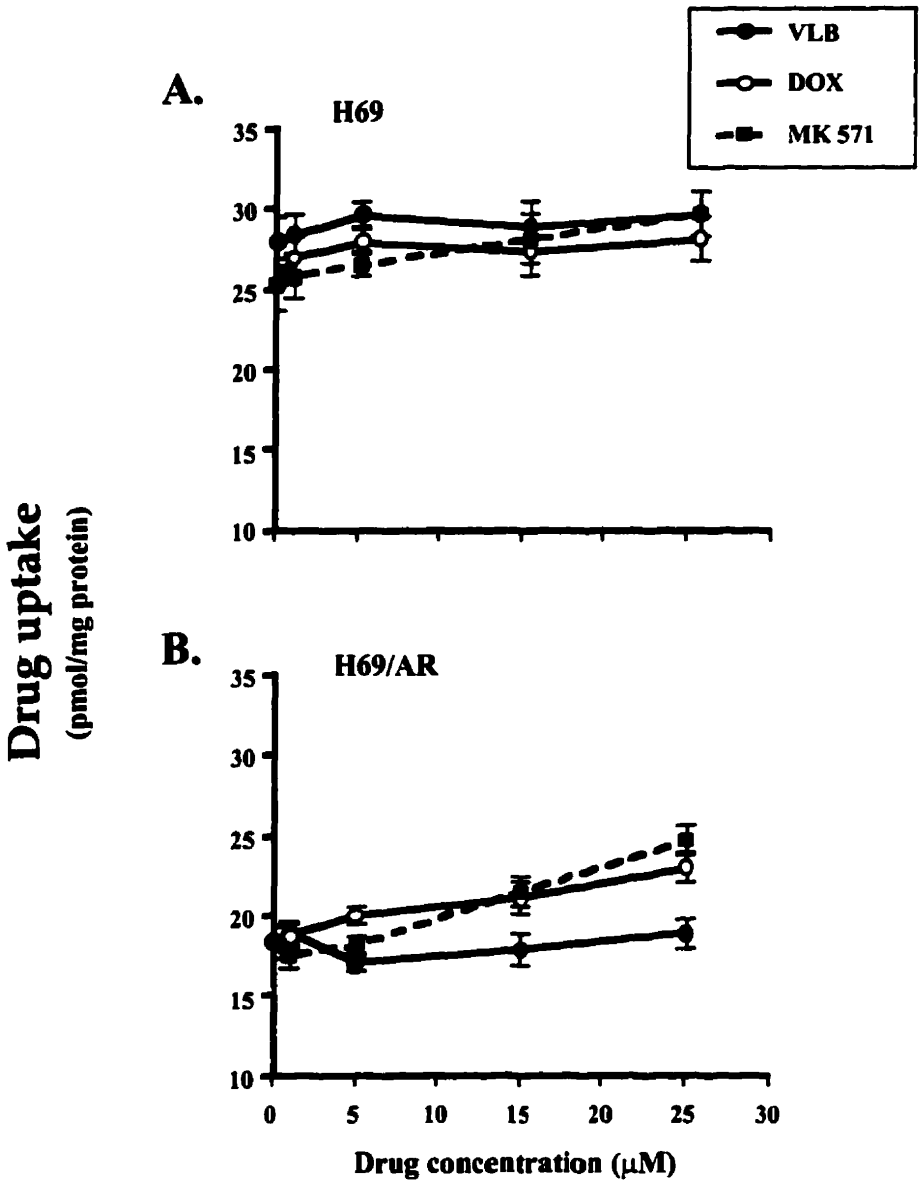


Figure 6. ASA-AQ drug uptake in H69 and H69/AR cells. H69 (A) and H69/AR (B) cells were preincubated in the presence of molar excesses (100 - 500-fold) of vinblastine (Vlb), doxorubicin (Dox) and MK 571 for 30 minutes at 37 °C. [^{125}I] ASA-AQ (50 nM) was added to each sample and the incubation of cells was continued for another 45 minutes at 37 °C. Cells were lysed and the amounts radiollabel accumulated were determined by scintillation countering. Each value is mean +/- SD of the two experiments in which triplicates were assayed.

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STATEMENT OF CONTINUITY

In the previous study (chapter II) we have characterized the interaction of a photoactive quinoline-based drug (ASA-AQ) with multidrug resistant protein (MRP). The cross-resistance of H69/AR cells to ASA-AQ is due to the direct binding of ASA-AQ to MRP1. Furthermore, MRP photoaffinity labelling with ASA-AQ did not require GSH conjugation. Competition of ASA-AQ photoaffinity labelling of MRP with MK571 and LTC_4 demonstrates an interaction of the aminoquinoline analogue at the physiologically relevant site.

The availability of a photoactive radioiodinated drug that binds specifically to MRP could facilitate analysis of MRP drug interactions. Due to structural similarities between ASA-AQ and some other quinine-related compounds (ie. chloroquine), it is of interest to examine biochemical interactions, between MRP and these compounds. Therefore, in the following study (chapter III) we have examined the ability of chloroquine to be transported in SCLC (small cell lung cancer) and human leukaemic cell lines expressing MRP.

CHAPTER III

The Antimalarial Drug, Chloroquine, Binds Directly to, and is a Substrate for the The Multidrug Resistance Protein (MRP1) in Tumor Cells

(Manuscript II)

Marko Vezmar, Leann Tilley and Elias Georges

(In preparation)

ABSTRACT

The multidrug resistance protein (MRP) have been shown to confer resistance to a broad spectrum of structurally and functionally dissimilar drugs that include the leukotriene C_4 (LTC_4), glutathione S-conjugates, amphiphilic anionic, neutral and cationic drugs. Recently, we have demonstrated a direct and specific binding of a quinoline-based photoactive drug to MRP1 in intact cells and plasma membranes. In this study, we show that tumour cell lines that overexpress MRP1 (H69/AR and HL60/AR) are cross-resistant to the antimalarial quinoline-based drug, chloroquine. Furthermore, we demonstrate that the LTD_4 antagonist (MK571) reverses the chloroquine resistance in a dose dependent manner. Using [^{14}C] chloroquine we show that both H69/AR and HL60/AR resistant cells accumulate less drug than their parental drug sensitive cells and that this reduced drug accumulation was due to the ATP-dependent drug efflux. Furthermore, molar excess of doxorubicin and MK571 but not vinblastine completely potentiated [^{14}C] chloroquine accumulation in resistant cells to the same level of that in drug sensitive cells. In addition, using a photoactive quinoline-based drug ([^{125}I] ASA-AQ), we show that molar excess of chloroquine, LTC_4, and MK571, but not vinblastine, inhibited the photoaffinity labelling of MRP by [^{125}I] ASA-AQ. Taken together, these results show for the first time that an important chemotherapeutic drug in

the treatment of malaria is a substrate for MRP1. Moreover, MRP1 mediates resistance to chloroquine by interacting directly with MRP1.

INTRODUCTION

The overexpression of two surface membrane proteins, the P-glycoprotein (P-gp1) and the multidrug resistance protein (MRP1) in tumour cell lines have been shown to confer resistance to a broad spectrum of structurally and functionally dissimilar drugs (for reviews see Lehnert, 1996; and Loe *et al.*, 1996). cDNA transfection studies have confirmed that P-gp1 or MRP1 alone are sufficient for the expression of MDR in otherwise drug sensitive cells (Ueda *et al.*, 1987; Gros *et al.*, 1986; Grant *et al.*, 1993; Cole *et al.*, 1992; Krishnamachary *et al.*, 1993; and Almquist *et al.*, 1995). Furthermore, drug transport studies using P-gp1 or MRP1 transfectant cells have demonstrated that the MDR phenotype is mediated by an energy dependent drug efflux (Gros *et al.*, 1986; Zaman *et al.*, 1994; Breuninger *et al.*, 1995). The normal physiological function of P-gp1 and MRP1 are presently not known; however studies on the tissue distribution of P-gp1 or MRP1 (Thiebaut *et al.*, 1987; Cole *et al.*, 1992; Kruh *et al.*, 1995; and Stride *et al.*, 1996) have suggested a role in the transport of normal cell metabolites and in removal of natural toxins (Chaudhary *et al.*, 1991; Cordon-Cardo *et al.*, 1990; Gottesman *et al.*, 1993). More recently, P-gp1 was shown to function as a lipid translocase of a broad specificity in epithelial LLC-PK1 cells (Van Helvort *et al.*, 1996), while MRP1 has been shown to mediate the transport of several organic anions, glutathione S-conjugates, certain *Vinca* alkaloids and metal oxyanions (Leier *et al.*, 1994; Jedlitschy *et al.*, 1994; Loe *et al.*, 1996; and Muller *et al.*,

et al., 1994).

P-gp1 and MRP1 are members of a large family of ABC-trafficking proteins that are evolutionary conserved (Higgins *et al.*, 1992). However, P-gp1 and MRP1 share little sequence and structural homology than expected based on their functional similarities with regards to drug resistance and substrate specificity (Cole *et al.*, 1992; Cole *et al.*, 1994). MRP1 shares high sequence similarity with the *Leishmania tarentolae* PgpA (Papadopoulou *et al.*, 1994; Callahan *et al.*, 1991) the yeast YCF1 (Szczypka *et al.*, 1994) and rat sulphonylurea receptor (SUR) (Aguilar-Bryan *et al.*, 1995). The latter homologues of MRP1 were shown to confer resistance to some metal oxyanions and antimony (Papadopoulou *et al.*, 1994; Callahan *et al.*, 1991). Moreover, the expression of human MRP1 in YCF1 lacking yeast mutant restored cadmium resistance and the transport of glutathione S-conjugate (Szczypka *et al.*, 1994). Similarly, P-gp1 homologues have been isolated from chloroquine resistant *Plasmodium falciparum (pfmdr1)* (Van Es *et al.*, 1994), vinblastine resistant *Leishmania* (Ouellete *et al.*, 1991), and emetine resistant *Entamoeba hystolytica* (Samuelson *et al.*, 1990). However, the role of P-gp1 homologues in the resistance to chloroquine, vinblastine and emiten in parasites has not been fully characterized. Of interest is the role of *pfmdr1* in chloroquine resistance which to date remains controversial.

We have previously demonstrated that a photoactive quinoline-based drug (ASA-AQ) binds specifically to MRP1 (Chapter 2). Furthermore,

the photoaffinity labelling of MRP1 with ASA-AQ was inhibited with molar excess of LTC_4 and MK571. Both of these compounds are substrates for MRP1 (Gekeler *et al.*, 1995). The inhibition of MRP1 photoaffinity labelling with MK571, also quinoline-based drug, lead us to speculate if other quinoline-based drugs are also substrates for MRP1. Furthermore, earlier reports by Cole *et al.* (1989) had demonstrated that MRP expressing cells show cross-resistance to chloroquine. However, it was not clear from those studies how chloroquine resistance was mediated in MRP expressing cells nor was it determined if MRP1 was involved in the chloroquine resistance. In this study it was of interest to determine how chloroquine resistance in H69/AR was modulated and if MRP1 plays a major role.

MATERIALS AND METHODS

Materials - Iodine-125 (100.7 mCi/ml) and [^{14}C] chloroquine (Specific act. 52 mci/mmol) were purchased from Amersham Biochemical Inc. (Mississauga, Ontario, Canada). The LTD_4 receptor antagonist MK571 was kindly provided by Dr. A.W. Ford-Hutchinson (Merck-Frosst Centre for Therapeutic Research, Point Claire-Dorval, Quebec, Canada). Leukotriene C_4 (LTC_4) was purchased from Cayman Chemical Co. (Ann Arbor, MI). The human Small Cell lung cancer lines H69 and H69/AR were a kind gift from Dr. Susan P. C. Cole (Cancer Research Laboratories, Queen's University, Kingston, Ontario, Canada).

Cell Culture and Plasma Membrane preparation - Drug sensitive (H69) and resistant cells (H69/AR) 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 essentially as described by Lin *et al* (1987). In brief, cells were collected by low speed centrifugation and washed three times with ice-cold Phosphate buffer saline, pH 7.4 (PBS). Cells were homogenized in 50 mM mannitol, 5mM Hepes and 10mM Tris-HCl, pH 7.4 (containing 2mM 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 precipitated by high speed centrifugation at 100,000X g for 1 hour at 4 °C in a Beckman SW28 rotor. The enriched plasma membrane pellet was washed with 10 mM Tris-HCl, pH 7.4 and resuspended in the same buffer containing 250 mM sucrose. Membrane fractions were stored at -80°C if not immediately used. Protein concentrations were determined by the Lowry method (Lowry *et al.*, 1951).

Photoaffinity labelling and SDS Gel electrophoresis - For photoaffinity labelling of cells, H69 or H69/AR cells were washed with PBS and preincubated for 20 minutes at 37°C in the presence of a molar excess of vinblastine, chloroquine, LTC_4 or MK571 before the addition of [^{125}I] ASA-AQ (0.25 μM). Cells were incubated at the room temperature in the dark for 30 minutes and then transferred to ice for 10 minutes. Following the latter incubation on ice, cells were irradiated for 10 minutes on ice with a UV source at 254 nm (Stratagene UV crosslinker, Stratagene, La Jolla, CA). Cells were centrifuged for 5 minutes at 2500 rpm in a microfuge, the hot supernatant was removed and cells were lysed in 20 μl of 50 mM Tris (pH 7.4) containing 1% NP40, 5 mM $MgCl_2$ and protease inhibitors (3 μg/ml of leupeptin and 2 mM PMSF). [^{125}I] ASA-AQ labelled proteins were isolated by

brief centrifugation at 4°C. Photoaffinity labelling of plasma membrane fractions, 15 μg aliquots of membranes prepared from H69 and H69/AR cell lines were photoaffinity labelled as described above. Proteins from intact cells photoaffinity labelled with [^{125}I] ASA-AQ or plasma membranes were mixed with 1:5 (V/V) of buffer I [10 mM Tris-HCl, pH 8.0 containing 2% SDS, 50 mM dithiothreitol (DTT), 1 mM EDTA] and equal volume of buffer II [2X buffer I and 9 M urea]. The solubilized proteins were then resolved by SDS PAGE using the Fairbanks gel system with some modifications (Fairbanks *et al.*, 1971) . Gel slabs containing the resolved proteins were fixed in 50% methanol, dried and exposed to XAR Kodak film at -70°C.

Cytotoxicity assay - Cells were harvested in the exponential growth phase and plated (1.0 - 2.0 10^4 cells/well) into 96-well plates. Cells were allowed to recover for 24 hours before the addition of chloroquine in the absence or presence of micromolar concentrations of MK571. Cells were allowed to grow in the dark for four days at 37 °C before the addition of the MTT dye (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide). The plates were incubated in the dark for 4 hours at 37 °C and the coloured crystals formed from the tetrazolium salt were solubilized by the addition of 50 μl of 10% Triton X-100 in 0.01 N HCl followed by vigorous pipetting. The 96-well plates were heated in the microwave oven for 1 minute at the low power setting and 10 μl of ethanol was added to disperse the formed bubbles. Plates

were read at 570 nm using an LKB microtiter plate reader.

Drug Transport - For drug accumulation, cells ($1X10^6$) were washed three times in PBS containing 5 mM D-glucose and incubated for 45 minutes with 1.0 μM [^{14}C] chloroquine (Specific act. 52 mCi/mmol) in the absence or the presence of a molar excess of vinblastine, MK571 or doxorubicin. The incubation was stopped with the addition of 1 ml of ice cold PBS and cells were then washed three times with the same solution. The cell pellets were lysed in 100 μl of 1M NaOH followed by neutralization with equal volume of 1M HCl and the accumulated radiolabel was determined by flowmetry using 1219 Racbeta model counters (LKB, Wallace).

For drug efflux, cells were loaded with 10 μM [^{14}C] chloroquine in the presence of 10 mM sodium azide for 30 minutes at 37°C. Cells were then washed with 5 mM D-glucose and incubation was continued for 60 minutes at 37 °C. Samples were collected at the different time intervals up to 60 minutes. Cells were washed in ice cold phosphate buffer saline (pH 7.2), and the cell pellets were lysed and processed as indicated above.

RESULTS

Cross-resistance to chloroquine in MDR cells with MRP1 - A previous study had shown that adriamycin selected SCLC cells (H69/AR) are cross-resistant to chloroquine (Cole *et al.*, 1989). However, it was not clear from that study how was the resistance to chloroquine modulated nor was it determine if MRP is involved in this cross-resistance. Our recent observation that a photoactive quinoline-based drug binds specifically to MRP1 in H69/AR cells promoted us to reexamine the cross-resistance of H69/AR cells to chloroquine. Figure 1 shows the effects of increasing concentrations of chloroquine on the growth of H69/AR cells and their parental H69 cell lines. The results in figure 1 show H69/AR to be more resistant to chloroquine compared to the H69 drug sensitive cells (IC_{50} of 135 μM versus 42 μM chloroquine, respectively).

The leukotriene LTD_4 antagonist MK571 has been previously shown to reverse MRP-mediate drug resistance (Gekeler *et al.*, 1995). To determine if chloroquine cross-resistance is mediated by MRP, cells were cultured in the presence of increasing concentrations of chloroquine and 10 μM or 30 μM MK571. The results in figure 1 show that MK571 reverses the resistance of H69/AR cells to chloroquine in a dose dependent manner. The resistance of H69/AR cells to chloroquine in the presence of 30 μM of MK571 was modulated by a factor of 1.65. Similar concentrations of MK571 did not affect

the sensitivity of H69 parental cells to chloroquine (data not shown).

Chloroquine transport in MDR cells with MRP1- Although the above results demonstrated a correlation between MRP overexpression and reduced sensitivity to chloroquine in H69/AR cells, it was not clear if this was due to differences in drug transport or metabolism. Figure 2a and 2b show the accumulation of [^{14}C] chloroquine in drug resistant (H69/AR and HL60/AR) and sensitive (H69 and HL60) cells. Interestingly, both H69/AR and HL60/AR accumulated less [^{14}C] chloroquine than H69 and HL60 cells (Figure 2a and 2b). Moreover, the decreased accumulation of [^{14}C] chloroquine in resistant cells was completely reversed when cells were preincubated for 30 minutes with sodium azide and 2-deoxy-glucose (Figure 2c and 2d, respectively).

To determine if the reduced chloroquine accumulation in resistant cells was due to enhanced efflux, cells were loaded with [^{14}C] chloroquine (see methods) and drug efflux was measured in the absence or in the presence of sodium azide and 2-deoxy-glucose. The results in figure 3a show a more rapid efflux of [^{14}C] chloroquine from HL60/AR than from HL60 cells (Figure 3a). By contrast, there was no enhanced efflux of [^{14}C] chloroquine from H69/AR cells compared to the parental H69 cells (Figure 3b). Interestingly, sodium azide inhibited the efflux of chloroquine from both sensitive (HL60 and H69) and resistant (HL60/AR and H69/AR) cells

(Figure 3). To determine if observed reduced accumulation but no efflux of [^{14}C] chloroquine in H69/AR is due to an enhanced metabolism of the drug, cells were incubated with [^{14}C] chloroquine and the [^{14}C] radiolabel was extracted as previously described (Nare *et al.*, 1994). The mobility of the extracted radiolabeled material from H69/AR or H69 cells was compared to the unaltered [^{14}C] chloroquine by HPLC using C_{18} column (Nare *et al.*, 1994). Analysis of the HPLC results showed identical elution times of the extracted radiolabel as that of [^{14}C] chloroquine (data not shown). Thus, differences in [^{14}C] chloroquine transport between H69/AR and HL60/AR is not due to the metabolism or organic modification of the drug.

The LTD_4 receptor antagonist has been shown to inhibit the transport of LTC_4, a normal substrate of MRP1 and reverse MRP1-mediated MDR (Gekeler *et al.*, 1995). In this study, figure 1, we have also demonstrated that MK571 reverses H69/AR cross-resistance to chloroquine in a dose dependent manner. Therefore, it was of interest to know if chloroquine accumulation in H69/AR cells is potentiated by MK571 and other substrates of MRP1. Figure 4 shows the effects of MK571, doxorubicin or vinblastine on [^{14}C] chloroquine accumulation in H69 and H69/AR cells. Molar excess of MK571 and doxorubicin but not vinblastine completely reversed the reduced accumulation of [^{14}C] chloroquine in H69/AR cells (figure 4b). The same drugs had no effect on [^{14}C] chloroquine accumulation in H69 drug sensitive cells (figure 4a).

Chloroquine interacts directly with MRP1 - We have previously demonstrated a direct and specific binding between MRP1 and a photoactive quinoline-based drug (Chapter 2). To determine if chloroquine interacts directly with MRP1, [^{125}I] ASA-AQ was used to photoaffinity label MRP1 in the presence of chloroquine, MK571, LTC_4 and vinblastine (figure 5). The results in figure 5a show specific photoaffinity labelling of MRP1 with [^{125}I] ASA-AQ in H69/AR but not in H69 cells. Moreover, molar excess of chloroquine, MK571, and LTC_4 but not vinblastine inhibited MRP1 photoaffinity labelling with [^{125}I] ASA-AQ in intact H69/AR cells (figure 5a). Similar photoaffinity labelling results of MRP1 were also obtained using plasma membranes from H69 and H69/AR cells (figure 5b). The latter results suggest that [^{125}I] ASA-AQ does not require glutathione S-conjugation for binding to MRP1 or efflux from H69/AR cells.

DISCUSSION

In this study we show that H69/AR SCLC are cross-resistant to chloroquine and that this resistance is reversible in the presence of the LTD_4 receptor antagonist, MK571. These results confirm an earlier observation with regard to the cross-resistance of H69/AR cells to chloroquine (Cole *et al.*, 1989) and extend that finding by demonstrating its reversibility with MK571. Given the role of MK571 as a modulating agent of MRP-mediated MDR (Gekeler *et al.*, 1995), the latter finding implicate MRP1 involvement in the cross-resistance to chloroquine in H69/AR cells. Further, we show that chloroquine accumulation is less in two MDR cell lines (H69/AR and HL60/AR) that overexpress MRP1 and that the reduced accumulation is energy-dependent. In addition, molar excesses of doxorubicin or MK571 but not vinblastine increased chloroquine accumulation in resistant cells to the same level as in the parental drug sensitive cells. The failure of vinblastine to potentiate [^{14}C] chloroquine accumulation to the same levels as aforementioned compounds is accounted for lack of interaction to MRP (Cole *et al.*, 1994).

The drug efflux results using H69/AR cells are consistent with the reduced energy dependent transport of chloroquine. However, rather unexpectantly were the efflux results from HL60/AR cells which showed energy-dependent decrease of chloroquine accumulation relative to the

parental HL60 cells but without detectable drug efflux (Figure 3). One interpretation of difference in chloroquine efflux between the cell lines could be accounted for differential subcellular distribution of MRP. While MRP in H69/AR cells has been reported to be predominantly in plasma membrane (Cole *et al.*, 1993; Coley *et al.*, 1993), although it can also be detected in some intracellular membrane fractions, MRP distribution in HL60/AR cells is mainly in the endoplasmic reticulum (Marquardt *et al.*, 1992). Since active efflux is an exclusive property of the plasma membrane (Gerlach *et al.*, 1986), it is believed that MRP acts as an energy-dependent plasma membrane pump (Zaman *et al.*, 1994) in H69/AR cells. Therefore, the lack of detectable chloroquine efflux from HL60/AR cells may be due to the accumulation of chloroquine in endosomal vesicles via MRP1 followed by endosomal transport. It is interesting to note in this respect that the association of reduced drug accumulation and enhanced drug efflux with the overexpression of MRP1 is less consistent than that seen for P-glycoprotein (Coley *et al.*, 1993; Marquardt *et al.*, 1992; McGrath *et al.*, 1989; and Marsh *et al.*, 1986).

Using a photoactive analogue of a quinoline-based compound (ASA-AQ), we have previously (Chapter 2) shown that ASA-AQ binds directly and specifically to MRP-1 in H69/AR cells. The demonstration in this study that chloroquine inhibits the photoaffinity labelling of MRP1 in intact H69/AR cells or plasma membranes similarly to LTC_4, or MK571 shows collectively

that chloroquine is a substrate for MRP1. Taken together, these results are the first demonstration that MRP-1 mediates the transport of the antimalarial drug, chloroquine, directly.

Earlier studies have shown tumour cell lines that overexpress P-gp also show cross-resistance to chloroquine (Lincke *et al.*, 1995). However, it is not known if the observed cross-resistance to chloroquine in previously examined P-gp1 positive cells do not co-express MRP1. Several reports have now demonstrated the co-expression of MRP1 and P-gp1 in *in vitro* selected MDR cell lines (Slapak *et al.*, 1994; Brock *et al.*, 1995). Furthermore, using several P-gp-positive MDR selected cell lines, we did not observe a significant and consistent cross-resistance to chloroquine (data not shown). Moreover, the cross-resistance to chloroquine was not reversible with verapamil or cyclosporin A (unpublished results).

It has been known for some time now that chloroquine resistant malaria accumulate less drug than sensitive ones. Furthermore, the reduced chloroquine accumulation was energy-dependent and was reversed with the calcium channel blocker verapamil, although the latter was not consistent (Martin *et al.*, 1987). However, the role of a P-glycoprotein homologue (pfmdr1) in chloroquine resistance in *Plasmodium falciparum* has been controversial. Therefore, the findings of this study with regard to the possible role of an MRP1 homologue in *Plasmodium falciparum* may be of interest to our understanding of chloroquine resistance in malaria.

In addition to the importance attached to chloroquine as an antimalarial drugs and the possible role of MRP-1 in malaria drug resistance to chloroquine, these finding also identify another quinoline-based drug, in addition to MK571 (Gekeler *et al.*, 1995) and ASA-AQ (Chapter 2), as a substrate for MRP1.

ACKNOWLEDGEMENTS

We thank Dr. Leann Tilley of the Walter and Eliza Hall Institute of Medical Research for providing the photoactive analogue N-{4-[1-hydroxy-2 (dibutylamino)- ethyl] quinolin-8-yl}-4-azido-2-salicylamide (ASA-AQ), and Dr. Susan Cole for providing Small Cell Lung Cancer (SCLC) cell lines (H69 and H69/AR). This research was supported by a grant from the National Cancer Institute of Canada.

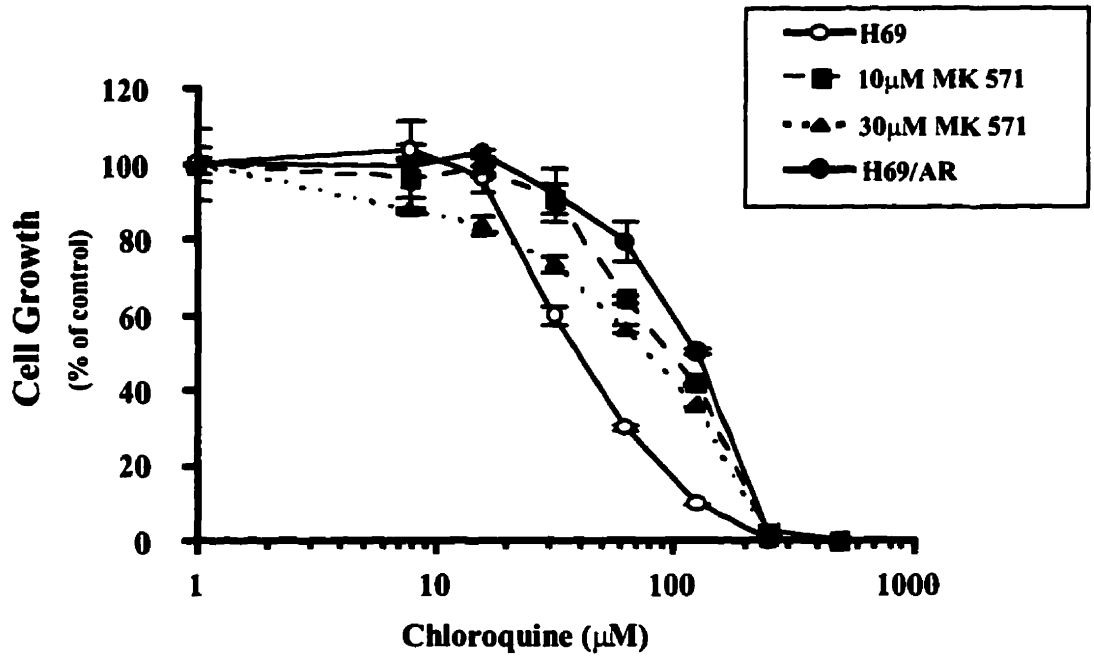

Figure 1. Chloroquine cross-resistance and reversal with MK571 in H69 and H69/AR cell lines. Cells were incubated in increasing concentration of chloroquine (1 μM to 500 μM) in the absence or presence of 10 μM MK571 and 30 μM MK571. The IC_{50} values were determined from the graph. Each value is mean +/- SD of the two experiments in which triplicates were assayed.

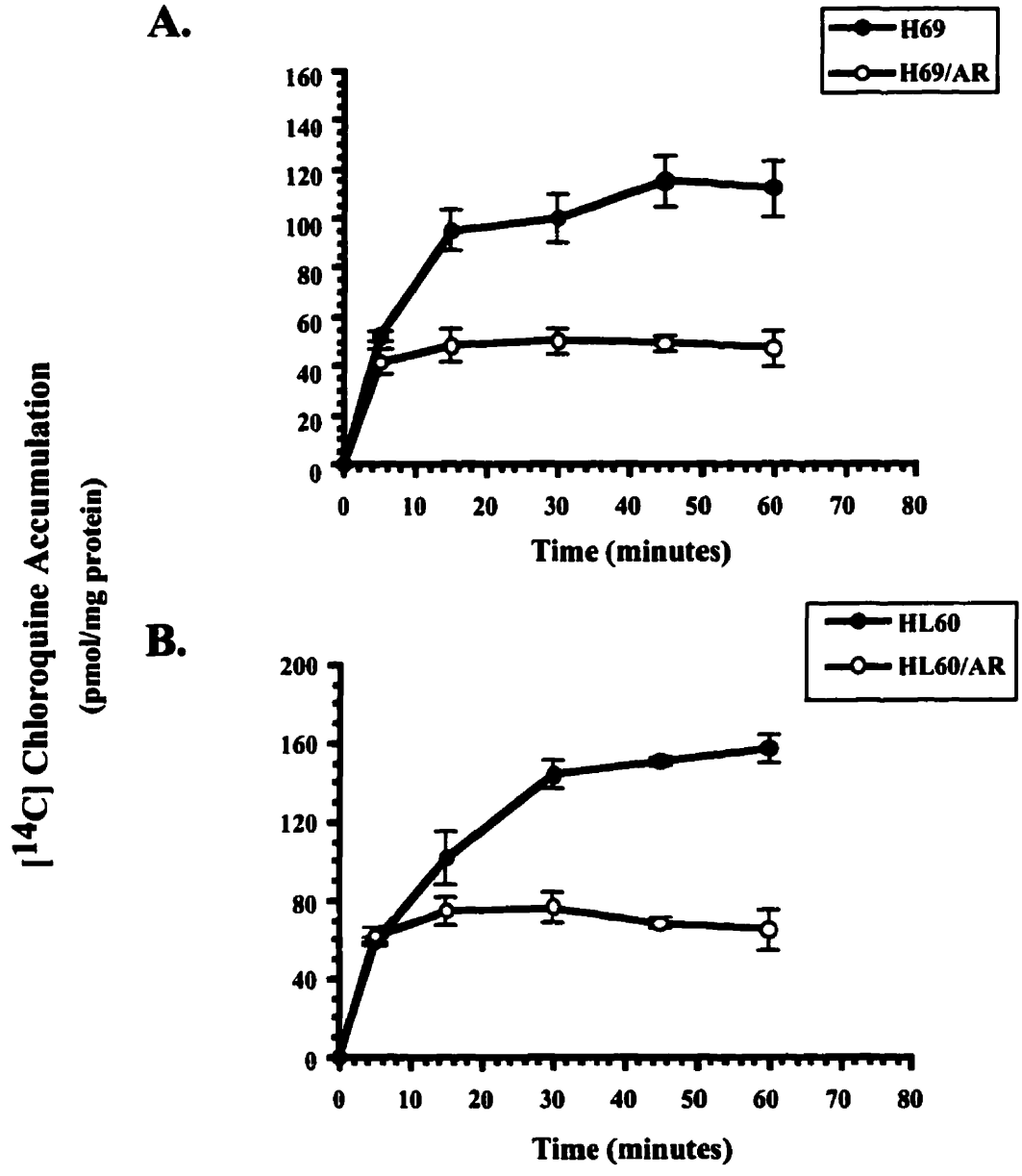

Figure 2 A,B. [14C] chloroquine transport in sensitive and resistant cells. Drug sensitive (H69) and -resistant H69/AR (**A**), and drug-sensitive HL60 and -resistant HL60/AR (**B**) cells were incubated in the presence of 1 μM [14C] chloroquine at 37°C. Samples were removed and the associated radioactivity was determined at five to sixty minutes by detection in scintillation counter. Each value is mean +/- SD of the three experiments in which triplicates were assayed.

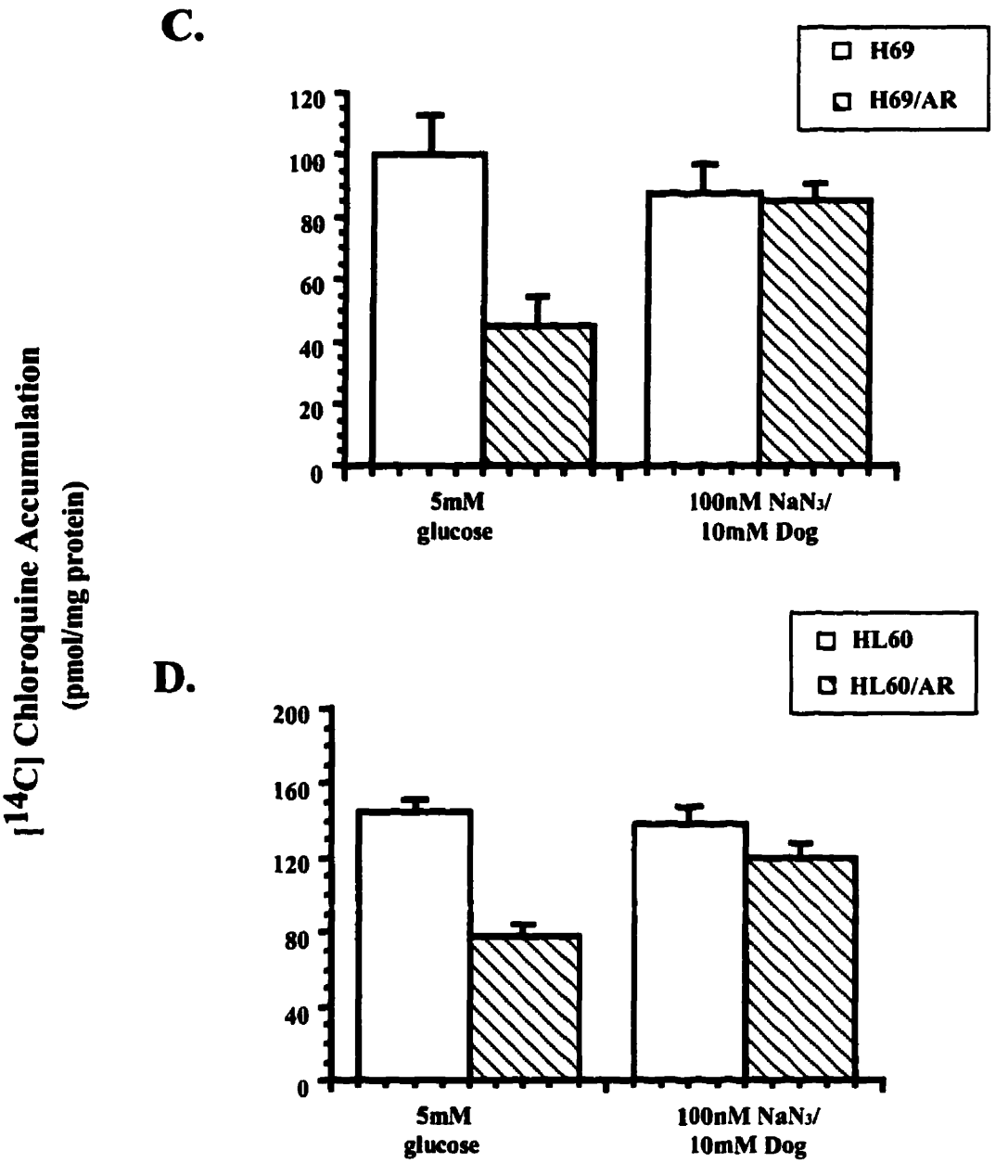

Figure 2 C,D. [14C] chloroquine transport in sensitive and resistant cells., Drug sensitive (H69) and -resistant H69/AR (C), and drug-sensitive HL60 and -resistant HL60/AR (D) cells were incubated in the presence of 1 μM [14C] chloroquine in the absence and in the presence of 100 nM sodium azide and 10 mM 2-deoxy-glucose. Each value is mean +/- SD of the two experiments in which triplicates were assayed.

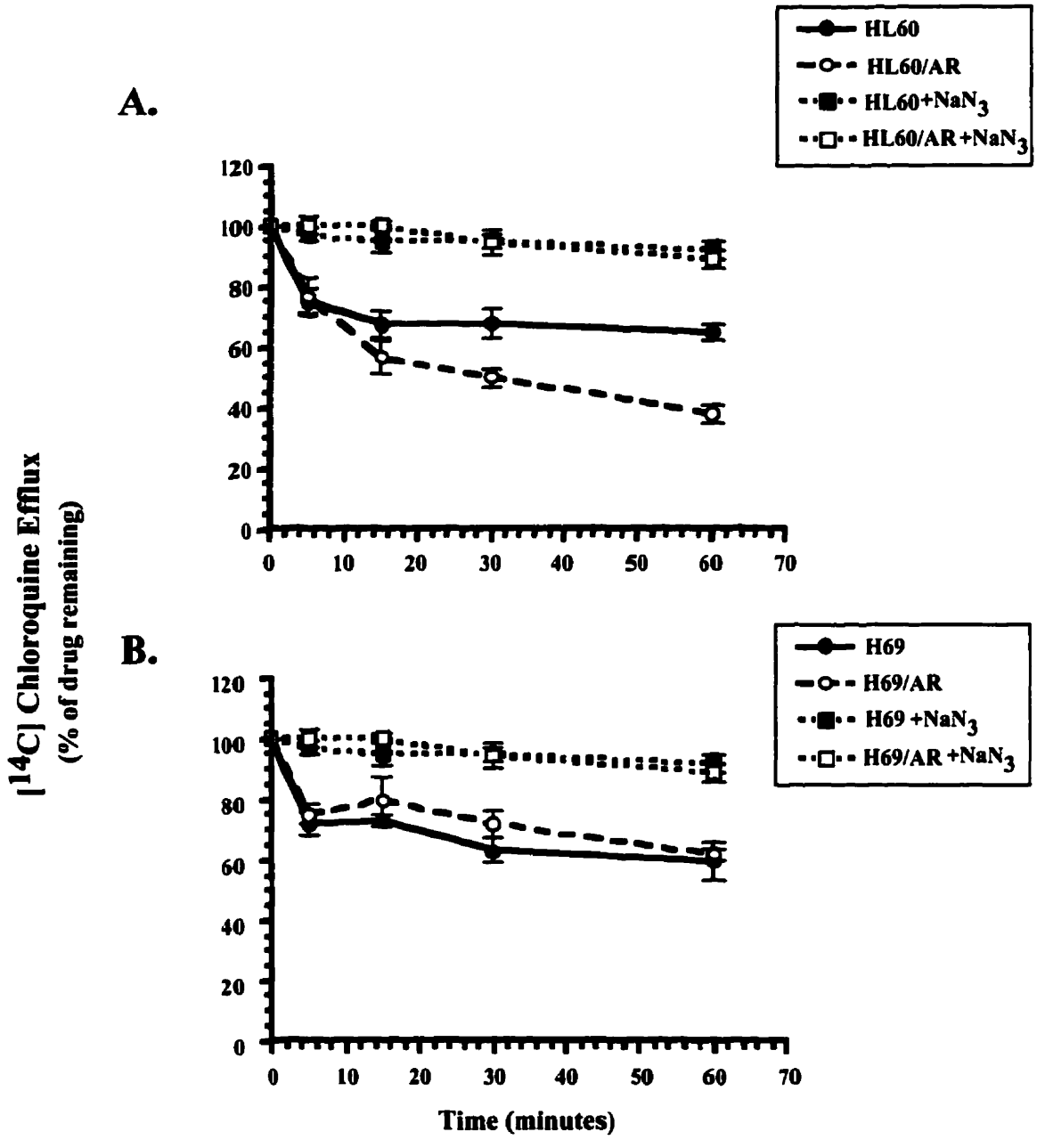

Figure 3. [14C] chloroquine efflux from drug sensitive and resistant cells. HL60 or HL60/AR (**A**), and H69 or H69/AR (**B**) cells were loaded with 10 μM [14C] chloroquine in the absence or presence of 10 mM sodium azide for 30 minutes after which drug extrusion was potentiated with administration of 5 mM D-glucose to one half of the sample. For the remaining half of the samples, [14C] chloroquine levels were determined in presence of sodium azide. Each value is mean +/- SD of the two experiments in which triplicates were assayed.

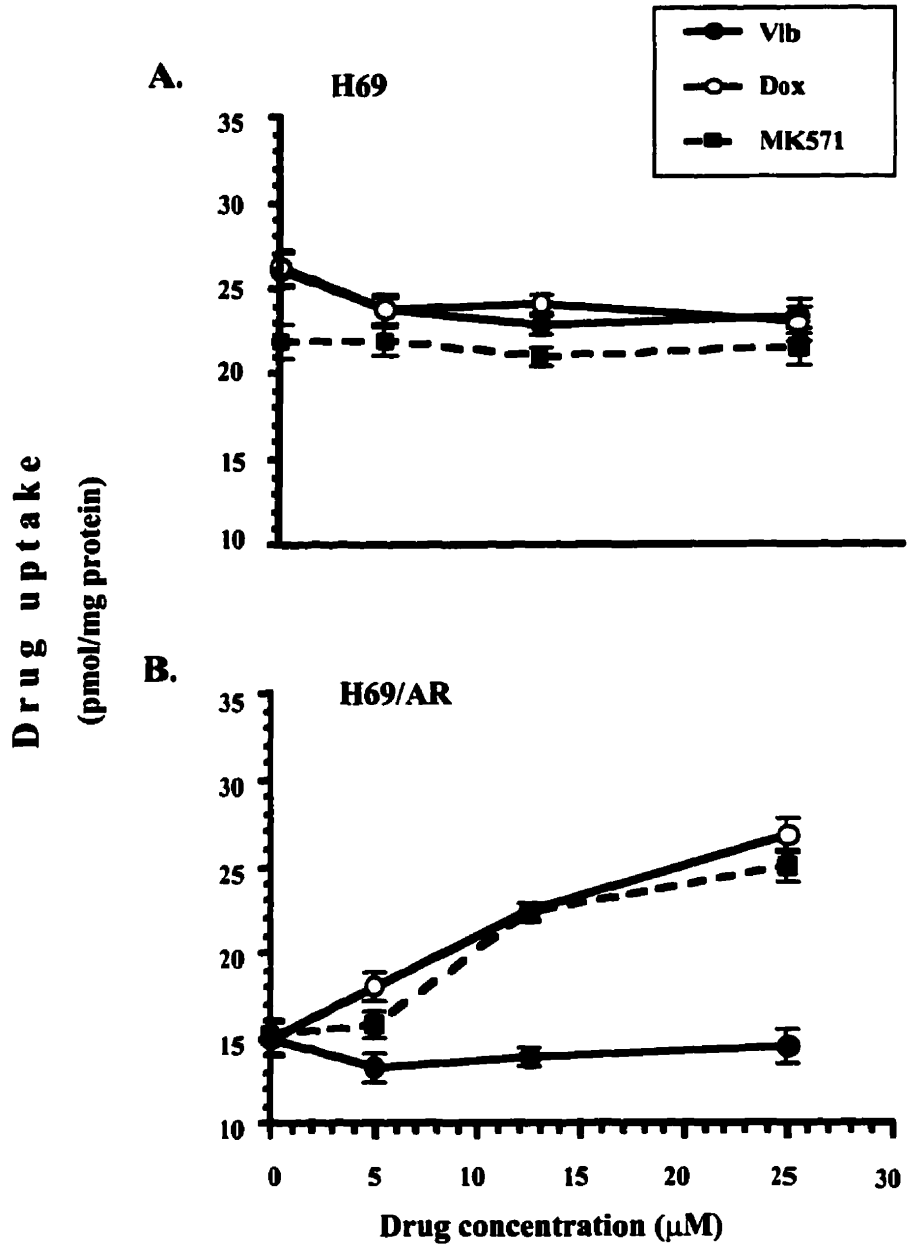

Figure 4. Effects of drugs on chloroquine transport in parental H69, and drug-resistant H69/AR cell lines. H69 (**A**) or H69/AR (**B**) cells were preincubated in the presence of a molar excesses (100-500-fold) of vinblastine (Vlb), doxorubicin (Dox) and MK 571 for 45 minutes prior to the addition of 1μM of [^{14}C] chloroquine. Total accumulation of [^{14}C] chloroquine was expressed as pmol of [^{14}C] chloroquine per mg of protein. Each value is mean +/- SD of the two experiments in which triplicates were assayed.

A.

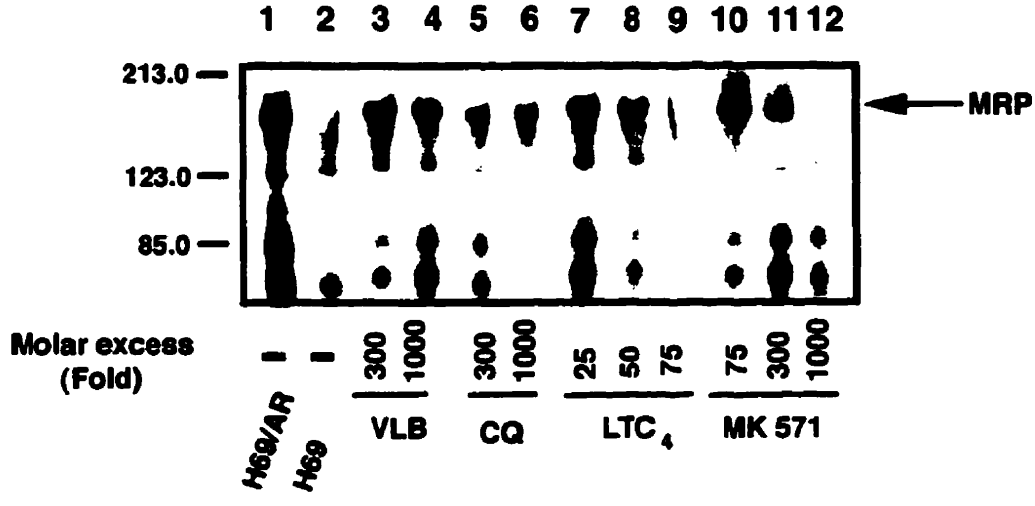

Figure 5a. Effects of chloroquine, vinblastine, MK 571 and LTC_4 on photoaffinity labelling of MRP in intact cells. H69 or H69/AR cells were photoaffinity labelled with 0.25 μM of [^{125}I] ASA-AQ in the absence or presence of 300 and 1000-fold molar excess of vinblastine (VLB) (lanes 3-4), chloroquine (CQ) (lanes 5-6), 25 - 75-fold of LTC_4 (lanes 7-9) or 75 - 1000 fold of MK571 (lanes 10-12). photoaffinity labelled proteins were isolated by brief centrifugation at 4°C and resolved by SDS-polyacrylamide gel (Fairbanks).

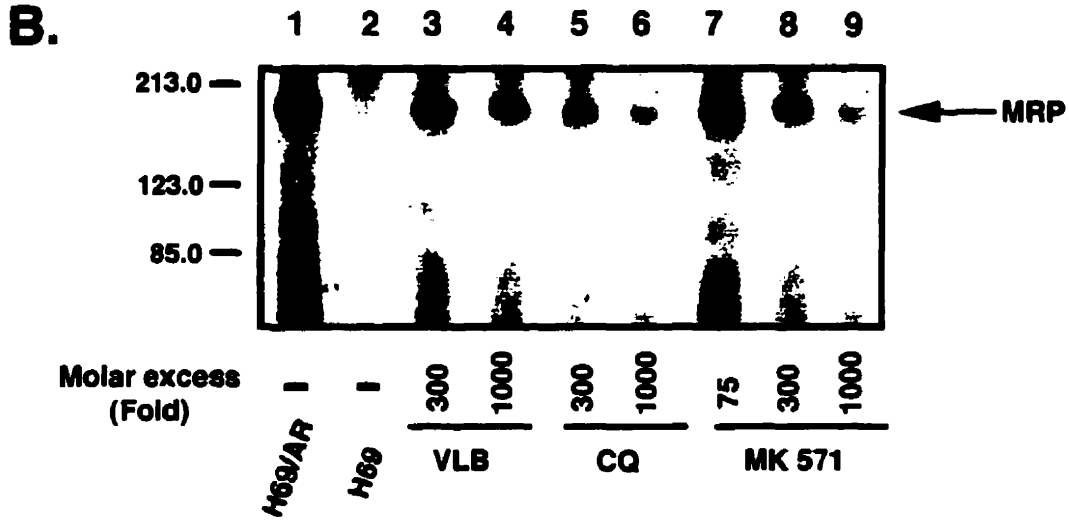

Figure 5b. Effects of chloroquine, vinblastine, MK 571 and LTC_4 on photoaffinity labelling of MRP in plasma membrane. Similarly, plasma membranes from H69 and H69/AR cells (15μg of protein) were photoaffinity labelled with 0.25 μM [^{125}I] ASA-AQ in the absence and the presence of 300 to 1000-fold molar excess of vinblastine (VLB) (lanes 3-4), chloroquine (CQ) (lanes 5-6) or MK571 (lanes 7-9).

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GENERAL DISCUSSION and FUTURE EXPERIMENTS

In the preceding studies we have evaluated the biochemical interaction of quinine-related compounds to the multidrug resistant protein (MRP1). The results of this thesis describe the first demonstration of direct binding of two quinoline-based compounds, aminoquinoline photoactive analogue (ASA-AQ) and antimalarial drug chloroquine, to MRP1.

Results presented herein, which suggest that quinoline containing compounds are likely to be substrates for MRP1, are consistent with evidence regarding MRP1 interactions with the anionic quinoline MK571 which is a substrate for MRP1. The ability of MK571 and LTC_4 to compete with ASA-AQ photoaffinity labelling of MRP1 suggests interaction of ASA-AQ with MRP1 at the physiologically relevant site, while competition of antimalarial drug, chloroquine, for photoaffinity labelling of MRP1 with ASA-AQ shows direct interaction to MRP1. The ability of quinoline-based compounds to interact with MRP1 is an interesting and provide direct evidence for the interaction of MRP1 with non-glutathione-S-conjugated drugs.

Clearly more research is required to determine if ASA-AQ binds to the same, or conformationally linked sequences as that of LTC_4 or MK571. The availability of photoactive radioiodinated drugs that bind specifically to MRP1 could facilitate future analysis of MRP drug interactions. For example,

it would be of interest to identify the exact amino acid sequence of a drug binding site(s) of MRP. Moreover, it would be of interest to determine if post-translational modifications of MRP1 (ie. phosphorylation) alters its ability to interact with ASA-AQ.

Finally, the findings of this study with regard to the possible role of an MRP homologue in *Plasmodium falciparum* may be of interest to our understanding of chloroquine resistance in malaria.

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IMAGE EVALUATION
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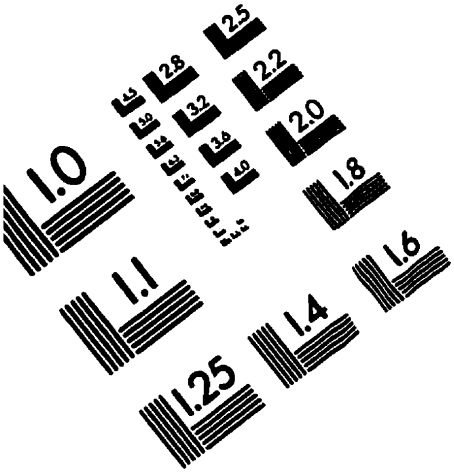

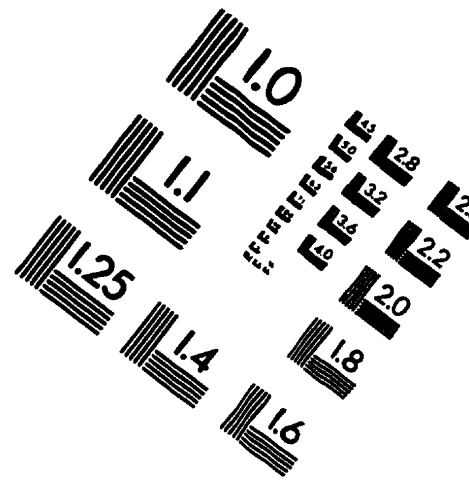

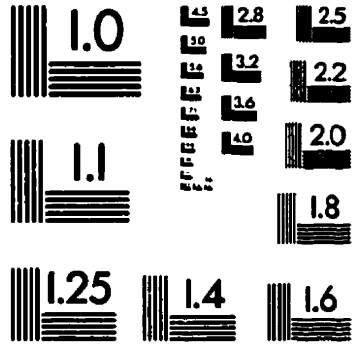

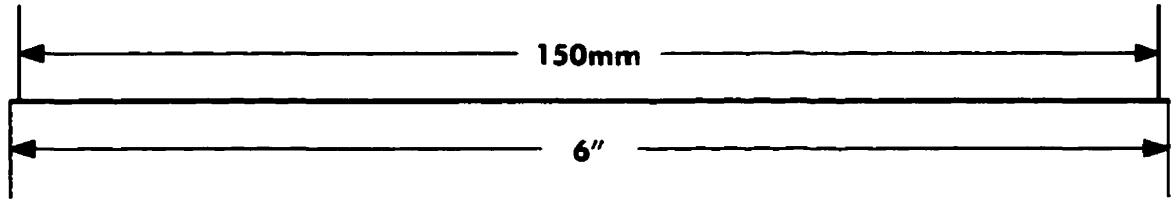

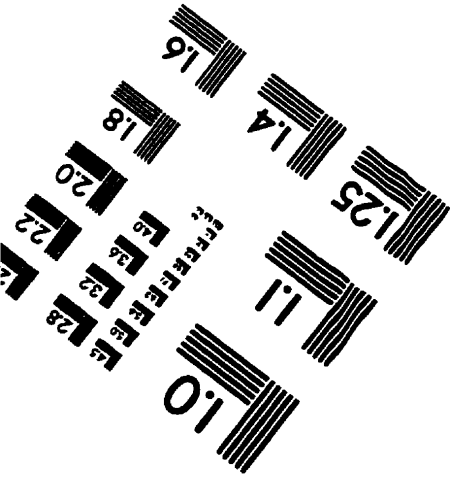

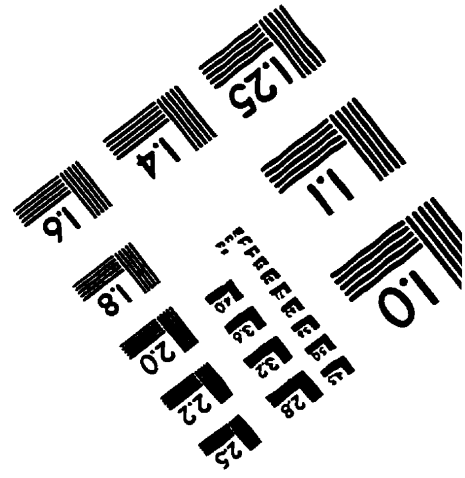

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