INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

IDENTIFICATION AND CHARACTERIZATION OF A NOVEL MECHANISM OF MULTIDRUG RESISTANCE IN TUMOUR CELLS

YING WANG

Institute of Parasitology

McGill University, Montreal

Submitted February, 1998

This thesis submitted to the Faculty of Graduate Studies
and Research in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

Short title

Novel Mechanism of Multidrug Resistance in Tumour Cells

ABSTRACT

The development of multidrug resistance (MDR) in tumour cells to a wide range of anticancer drugs has become a major obstacle in the chemotherapeutic treatment of cancer. Molecular characterization of MDR tumour cells has led to the identification of several cell-based genetic alterations including the overexpression of a membrane protein, P-glycoprotein (P-gp). P-gp is a ATP dependent drug efflux pump and P-gp ATPase activity has been demonstrated to be essential in drug transport. In an effort to understand how P-gp ATPase activity is coupled to drug binding and transport, we examined the effects of N-ethylmaleimide (NEM) , a potent inhibitor of P-gp ATPase, on P-gp drug binding and transport. Our results show that short term treatment of MDR cells with NEM led to a concentration-dependent increase in P-gp drug binding and phosphorylation. In addition, NEM increases [^{3}H]-vinblastine accumulation in drug resistant cells but not in sensitive cells. Our study suggests that inhibition of P-gp ATPase activity, and not increased phosphorylation of P-gp by NEM, is responsible for the observed increase in P-gp-drug binding.

Selection of tumour cell lines *in vitro* has led to multiple cellular changes that may mediate drug resistance to anticancer drugs. The role of other mechanisms, in addition to P-gp and multidrug resistance protein (MRP) in drug resistance, is supported by evidence from studies with tumour cell lines and clinical tumours. In an effort to identify other cellular changes that may be important in tumour drug resistance to anticancer drugs, we have used a differential immunodot blot method to isolate monoclonal antibodies that bind to proteins in drug resistant but not in drug sensitive cells. By using the immunodot blot method, we have isolated a monoclonal antibody (IPM96) which recognized a 40 kDa protein (P-40) in several MDR cell lines. The expression of P-40 is concurrent with the level of drug resistance. Biochemical characterization showed P-40 to be associated with the cell membrane and in the soluble fraction. Molecular cloning of P-40 cDNA revealed that P-40 is identical to annexin I, a substrate for the epidermal growth factor receptor tyrosine kinase. The observed increase in P-40 (or annexin I) protein levels in drug resistant cells is due to the elevation of P-40 transcripts. The pharmacological characterization of P-40 cDNA transfectants (P-40-MCF-7) has demonstrated that overexpression of P-40 in drug sensitive cells is capable of conferring drug resistance to adriamycin, actinomycin D , Taxol and cisplatin. Taken together, our study provides convincing evidence that annexin I is important in the development of drug resistance in cancer cells. In addition, it suggests a novel mechanism of drug resistance that is different from the ATP-dependent drug efflux pumps that mediate P-gp- and MRP-associated MDR

ABRÉGÉ

Le développement, chez les cellules cancéreuses, d'une résistance multiple à un vaste nombre de médicaments anticancéreux est devenu un obstacle important au traitement chimiothérapeutique du cancer. La caractérisation moléculaire des cellules cancéreuses résistantes a permis d'identifier plusieurs altérations génétiques comme la surexpression d'une protéine membranaire: la P-glycoprotéine (P-gp). La P-gp est une pompe d'écoulement de composés chimiques en fonction de l'ATP, et l'activité de la P-gp ATPase est essentielle au transport de ces composés. Pour comprendre comment l'activité de la P-gp ATPase est jumelée à la fixation et au transport de substances chimiques, nous avons examiné les effets de N-éthylmaléinide (NEM), un puissant inhibiteur de la P-gp ATPase, sur la fixation et le transport de composés au P-gp. Nos résultats démontrent qu'à court terme le traitement de cellules résistantes avec NEM amène une augmentation de la fixation et de la posphorylation des composés chimiques au P-gp en fonction de la concentration. De plus, NEM augmente l'accumulation de la vinblastine tritiée dans les cellules résistantes, mais non dans les cellules sensibles. Notre étude suggère que l'inhibition de l'activité de la P-gp ATPase est responsable de l'augmentation de la fixation P-gp-composé chimique et non l'augmentation de la phosphorylation de la P-gp par NEM.

Le processus de sélection de lignées cellulaires cancéreuses *in vitro* a engendré de multiples changements cellulaires qui peuvent moduler la résistance aux médicaments anticancéreux. En plus de la P-gp et de protéines multirésistantes, d'autres mécanismes de résistance sont révélés par des études sur des lignées de cellules cancéreuses et des cas cliniques. Pour identifier d'autres altérations cellulaires importantes à la résistance cellulaire aux médicaments anticancéreux, nous avons utilisé la méthode par immunodot blot pour isoler des anticorps monoclonaux s'attachant aux protéines de la résistance médicamenteuse mais non aux cellules sensibles. Grâce à cette procédure, nous avons isolé un anticorps monoclonal (IPM96) qui reconnaît une protéine (P-40) de 40 kDa dans plusieurs lignées cellulaires résistantes. L'expression de P-40 est concourante au niveau de résistance médicamenteuse. La caractérisation biochimique de P-40 a démontré qu'il est associé à la fraction soluble de la membrane cellulaire. Le clonage de P-40 ADN-c a démontré qu'il était identique à annexin I, un substrat d'un facteur de croissance épidermique récepteur de la tyrosine kinase. L'augmentation observée du niveau de P-40 (ou annexin I) dans les cellules résistantes est due à l'élévation du nombre de transcrits de P-40. Le profile pharmacologique de cellules transfectées au P-40 ADN-c (P-40-MCF-7) a démontré que la surexpression de P-40 dans les cellules sensibles aux médicaments est capable de créer une résistance à l'adriamycine, l'actimomycine D, le taxol et la cisplatine. Dans l'ensemble, notre étude prouve de façon convaincante que annexin I est important au développement de la résistance médicamenteuse des cellules cancéreuses. De plus, elle suggère un nouveau mécanisme de résistance médicamenteuse différent des pompes d'écoulement de composés chimiques en fonction de l'ATP qui module la P-gp et autres protéines associées à la résistance multimédicamenteuse.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my research supervisor, Dr. Elias Georges, for his constant support, advice, encouragement and understanding for making this work possible.

During my studies at the Institute of Parasitology, I received help and friendship from many people. It is difficult to credit each person in turn, but I would like to thank:

The professors of Institute for sharing their time and knowledge with me, especially, Dr. Greg Matlashewski and Dr. Paula Ribeiro for their invaluable suggestions and help,

Students and technicians in my lab for their friendship and assistance,

Supporting staffs, especially Shirley Mongeau, Christiane, Gord Bingham Sylvie Labrecque for their sincere help and advice,

A special thank is given to my husband, Weiqing Chen, for his enduring love, caring , and patience. His consideration and love is so important for me to finally make my study complete.

Finally, I would like to thank my parents for their love and encouragement in everything that I have done in all these years. I thank my sisters, Yan Wang and Xiaogung Wang, for their making me happy all the time.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER III. MANUSCRIPT I.

CHAPTER IV. MANUSCRIPT II.

CHAPTER V. MANUSCRIPT III.

LIST OF TABLES

CHAPTER II. LITERATURE REVIEW.

CHAPTER IV. MANUSCRIPT 2.

STATEMENT OF ORIGINALITY

Manuscript I.

1.The inhibition of P-gp ATPase activity does not inhibit the capacity of P-gp to bind drugs.

In P-gp expressing MDR cell lines, the abolishment of P-gp ATPase activity by a V-type ATPase inhibitor (N-ethylmaleimide) leads to an increase in photolabeling with Iodoaryl-Azioprazosin (IAAP) and [^{3}H]-vinblastine accumulation in MDR cells. These effects are largely due to the inhibition of P-gp drug transport, which in turn leads to the saturation of P-gp drug binding sites.

2. Persistent phosphorylation of P-gp does not affect P-gp drug binding capacity.

Super-phosphorylation of P-gp in the presence of N-ethylmaleimide in MDR cells is due to its inhibition of P-gp phosphate turnover. Using a phosphatase inhibitor (calyculin A) to mimic the effect of N-ethylmaleimide, P-gp drug binding can not be affected.

Manuscript II.

1. Overexpression of a 40 kDa protein in human multidrug resistant (MDR) cell lines has been identified.

By using immuno-dot blot method, we have isolated a monoclonal antibody which recognizes a 40 kDa protein (P-40) in several MDR cell lines (MCF-7/Adr, SKOV/VLB$^{1.0}$, H69/AR and HL60/AR). Furthermore, P-40 levels dropped significantly in one revertant cell line (H69/PR) derived from H69/AR cell line. The low level of P-40 was also detected in SKOV3, a cell line that was previously shown to be clinically resistant to several cytotoxic drugs (cisplatin and adriamycin). Since the expression of P-40 is also higher in two tumour cell lines (SKTax6a and A2780^{CP}) that were selected with paclitaxel and cisplatin but do not express P-gp or MRP, the overexpression of P-40 is not the result of co-amplification of P-gp or MRP.

2. P-40 is a membrane-associated intracellular protein.

We have demonstrated that two pools of P-40 exist in MDR cells. The membrane-associated P-40 is highly resistant to extraction with high salt (disruption of the ionic force) and EDTA (cationic chelating agent) but not resistant to extraction with detergent and urea.

Manuscript III.

1. Molecular cloning of P-40 demonstrated that P-40 is identical to annexin I, a substrate for epidermal growth factor receptor kinase.

The identity of P-40 and annexin I has also been established based on (a) the similarity of the molecular mass of P-40 and annexin I, (b) the cross-reactivity of IPM96 monoclonal antibody and (c) the distribution pattern of P-40 and annexin I in mammalian cells.

2. The expression of P-40 confers low levels of drug resistance to several cytotoxic drugs.

The P-40 cDNA stable transfectant has been established by transfecting P-40 cDNA into MCF-7 drug sensitive cells. Characterization of P-40 stable transfectant shows that the expression of P-40 in drug sensitive MCF-7 cells confers low levels of drug resistance to a variety of anticancer drugs.

3. Annexin family may be very important in the development of multidrug resistant phenotype in cancer cells.

The expression of several members of the annexin family (eg. II and IV) is increased in MDR cell lines relative to the parental cell lines, but to lesser extent as that of P-40 (or annexin I). The levels of P-40 (or annexin I), annexin II and IV are decreased in a revertant cell line (H69/PR). These facts are critical for future evaluation of the functions of the annexin family in MDR.

THESIS OFFICE STATEMENT

In accordance with the regulation of the Faculty of Graduate Studies and Research of Mcgill University, the following is included in this thesis.

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

If this option is chosen, connecting texts, providing logical bridges between the different papers are mandatory.

The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that s more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: (1) a table of contents, (2) a general abstract in English and French, (3) an introduction with clearly states the rational and objective of the study, (4) a comprehensive general review of background literature to subject of the thesis, when ths review is appropriate, and (5) a final overall conclusion and /or summary.

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

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

STATEMENT OF AUTHORSHIP

This thesis consists of three manuscripts coauthored with my supervisor, Dr. Elias Georges. All the experiments described herein were designed and performed by myself under the guidance of my supervisor, with the exception of the production of the monoclonal antibody and the drug accumulation experiments. Dr. Pan participited intially in the genetration of monoclonal antibodies. Ms. F. Lheureux worked on the screening and culturing of the hybridoma clones. The drug accumulation experiments were executed by Ms. Z.Liu. I aided her in their design and data analysis.

My supervisor, Dr.Elias Georges, provided advice and feedback on the designs and analyses of all three studies, and on preparation of manuscripts for publication.

ABBREVIATION

ABC	ATP binding cassette
AML	acute myelogenous leukemia
ANLL	acute lymphocytic leukemia
ATPase	adenosine triphosphatase
BCLL	B cell lymphoblastic leukemia
Bcl-2	Bell cell lymphoma 2 gene
Bcl-xl	long form of BCL2 homologue
Bcl-xs	short form of BcL 2 homologue
BSO	buthionine sulphoximine
cDNA	complementary DNA
CFTR	Cystic fibrosis transmembrane conductance regulator
CHAPS	3,[(3-cholaidopropyl)dimethylammonio]-1-propane-sulfonate
CML	chronic myelogenous leukemia
CMV	cytomegalovirus
EGF	Epidermal growth factor
EDTA	ethylenediaminetetraacetate
FITC	Fluorescein isothiocyanate
GSH	Glutathione
GS-X pump	ATP dependent Glutathione S-conjugate export pump
GST	glutathione S-transferase
LRP	Lung Cancer Resistant Protein
LTC4	Leukotriene C4
mAb	monoclonal antibody
MDR	multidrug resistance
MDM2	mouse double minute DNA 2
MK571	3([{3-(2[7-chloro-2-quinolinyl)phenyl}-3-dimethyl-amino-3-oxopropyl)-thio}-methyl]thio)propanic acid

MRP	multidrug resistant associated protein
MOAT	multispecific organic anion transporter
NEM	N-ethylmaleimide
NHL	Non-Hodgkin's lymphomas
NSCLC	non small cell lung cancer
TM	transmembrane
P-40	a 40 kDa protein
p53	a 53 kDa nuclear phosphoprotein
PCR	polymerase chain reaction
P-gp	P-glycoprotein
PKA	cyclic AMP dependent protein kinase
PKC	Ca^{2+} activated protein kinase C
PMSF	phenylmethyanesulfonyl fluoride
PSC 833	[3'-oxo-4-butenyl-4-methyl-THr^1Val^2]cyclosporin
PVDF	polyvinylidene difluoride
SCLC	small cell lung cancer
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
V-ATPase	vascular proton-translocating adenosine triphosphate

CHAPTER I.

LITERATURE REVIEW

PART I.

THE MECHANISMS OF MULTIDRUG RESISTANCE IN CANCER CHEMOTHERAPY

1.INTRODUCTION

The ability of malignant cells to develop resistance to cytotoxic drugs is a major obstacle in the chemotherapeutic treatment of cancer patients. Clinically, a single drug treatment of cancer patients results in the development of tumours that are resistant to other drugs that are structurally and functionally dissimilar. Interestingly, a similar phenomenon has also been observed *in vitro* using a cell culture system. When tumour cell lines grown in culture are challenged with a single cytotoxic drug, they become resistant to many structurally and functionally unrelated compounds (Endicott and Ling, 1989; Gottesman and Pastan, 1993). Several mechanisms have been described during the past decades to explain this pleotropic phenotype. For example, most multidrug resistant tumour cell lines that are selected with natural product toxins overexpress one of two plasma membrane proteins, P-glycoprotein (P-gp) or the Multidrug Resistance Protein (MRP). P-gp and MRP are members of the ATP binding cassette superfamily of membrane trafficking proteins (Higgins ,1993). The mechanism by which P-gp mediates multidrug resistance (MDR) has been extensively studied and it is now accepted that P-gp functions as an energy-dependent drug efflux pump. The mechanism by which MRP mediates the multidrug resistance phenotype is similar to that for P-gp. However, more work is required to elucidate the molecular basis by which MRP confers drug resistance.

The realization that MDR in cancer cells, especially in the clinical situation, is a multifactorial process has led to an intensive investigation of other potential MDR-associated mechanisms in tumour cells. These studies have demonstrated that the development of drug resistance in malignant tumours could be associated with a number of cellular changes. Such changes can either act separately or together to confer drug resistance. Some of the cellular changes that have been identified in MDR tumour cells include changes in, (a) lipid composition (e.g., ganglioside expression; Biedler and Peterson, 1981),(b) enzymes involved in the metabolism of drugs and xenobiotics (e.g., cytochrome P450 and GST isoenzymes; Vickers et al, 1989; Tew 1994; Batist et al, 1986), © nuclear proteins (e.g., topoisomerase II; Evans et al, 1994) and the expression of cytosolic proteins (e.g., sorcin; Van der Bliek et al, 1986). More recently, it has been demonstrated that changes in the susceptibility of tumour cells to apoptotic cell death following chemotherapy also lead to the MDR phenotype (Lowe et al, 1993). Furthermore, mutation or allelic disruption of p53,

overexpression of Bcl-2 and its homologoue Bcl-x, or MDM2 are all able to induce drug resistance in certain types of tumour cells (Symmans et al, 1993; Kamesaki et al, 1993; Kitada et al, 1994; Lowe et al, 1993; Venil et al, 1995; Perego et al, 1996; Kondo et al, 1995). This literature review is a brief summary of the current understanding of the MDR phenotype and the mechanisms that contribute to this phenotype.

1.1 The MDR phenotype

The MDR phenotype was first described in tumour cell lines grown *in vitro* in the presence of sublethal concentrations of cytotoxic drugs. For example, P388 murine leukemic cells selected for resistance to vinblastine showed cross-resistance to actinomycin D, vincristine and daunorubicin , and hence an MDR phenotype (Kessel et al ,1968). The phenomenon of MDR is exemplified by the expression of resistance to a wide range of structurally and functionally unrelated drugs. This phenotype occurs frequently in cultured mammalian cells exposed to sublethal concentrations of cytotoxic drugs and in cancer patients following chemotherapeutic treatment with adjuvant anticancer agents.

1.2. MDR tumour cell lines

To gain a better understanding of the MDR phenotype, several MDR tumour cell lines were isolated by exposing drug sensitive tumour cells to sublethal concentration of anticancer drugs (Bech-hansen et al, 1976; Ling et al, 1983; Gazdar et al, 1980; Biedler and Riehm, 1970; Siegfried et al, 1983; Mirski et al, 1987; Twentyman et al, 1986; McGrath and Center, 1987). Some of the characteristics of MDR cell lines are listed in Table 1.

Table 1.Characteristics of the MDR phenotype

Increased resistance to unrelated cytotoxic drugs
Decreased intracellular accumulation of drugs
Enhanced drug efflux
Collateral sensitivity to membrane-active agents (eg. Triton X-100 and local anaesthetics)
Overexpression of a membrane transport protein or P-glycoprotein (P-gp)
Reversal of drug resistance by different classes of drugs known as reversing agents (eg. Verapamil and cyclosporin A)

Georges et al, 1992

One of the most interesting characteristics of the above drug resistant cells, which provided the impetus to the study of MDR cell lines as a model for clinical drug resistance, is the development

of drug resistance to a broad spectrum of drugs upon exposure to a single cytotoxic drug (Bech-Hansen, et al 1976). Other characteristics included reduction in the cellular accumulation of cytotoxic drugs (Inaba et al, 1982; Willingham et al, 1986) and collateral sensitivity to various membrane-active agents such as calcium channel blockers, steroids and local anaesthetics (Biedler and Riehm, 1970). The mechanism by which multidrug resistant cells acquire collateral sensitivity to membrane active agents remains unclear. However, it has been proposed that changes to the properties of the cell membrane following treatment by membrane active agents, may influence the fluidity of the lipid bilayer and induce programmed cell death (Lincoln and Georges, 1998 submitted).

Overexpression of P-gp is the most consistent alteration identified in MDR cells. The levels of P-gp expression have been shown to correlate with the degree of drug resistance in many separately derived MDR cell lines (Ling et al, 1983; Kartner and Ling ,1984). Moreover, the role of P-gp in the development of the MDR phenotype has been confirmed by single gene transfer studies (Gros et al, 1986; Ueda et al, 1987). Another important characteristic of P-gp-expressing MDR cells is the reversal of the drug resistance phenotype in the presence of nontoxic compounds collectively known as reversing agents(for review, Georges et al., 1990).

1.3. Clinical MDR

The occurrence of drug resistance during the chemotherapeutic treatment of cancer patients is a major obstacle limiting the success of cancer treatment. It is believed that chemotherapy can exert strong selective pressure on the malignant tumour cells leading to the outgrowth of chemo-resistant cells. The ability of this small population of malignant cells to survive, when exposed to sublethal doses of anti-cancer agents and to develop an MDR phenotype results in untreatable clinical relapse. Recent findings have established a correlation between P-gp levels in tumour samples and failure of the chemotherapy in hematopoietic and childhood malignant tumours (Chan et al, 1991; Chan et al, 1990; Bordow et al, 1994; Goldstein et al, 1989; Sato et al,1990). However, the prognostic value of P-gp in other types of cancers is less certain. The recently identified membrane protein, MRP, in small cell lung cancer (SCLC) cells, in the absence of P-gp, may be of prognostic value in P-gp-negative MDR tumours. In the majority of cases of clinical drug resistance, especially in solid tumours, it is likely that other cellular factors play critical roles. These cellular factors may include drug uptake (Kaye, 1995), failure of prodrug activation (Powis and Prough,

Table 2 Frequently encountered neoplasms

Oral cancer	Oesophageal cancer
Gastric cancer	Pancreatic cancer
Hepatoma	Colon and rectal cancer
Prostate cancer	Kidney cancer
Breast Cancer	Bladder cancer
Lung cancer	

In practice, these tumours are rarely cured by chemotherapy and account for over 90% of cancer related deaths. (Spiers, 1994)

1987; Nebert et al, 1991), changes in intracellular detoxification pathways (Hamilton et al, 1985; Perez et al, 1990), changes in target enzymes (Pommier et al, 1986; Kim et al, 1991) and DNA repair processes (Masuda et al, 1988). In addition, other intracellular molecules may impact drug delivery and genetic controls for cellular mechanisms such as apoptosis (Miyashita et al, 1992; Lowe et al, 1993). Therefore, it is likely that these changes, in addition to P-gp and MRP, confer drug resistance onto cancer cells. Furthermore, given that P-gp or MRP transfectants expressed lower levels of drug resistance when compared to cells selected *in vitro* that express same amount of these proteins strongly suggests that other mechanisms are involved in drug resistance.

2. MDR and overexpression of P-gp or MRP

Several studies have provided compelling evidence that overexpression of P-gp or MRP is sufficient to confer multidrug resistance (Gros et al, 1986; Ueda et al, 1987; Grant et al, 1994). Analysis of the predicted amino acid sequences of these integral membrane proteins revealed structural motifs characteristic of the superfamily of ABC membrane transporters. Thus, at least *in vitro,* the role of P-gp and MRP in MDR is well established (Higgins, 1992). Moreover, given the potential roles of these proteins in the MDR phenotype in some cancers, a major effort to develop drugs that reverse P-gp- or MRP-mediated MDR has led to the development of a group of reversing agents (e.g., cyclosporin A, BIBW 22 and PSC 833, etc.). Clinical trials using such reversing agents in the treatment of P-gp- mediated MDR in tumours are in progress.

2.1. P-gp and multidrug resistance

Overexpression of P-gp has been identified in many MDR cells or tissues using P-gp-specific monoclonal antibodies against different epitopes in P-gp (Kartner et al, 1985; Hamada and Tsuruo,

1986). Moreover, a cDNA fragment that encodes a small domain in P-gp was also used as a probe to measure P-gp mRNA and gene amplification (Riordan et al 1985). In another study, Ling and Baker (1978) have demonstrated that the expression of the MDR phenotype segregates with the acquisition of the P-gp gene. The demonstration that P-gp alone is sufficient for the expression of the MDR phenotype was shown in transfection experiments using full-length clones of P-gp cDNA (Gros et al, 1986). In this study, Gros et al (1986) provided conclusive evidence that the overexpression of P-gp alone in drug sensitive cells can confer an MDR phenotype. The latter conclusion was further confirmed by others using cDNAs encoding human and hamster P-gp (Devaut and Gros, 1990; Ueda et al, 1987).

Using a highly conserved fragment of P-gp gene, it was later shown that P-gp is encoded by a small gene family of three genes (mdr1, mdr2, and mdr3) in rodents (hamster and mouse) and two (MDR1 and MDR3) in humans (Ng et al, 1989; Van der Bliek et al, 1987). Interestingly, only MDR1 in human and mdr1 and mdr3 in rodents were shown to confer the MDR phenotype when full length clones of P-gp cDNAs were transfected into drug sensitive cells. The transfection of cDNAs encoding the human MDR3 and rodent mdr2 isoform of P-gp from human or rodents did not confer the MDR phenotype (Schinkel et al, 1991; Buschman and Gros, 1991).

P-glycoprotein structure and function: Analysis of the amino acid sequence of P-gp revealed a tandemly duplicated structure of hydrophobic and hydrophilic domains. The hydrophobic domains are thought to encode for six putative transmembrane sequences linked via a stretch of 60 amino acids. The hydrophilic domains encode for a large cytoplasmic domain each containing one ATP binding site (Gerlach et al, 1986; Gros et al, 1986; Chen et al, 1986). As an ABC transporter, P-gp was shown to bind to and hydrolyse ATP. Moreover, several studies have demonstrated a basal ATPase activity using purified P-gp (Shapiro and Ling, 1995). Furthermore, P-gp ATPase activity was shown to be stimulated in the presence of some anticancer drugs (Cornwell, et al., 1987; Schurr, et al. 1989; Germann et al, 1990; Georges et al, 1992). Although the molecular mechanism by which ATP hydrolysis is coupled to drug binding or transport is not clear, it has been demonstrated that both ATP binding sites in P-gp are required for the function of P-gp as a drug efflux pump(Azzaria et al, 1989). Immunohistochemisty studies with monoclonal antibodies (MRK-16 ,Georges et al, 1993; C219, Kartner et al, 1985; MM4.17, Cianfriglia et al, 1994) have

confirmed the cytoplasmic location of ATP-binding domains and the extracellular location of the loop linking the putative transmembrane domains TM1 and TM2 and the loop linking the putative domains TM7 and TM8. However, alternative topologies of P-gp have been observed in cell free system (Zhang and Ling, 1991; Zhang et al, 1993; Skach et al, 1993), Xenopus oocytes(Skach et al, 1993) , and bacterial expression system (Bibi and Beja, 1994; Beja and Bibi,1995). More recently, the topological studies of P-gp have shown that P-gp undergoes major changes in its tertiary structure upon the addition of Mg-ATP or Mg-ATP and verapamil (Sonveaux, et al 1996). Thus, although the three dimensional structure of P-gp is not yet known, in a recent study using a two dimensional crystalline array of detergent-solubilized and lipid reconstituted P-gp, the structure of P-gp was determined by electron microscopy and image analysis to a resolution of 2.4 nm (Rosenberg et al, 1997). In that study, P-gp was determined to be a monomer with an overall shape of a cylinder of 10 nm in diameter and 8 nm in height viewed from the extracellular surface. P-gp appeared as a toroidal with a large central pore of 5 nm in diameter.

The role of P-gp in MDR was initially based on its high sequence and structural homologies to bacterial periplasmic membrane transport proteins (Doige and Ames, 1993). The structural homology of P-gp was extended to include other membrane proteins such as the cystic fibrosis transmembrane regulator (Riordan et al, 1989), the MHCII endoplasmic peptide transporter (Spies et al, 1990), and the a-mating factor transporter in yeast (STE6) (McGrath et al, 1989). In fact, based on evidence from the yeast genomic project, the ABC family of proteins appears to be the largest family of functionally or structurally conserved proteins when compared with other protein families (Hartwell et al, 1997).

Further confirmation of the role of P-gp in the expression of the MDR phenotype of tumours has been supported by the isolation of a number of P-gp homologues from drug resistance parasites. For example, it was shown that membrane proteins encoding 12 putative transmembrane domains and two ATP binding sites are overexpressed in drug resistant *Plasmodium falciparum* (Wilson et al, 1992), *Leishmania* (Hendrickson et al, 1993), and *Entamoeba histolytica* (Descoteaux et al, 1992). However, direct and conclusive evidence for the roles of these proteins in drug resistance remains to be demonstrated. Taken together, it appears that P-gp functions as an energy-dependent drug efflux pump that extrudes cytotoxic drugs from tumour cells *in vitro*. The molecular mechanism

of drug efflux via P-gp is currently not clear. Using photoactive analogues of cytotoxic drugs, it was shown that P-gp interacted directly with various lipophilic compounds including most cytotoxic drugs (Nogae et al, 1989; Pearce et al, 1989; Safa et al 1987; Safa et al, 1989; Nare et al, 1994; Liu et al ,1996). In addition, P-gp with a single point mutation in the putative transmembrane domains 6 ,11 or 12 was shown to display altered drug specificity (Devine, et al 1992; Loo and Clark et al, 1993; Gros et al, 1991). The latter observation clearly suggests that P-gp is directly involved in the efflux of cytotoxic drugs from MDR cells.

P-glycoprotein expression in normal and tumour tissues: P-gp expression has been shown in normal and tumour tissues (Weinstein et al, 1991). Studies on the normal tissue distribution of P-gp in rodents and humans have indicated a restricted expression (Sugawara et al, 1988; Mukhopadhyay et al, 1988). For example, it has been shown that certain normal tissues have intrinsically high levels of P-gp, while other normal tissues do not express detectable levels of P-gp (Bradely et al, 1990). In light of the possible role of different isoforms of P-gp, it was shown via two independent approaches, that different isoforms of P-gp are differentially expressed. In one such study, using epitope-specific monoclonal antibodies, Georges et al., (1990) demonstrated the expression of class I isoform in colonic epithelial cells while the class II isoform was overexpressed to high levels in the adrenal cortex. The class III isoform was expressed only in a small subset of skeletal muscle fibers and in smooth muscle. Using a similar approach, Bradely et al., (1991) extended the above tissue distribution of P-gp isoforms in normal hamster tissues (see Table 3). In addition, the above study also demonstrated that the distribution of P-gp isoforms in some tissues appears to be sex-dependent and was hormonally-regulated. Based on the normal tissue distribution of P-gp isoforms, it was speculated that P-gp is likely to mediate a specialized transport function in different tissues. The above differential expression of P-gp isoforms was confirmed by using isoform-specific cDNA probes that encode the mouse P-gps (Croop et al, 1989).

The normal function of P-gp, apart from its role in MDR, is not known. Although possible endogenous substrates for P-gp have not been identified, the tissue distribution studies, especially the polarized location of P-gp in some tissues (e.g., kidney and colon), have led some to speculate about the roles of P-gp in the transport of normal cell metabolites and detoxification of xenobiotics

Table 3. Detection of P-gp in normal hamster tissues

P-gp detected	P-gp not detected
Cerebrum and cerebellum	Salivary gland
Heart	Mammary gland
Small and large intestines	Trachea and lung
Liver	Buccal pouch, oesophagus, stomach
Ovary, oviduct, uterus, vagina	Pancreas, spleen
Testis	Kidney
Adrenal cortex	Adrenal medulla
Gravid uterus	Placenta
Striated muscle	Smooth muscle

Bradely et al, 1990

(Thiebaut et al, 1987; Rao et al, 1994; Kalken et al, 1993). To gain a better insight into the normal function of P-gp, Smit et al., (1993) have developed knockout mice with disrupted mdr2 gene (the class III isoform). These mice showed severe liver disease and the major biochemical defect appeared to be the secretion of phospholipid into bile. This study strongly suggested that the mdr2 gene product is involved in the transport of phospholipids. The latter conclusion was further supported by a later study using a mdr2 heterozygous yeast expression system (Ruetz and Gros,1994). In contrast, knockout mice with P-gp mdr1 (class I isoform) disruptions showed normal liver phospholipid transport but were hypersensitivity to cytotoxic drugs at the level of the blood-brain barrier (Schinkel et al, 1994). For example, mice with both alleles of mdr1 disrupted showed 80 fold increase in the accumulation of drugs in tissues that express high levels of P-gp (e.g., brain , intestine etc.). These results demonstrated the role of mdr1 in the extrusion of cytotoxic compounds. It should be pointed out that although high levels of P-gp (mdr1) were observed in other normal tissues (other than the endothelial cells of brain blood capillaries), no preferential drug accumulation was observed in those tissues. These results may suggest that other drug efflux mechanisms are operative in those tissues. More recently, another study from the above group showed that human MDR1 or mouse mdr1 *pgp* is able to transport short chain phospholipid analogues with broad specificity *in vitro* (Van Helvoort et al, 1996). Collectively, these results have led to the speculation that the class I isoform of P-gp (human MDR1 and mouse mdr1 *pgp)* may have a physiological function as a flippase in translocating certain type of phospholipids within the plasma membrane. Taken together, it appears that class I and class II isoforms of P-gp (mdr1 and

mdr2 gene) mediate the transport of xenobiotics and normal cell metabolites, while the class III isoform (hamster mdr 3 and murine mdr2) is involved in the transport of phosphatidylcholine.

Table 4. P-gp expression in cancer

	Tumour type.
Constitutive expression	Colon carcinoma
	Adrenal cancer
	Renal carcinoma
	Hepatoma
	Pancreatic islet cell tumour
	NSCLC with neuroendocrine properties
	CML in blast crisis
	AML
Increased expression after chemotherapy	NHL
	BCLL
	ANLL
	Neuroblastoma
	Breast cancer
	Multiple myeloma
	Rhabdomyosarcoma

Trambas et al, 1997

Using the same molecular probes, investigation of P-gp expression in human tumours was also undertaken. The study by Bell et al., (1985) was the first demonstration of high P-gp levels in tumour samples from patients with advanced drug resistant ovarian cancer. Later studies by Fojo et al., (1987) demonstrated high P-gp mRNA in various tumours. For example, mdr1 (or class I isoform of P-gp) transcripts were present at high levels in many untreated tumours including adrenal gland and colon tumours in addition to treated and nonresponsive tumours in other organs. Recently, (Fojo et al, 1987b; Gerlach etal, 1987; Goldstein et al, 1989, Salmon et al, 1989; Schlaifer et al, 1990) high levels of P-gp or its mRNA have been reported in a large number of different tumour samples (see Table 4). More recently, Abolhida et al (1997) described an acute transcriptional up regulation of MDR1 gene in a human solid tumour from patients with undessectable sarcoma pulmonary metastasis undergoing lung perfusion with adriamycin.

Post-translational modification of P-gp P-gp is post-translationally modified by glycosylation and phosphorylation (Carlsen et al, 1977; Hamada et al 1987, Mullar et al 1987, Ichikawa et al 1990).

Earlier studies have conclusively demonstrated that glycosylation does not significantly affect the drug transport function of P-gp. However, the role of phosphorylation on P-gp function remains controversial.

Analysis of the primary amino acid sequence of P-gp revealed several potential protein kinase A (cyclic AMP-dependent protein kinase, PKA) and protein kinase C (Ca^{2+} activated kinase, PKC)phosphorylation sites (Kennelly and Krebs, 1991). The covalent modification of P-gp by phosphorylation has been established as a general feature of native P-gp in drug-selected MDR cell lines and in recombinant P-gp expressed in mdr-transfected mammalian cells (Schurr, et al 1989) or in mdr1 baculovirus-infected insect cells (Germann et al, 1990). The Linker region of P-gp1 has been characterized by its high content of charged amino acids clustered into two regions with predominately negative and positive domains. The identified phosphorylation sites of human P-gp found in the basic domain are serine 661, 667 and 671 for PKC and serine 667, 671 and 683 for PKA (Chambers et al, 1993; Chambers et al, 1994). More recently, a cluster of serine /threonine residues that can be phosphorylated by the acidic-directed protein kinase in the acidic segment (predominantly negative domain) of the linker region has been identified (Glary et al, 1997). Given the importance of protein phosphorylation in general, the effects of phosphorylation on the function of P-gp in drug transport has been thoroughly investigated. In the early stages, efforts were focussed on the investigation of the level of expression of various kinases in MDR cells and the regulation of multiple protein kinases by their activators and inhibitors. An assumption had been made that changes in the levels and activities of P-gp-related protein kinases might correlate with the levels of drug resistance (Center et al, 1985; Bates et al, 1992; Mellado and Horwit,.1987; Staats et al, 1990; Chamber et al, 1990; Fine et al, 1988; Abraham et al 1990). However, the nonspecific effects of these regulators in intact cells made it difficult to link the activation of protein kinases to P-gp phosphorylation and to MDR in tumour cells. Later studies to assess the role of the phosphorylation in P-gp-mediated MDR have embarked on the identification of actual phosphorylation sites within P-gp. Site-directed mutagenesis of P-gp phosphorylated sites (Germann et al 1996) has demonstrated that unphosphorylated P-gp confers drug resistance and shows ATP-dependent drug transport. Thus, phosphorylation /dephosphorylation of P-gp does not play an essential role in mediating MDR (Goodfellow et al, 1996; Buschman and Gros, 1991). However, P-gp phosphorylation may

contribute to the regulation of its substrate specificity (Bates et al, 1992) and chloride channel activity (Goodfellow et al 1996). The P-gp phosphorylation sites located in the acidic domain of linker region have been recently identified and the role of phosphorylation of these sites is under intense investigation (Glarys et al, 1997). The physiological role of P-gp phosphorylation remains to be elucidated.

P-gp as a marker of multidrug resistance : Clinically, the most important question relating to the MDR phenotype is whether or not P-gp is a prognostic of MDR. To address this question more accurately, sequential tumour samples from the same patients before and after chemotherapeutic treatment are required. This however, is technically and morally difficult and has been a major obstacle in yielding an accurate and consistent answer to the prognostic value of P-gp in the development of MDR in tumours from patients. In spite of these difficulties, clinical data appear to be split on the prognostic role of P-gp in clinical drug resistant tumours. The first report by Ma et al.,(1987), using sequential testing of ANLL leukemic cell samples from two patients with progressively non-responsive disease, showed an increase in P-gp levels and the proportion of tumour cells which express P-gp. A larger scale study by Chan and her coworkers using immunohistochemical staining technique demonstrated a prognostic correlation of P-gp in soft tissue sarcoma in children (Chan et al, 1990). Further study by the same group reported a correlation between P-gp levels and the survival of children with Neuroblastoma (Chan et al, 1991). In both studies, patients whose tumours contained no detectable levels of P-gp had better prognosis and longer survival curves following chemotherapy.

Reversal of Pgp-associated multidrug resistance by reversing agents : Studies in support of the role of P-gp in MDR have been based on the capacity of certain reversing agents to potentiate the cytotoxic effects of chemotherapeutic treatment in previously drug resistant cells. One of the important characteristics in P-gp-mediated multidrug resistance is that the activity of P-gp could be blocked pharmacologically by a large number of non-toxic compounds including calcium channel blockers, calmodulin inhibitors, steroidal agents, immunosuppressive drugs, antibiotics and other synthetic chemical agents (Ford and Hait, 1990; Ford and Hait, 1993; Lumb et al, 1993; Sikic et al, 1997). It has been proposed that these MDR modulators can block the function of P-gp as drug transporter by either occupying the drug binding sites that results in a competitive inhibition, or a

reversing agent binding sites at P-gp as non-competitive inhibitors to disrupt drug efflux (Ford, 1995). Clinical trials using anti-cancer drugs plus MDR-reversing agents provided good evidence for the role of P-gp in clinical drug resistance (Kloke and Osieka, 1985; Verweij et al, 1991). In one such study, Dalton et al (1988) obtained a significant clinical response when verapamil was included with cytotoxic drugs in the treatment of patients previously refractory to multiple drug treatment. The potentiation of chemotherapeutic treatment in those patients in the presence of verapamil was proposed to be due to the presence of P-gp. A phase I clinical trial using a P-gp reversal agent, Cyclosporin A or its analogue PSC 833, also showed that the combination treatment resulted in a rapid decrease in circulating leukemic cells that expressed MDR1, thus restoring the drug sensitivity and prolonging myelosuppression (Kloke and Oksiek et al, 1985; Sonnevel and Nooter, 1991; Jachez et al, 1993; Boesch et al, 1991). The potential clinical importance of MDR1 gene expression in some malignancies has led to numerous strategies to overcome the MDR phenotype.(Table 5).

Table 5. Strategies to overcome P-gp-mediated MDR

1.Using currently available chemotherapeutic drugs which are not substrates for P-gp.
2.Development of structurally modified analogues of MDR related drugs that retain their cytotoxicity but are no longer substrates for P-gp.
3.Delivery of cytotoxic drugs via liposomal carriers.
4.Concurrent administration of P-gp reversing drugs and chemotherapeutic agents.

Fisher et al 1995

Although there is a considerable body of evidence to demonstrate the role of P-gp in the development of MDR phenotype during the chemotherapeutic treatment, the involvement of this protein in clinical drug resistance in solid tumours remains unclear and controversial. It has been estimated that P-gp is increased by as much as 50% in all human tumours at some stage of treatment with anticancer agents (Gottesman, 1993), but the relevance of its expression and outcome of the chemotreatment remains equivocal. Therefore, it is now believed that other mechanisms exist and may explain the MDR phenotype in solid tumours that are P-gp negative.

2.2. MRP and MDR

Given the role of P-gp in the MDR phenotype of tumour cell lines, it was predicted by some that P-gp would play a major role in the responsiveness of tumour cells to chemotherapy.

Unfortunately, results from clinical studies with solid tumours, which account for 90% of cancer-related deaths, have not established P-gp as the major diagnostic/prognostic marker of MDR (Doyle 1993; Linn et al, 1995). Moreover, there are many examples of multidrug resistant cell lines where overexpression of neither P-gp nor its mRNA could be detected (Danks et al , 1987; Mirski et al 1987; Twentyman et al, 1986; Versantoort et al,1990). These MDR cell lines share many features associated with the classical MDR mechanism such as an energy-dependent decrease in drug accumulation and cross-resistance to structurally dissimilar drugs (Cole, 1992). The characterization of some of these cell lines led to the identification of a 190 kDa plasma membrane protein that was overexpressed in several non-P-gp tumour cell lines (McGrath anc Center, 1988; Center et al, 1990; Marquardt et al, 1990; Versantvoort et al, 1992). The gene encoding for the 190 kDa protein (Multidrug Resistance Protein or MRP) was subsequently cloned by Cole and her collogues from a Small Cell Lung Cancer (SCLC) drug resistant cell line (H69/AR) using a differential hybridization technique (Cole et al, 1992). MRP gene (*mrp1*) encodes a 1531 amino acid protein. Analysis of MRP amino acid sequence showed strong homology to other membrane transporters of the ABC superfamily (Cole, et al, 1992). Interestingly, the amino acid identity between MRP and another member of ABC superfamily (P-gp) is only 15% with the highest sequence identity confined to the nucleotide binding domains (Stride et al 1996).

Biochemical characteristics of MRP: MRP is 190 kDa glycophosphoprotein, originally isolated from SCLC cell line (H69/AR). Northern blot analysis using a cDNA fragment which encodes part of the MRP gene showed 100-200 fold increase in MRP mRNA in drug resistant H69/AR cells versus the parental drug sensitive cells (Cole et al, 1992). The overexpression of MRP mRNA was shown to be due to the amplification of the cognate gene located on chromosome 16 at band 13.1. Transfection of MRP cDNA into drug sensitive cells conferred an MDR phenotype to some but not all P-gp associated drugs (Grant et al, 1994). Nevertheless, several studies have now shown that overexpression of MRP alone is sufficient to cause MDR onto otherwise drug sensitive cells (Cole et al 1994; Kurt et al, 1995; Kruh et al, 1994; Stride et al, 1996).

The predicted secondary structure of MRP consists of 11 transmembrane domains in the amino proximal half and 6 transmembrane domains in carboxyl proximal half. Both halves of MRP are thought to be N-glycosylated (Loe et al, 1995; Bakos et al 1996). Moreover, similar to other

members of the ABC family, MRP contains two cytoplasmic nucleotide binding domains located in each half of the transmembrane domains. MRP contains multiple consensus sequences that can be phosphorylated by protein kinases. Analysis of *in vitro* and *in vivo* phosphorylated MRP showed MRP to be highly phosphorylated on serine residues (Ma et al, 1995). The effects of phosphorylation of MRP on its function remain to be elucidated.

Recently, several homologues of MRP were identified by using different approaches. Other MRP homologues include the GS-X pump with 49% amino acid sequence identity to the human MRP1 , and the cMOAT (canalicular multispecific organic anion transporter) (Paulusma et al, 1996 ; Taniguchi et al, 1996; Kool et al 1997). By screening databases of human expressed tags, Kool et al., (1997) identified several other MRP homologues (MRP-3, MRP4 and MRP5) with different tissue specificity and expression pattern in cisplatin resistant cell lines. The functions of the latter MRP homologous remain to be determined (Kool et al, 1997).

The mechanism by which MRP confers MDR in cancer cells is not clear. Current evidence suggests that MRP acts as an energy dependent efflux pump at the cell surface in some cell lines (Zamans et al 1994), while in other cell lines, MRP functions to sequester drugs in membrane vesicles (Breuninger et al , 1995; Cole et al, 1994; Pauls et al, 1996; Krishnamachary and Center, 1994; Flen et al, 1994). In both cases, MRP is thought to reduce the free intracellular concentrations of cytotoxic drugs. Therefore, cancer cells that express MRP are able to maintain nontoxic levels of anticancer drugs.

MRP expression and multidrug resistance: High levels of MRP have been detected in several *in vitro* selected cell lines including fibrosarcoma, SCLC, NSCLC, breast, prostate and bladder carcinomas (Loe et al, 1996). Characterization of these MDR cell lines strongly suggests that the decreased drug accumulation in these cells correlates with the overexpression of MRP (Loe et al, 1996). Immunocytochemistry and immunoelectron microscopy studies indicated that MRP is predominantly located in the plasma membrane of MRP-expressing cells. Interestingly, MRP was also detected in the membrane of Golgi network, endoplasmic reticulum and intracellular membrane vesicles (Hipfer et al, 1994). However, it is not clear what factors govern the subcellular localization of MRP in different MDR cell lines (Kavallaris et al, 1997). Furthermore, differences in MRP expression among the various membrane compartments may explain the observed differences in the

drug efflux kinetics seen in different MRP expressing cell lines (Loe et al, 1996).

The clinical relevance of MRP-mediated MDR in human cancers remains largely unknown. Using immunohistochemistry, Nooter et al (1996) examined different stages of NSCLC samples resected from primary NSCLC with a variety of cell types. The results of that study showed that 35% of the samples had elevated MRP mRNA, 40% of the tumour samples have clear cytoplasmic staining and 34% of the tumour samples have strong cytoplasmic as well as plasma membrane staining. In another study (Nooter et al, 1997), using series of 259 resected invasive primary breast carcinomas, MRP expression was associated with increased risk for failure of treatment in patients with small tumours, in node-negative patients and in node-positive patients who received combinational chemotherapy. Hence, it was concluded that MRP overexpression could lead to a more aggressive phenotype and therefore may be prognostic of MDR.

The overexpression of MRP has been observed in tumour cells that respond poorly to chemotherapy (e.g., anaplastic carcinoma of the thyroid and human glioma; Sugawara et al, 1994; Abe et al, 1995). However, the relationship between MRP expression and the outcome of chemotherapeutic treatment is not clear. Burger et al (1994) observed high levels of MRP expressed in treated or untreated tumours from 80 patients with chronic B-cell malignancies. However, further analysis showed no correlation between the levels of MRP expression in treated and untreated tumours. Taken together, the clinical relevance of increased levels of MRP is yet to be resolved.

Substrate specificity and chemosensitisation of MRP-mediated MDR : Studies with *in vitro* selected MRP-expressing cell lines and MRP transfectants have suggested that MRP is an ATP-dependent drug efflux pump (Cole et al, 1994). Overexpression of MRP results in an increased level of resistance to natural anticancer drugs but not to taxol, mitoxanthrone and cisplatin. MRP overexpression has also been implicated in the resistance to some heavy metal ions such as arsenate and antimony (Cole et al, 1994). Furthermore, MRP-mediated MDR can not be reversed by drugs such as verapamil and cyclosporin A that are potent reversing agents for Pgp-mediated MDR. In particular, unlike Pgp, there is no evidence for the direct interaction between MRP and unmodified anticancer drugs. It has been suggested that MRP functions as GS S-conjugate carrier (GS-X pump) and transports amphiphilic anionic conjugates.

GSH-conjugates are actively transported by GS-X pump in an ATP-dependent manner. This pump was shown to have broad substrate specificity toward drugs that contain a hydrophobic portion and at least two negative charges (Ishikawa 1992; Heijn et al, 1992; Ishikawa 1989). Using membrane vesicles from MRP expressing MDR cells and Hela cell transfected with MRP cDNA, Leier et al. (1994) and Muller et al. (1994) independently showed that MRP is able to transport leukotriene C4 (LTC4) and other compounds conjugated with glutathione, glucuronate or sulfate. Consequently, depletion of cellular GSH by exposure of cells to buthionine sulphoxmine (BSO) , a potent inhibitor of GSH synthesis, led to the inhibition of MRP transport activity and may act as a reversal agent (Kramer et al, 1988; Dusre et al, 1989). These studies strongly suggested that MRP is either identical to GS-X pump or is able to activate endogenous GS-X pump activity that extrude GSH-conjugated anticancer drugs.

Cellular GSH modulates a variety of physiologically important functions in cellular defence and metabolism, including the modulation of thiol-disulphate status of proteins, protection of cells from oxidative stress, detoxification of electrophilic compounds, synthesis and transport of biologically active and endogenous substrates (Tew 1994). Although the evidence of GSH co-transport by MRP is equivocal, *in vitro* studies suggested that GSH facilitates drug transport by MRP. Using chemically synthesized GSH-adriamycin conjugates, Priebe et al (1997) observed that GSH conjugated daunorubicin and adriamycin showed great inhibition of LTC4 transport up to 60% compared to the unconjugated drugs. Using vincristine, Loe et al (1996) showed that ATP-dependent uptake of unmodified drug by membrane vesicles derived from MRP transfectants can be demonstrated only in the presence of GSH. This group also showed that MRP mediated an ATP dependent transport of aflatoxin B1, a well characterized hepatotoxin and hepatocarcinogen, in the presence of GSH (Loe et al, 1997). The mechanism by that mediates GSH-dependent vincristine and aflatoxin B1 transport occurs, remains unclear. Since there is no evidence that these compounds form conjugates with GSH *in vitro*, it is possible that GSH interacts directly with MRP and this interaction is necessary for transport (Loe et al, 1996; Loe et al, 1997).

Studies in support of MRP as an GS-X pump have been based on the capacity of certain modulators to potentiate the cytotoxic effects of chemotherapeutic agents in drug resistant cells. Most agents that are effective in reversing the drug resistance of P-gp- mediated MDR are unable to

reverse MRP- mediated MDR. The ability of MRP to transport LTC4 and other GSH conjugates has led to the suggestion that compounds that alter GSH levels may regulate MRP mediated drug resistance. In an effort to investigate whether lowering cellular GSH can increase the drug sensitivity of MRP expressing cells to cytotoxic drugs, the cultured cells were incubated with buthionine sulphoximine (BSO) and cytotoxic drugs. Studies from different groups showed that resistance to daunorubicin, vincristine, and rhodamine 123 was partially reversed in MRP expressing cells following exposure to BSO (Versantvoort et al, 1995a). In particular, an increase in the cellular level of GSH in BSO treated cells resulted in restoration of daunorubicin accumulation. Other compounds, such as MK571, an LTD4 receptor antagonist , were also able to increase the drug accumulation and modulate the drug resistance of MRP expressing cells (Leier et al 1994; Gekeler et al, 1995). In addition, treatment of MRP expressing cells with antisense oligonucleotides showed enhanced sensitivity to adriamycin (Stewart et al, 1996). The latter approach to reverse drug resistance in MRP associated MDR cells suggests an alternative method of MDR reversal (Stewart et al, 1996).

3.Multidrug resistance caused by alteration of intracellular proteins.

Several cell lines have been developed that display resistance to different classes of anticancer drugs in the absence of P-gp or MRP expression (Gabral et a, 1997; Withoff et al, 1996; Minderman et al, 1996). Drug resistance in those cell lines shows various cellular alterations including decatenation of topoisomerase II (Takano et al, 1991), changes in GSH levels, increased GST activity (Tidefelt et al, 1992; Kim et al, 1991), reduced expression of cytochrome P-450 (Batist et al, 1986; Vickers et al 1989) and overexpression of specific isoforms of β-tubulins (Gabral et al, 1981; Kavallaris et al, 1997; Monzo et al, 1997). Most of these proteins are either the targets for many natural products of anticancer drugs or essential enzymes for drug metabolism. Studies with some of these cell lines suggested that they are cross resistant to structurally, but not necessarily functionally different anticancer drugs. Interestingly, during the selection of drug resistant cell lines, some of the above alterations (e.g., changes in topoisomerase II activity) occur in the early stages of drug selection and cause low levels of drug resistance (Matsumoto et al, 1997). Hence, it has been widely accepted that cellular alterations described above may act together with

well defined mechanisms to cause MDR *in vivo*.

A 110 kDa cytosolic protein, termed lung-resistance protein (LRP) was overexpressed in several non-P-gp cell lines (Scheper et al, 1993; Rik et al, 1993; Izquierdo et al, 1996). Sequencing of the LRP cDNA revealed that LRP is a major component of human vaults which are highly conserved cytoplasmic, ribonucleoprotein particles and thought to mediate intracellular transport processes (Scheffer et al, 1995; Rome et al, 1991). Although transfection of LRP into drug sensitive cells did not confer an MDR phenotype (Scheffer et al, 1995), the role of vault proteins in MDR has not been excluded. The role of LRP in MDR and the chemotherapeutic outcome is under intensive investigation.

4. Mutidrug resistance caused by dysregulation of apoptosis

Apoptosis or programmed cell death plays a critical role in the regulation of tissue homeostasis (Hartwell and Kastan 1994; Kerr et al, 1994). This pathway is highly regulated by a set of genes that promote apoptosis and those that block it. There is now increasing evidence that dysfunction of the apoptotic pathway plays an important role in cancer and the outcome of cancer chemotherapy. As most chemotherapeutic drugs directly or indirectly induce DNA damage, mutations in several genes required for the activation of cell death affects the efficiency of chemotherapy (Fisher 1994; Lowe et al, 1993). Several studies have now shown a direct link between dysfunction of genes in cell death pathway and the expression of drug resistance to chemotherapeutic drugs in cancer (Lowe et al, 1993; Perego et al, 1996).

4.1. p53 and drug resistance

p53 is a DNA binding protein that functions as a transcriptional factor to induce cell growth arrest and apoptosis in response to DNA damage induced by chemotherapeutic drugs and radiation (Fisher, 1995). The fact that cancer cells frequently carry aberration of p53 has led some to suggest a link between p53 function and the outcome of cancer chemotherapy (Bartek et al 1990). Using a p53-deficient embryonic mouse fibroblast cells, Lowe et al (1993) investigated the malfunction of p53 on the impact of chemotherapy. They observed that p53 deficient embryonic fibroblast cells are cross resistant to 5-FU, adriamycin and VP-16. Moreover, mutations in p53 sequence have been associated with cisplatin resistance in some drug resistant ovarian cancer cell lines (Perego et

al, 1996). As a consequence of losing the ability of p53 as trans-activator to its down stream targets, reduced expression of Bax was seen in these drug resistant cell lines. These *in vitro* studies demonstrated that p53 gene is required for the efficient activation of apoptosis following chemotherapy and thus, lack of p53 expression leads to a dramatic increase in cellular drug resistance. Since mutant p53 is frequently found in solid tumours, one may speculate that the expression of mutant p53 or null p53 could be related to the lack of sensitivity to chemotherapy in the absence of P-gp. However, the observation from clinical tumours appears to be controversial (Wu and El-diry 1996; Hawkins Ds et al, 1996). Clearly, more studies are required to resolve the role of p53 in the MDR phenotype in solid tumours.

4.2. Overexpression of Bcl-2/Bcl-xl and drug resistance.

Bcl-2 was originally described in non-Hodgkins lymphomas (Tsuyimoto et al 1986) as a chromosomal translocation event from chromosome 18 into a juxtaposition to γ-globulin heavy chain gene locus on chromosome 14. As an important anti-apoptotic regulator, Bcl-2 interacts with other proteins to induce apoptosis (Reed 1995; Nunez et al, 1990; Conner et al, 1997). Single gene transfer studies of Bcl-2 into leukemic cells that express low levels of this protein showed a correlation between the overexpression of Bcl-2 and drug resistance (Cathode et al, 1994)

Many *in vitro* studies and animal models had provided perfect correlation between the alteration of the genes in apoptotic pathway (e.g., mutation of p53 and dysregulation of expression of Bcl-2/ Bcl-xl) and multidrug resistance in chemotherapy and radiotherapy. This correlation is consistent with the mode of action of anticancer drugs leading to apoptosis. More efforts are now directed towards understanding the effects of genes in the apoptotic pathway and multidrug resistance in clinical tumours.

PART II.

BIOLOGY OF ANNEXINS

1.INTRODUCTION

Annexins are a family of structurally related Ca^{2+}- dependent phospholipid binding proteins (Ranal and Pollard, 1994). Annexins are evolutionarily conserved and are found in many life forms from mammals to molds and plants. Several different annexin gene products are expressed in all mammalian cells except red blood cells, and are thought to play important roles in numerous intra- and extracellular processes. Although the normal biological functions of annexins are not known, they have been implicated in membrane vesicular trafficking(Gruenberg and Emans ,1993; Burgoyne and Glague, 1994), membrane fusion during exocytosis (Creutz 1992), transmembrane channel activity (Demange 1994; Pollard and Rojas E 1988; Rojas et al, 1990), organization of membrane phospholipid domains (Geisow et al, 1987), mitogenic signal transduction (Moss 1992), settlement ofcell-matrix interaction (Mollenhauer and Vondermark1993;Fernandez et al, 1988;Wirland Schwartz 1990), and inhibition of phospholipase A2 (Huang et al, 1986; Flower and Blackwell, 1979).

2. Discovery of annexins.

As a distinct family of intracellular Ca^{2+} binding proteins, annexins have emerged from multiple areas of investigation during 1980s. Several groups have identified individual members of the annexin family from different tissues. All members of the annexin family are characterized by their calcium-dependent binding to biological lipids and dexamethasone-induced inhibition of eicosanoid production (Flower, 1985; Flower et al, 1988; Geisow et al, 1982; Creutz et al, 1983; Moore et al, 1982; Moore et al, 1984). Annexins constitute a family as evidenced by their cross-reactivity with specific antisera, amino acid and cDNA sequence homology, "annexing" of phospholipid membranes and their inhibitory activity on phospholipase A2 (Pepinsky et al, 1988; Crumptom and Dedman,1990). Studies from several avenues have clearly demonstrated that the structural framework defined by annexin I and II is the norm for the annexin family.

Efforts to identify proteins that participate in intracellular calcium signalling and macromolecular transport, led Creutz and his coworkers (1978) to purify a 47 kDa protein (Synexin, now termed annexin VII) from bovine adrenal medulla that promotes aggregation of secretory granules from chromaffin cells *in vitro* in the presence of Ca^{2+}. Subsequently, several other soluble proteins that bind to phospholipids in a Ca^{2+}- dependent manner were identified.

Using rat peritoneal exudate, Pepinsky et al (1986) first reported partial peptide sequence

of a 37 kDa protein (initially named licoportin, and now known as annexin I) that was able to inhibit the activity of phospholipase A2. Wallner et al (1986) subsequently isolated the corresponding human gene which encoded a protein of 346 amino acids. Cytogenetic studies showed that human annexin I was coded by a single gene, located on chromosome 9 and mapped to the region 9q11 - 9q22 (Wallner et al, 1986; Huebner et al, 1988). Later studies showed that annexin II is also located on chromosome 9, close to the annexin I gene (Huebner et al ,1988).

Following these findings, Wallner's group isolated five other distinct calcium and phospholipid binding proteins from rat and bovine that shared more than 50% identity with human lipocortin I and II (Pepinsky et al, 1988). The annexin family is now composed of 18 members that are widely distributed throughout higher plants and animal cells. Up to 10 members are found in mammalian cells from different organs and tissues (Burgoyne and Geisaw 1989; Kaetzel et al, 1989; Pepinsky et al, 1988; Raynal et al, 1994; Russo-Marie et al, 1991; Creutz et al, 1996). In addition , the expression of certain annexins is developmentally regulated (Hofmann et al,1992). Moreover, annexins are differentially expressed between normal and malignant cells in several tissues (Masaki et al, 1996; Sato-Matsumura et al 1996).

3. Structure and functions of annexins

The biochemical properties of annexins have been extensively studied in the past decades. As mentioned above, proteins belonging to the annexin family share high degree of sequence and structural homologies. Analyses of the primary amino acid sequences of annexin proteins suggested that each annexin is made up of two different regions: a unique N-terminal domain (termed the tail domain) and a C-terminal domain (termed the core domain) (Geisow 1986; Geisow et al, 1986; Huber et al, 1992; Pepinsky et al, 1988). The C-terminal domain is conserved and is a hallmark feature of the annexin family.

3.1. C-terminal domain of annexins.

Amino acid sequence analyses of the C-terminal domain of annexins showed that each protein contains four repeats of about 70 amino acids in length with a molecular mass of 34 kDa (in the case of annexin VI, 8 repeats) (Geisow 1986). These annexin repeats show 40 to 60% intra- and inter-sequence identities (Barton et al, 1991). The similar properties displayed by the annexin family regarding the calcium and phospholipid binding are related by this unique primary sequence.

A highly conserved 17 amino acid consensus sequence with its characteristic GXGTDE motif for calcium binding is invariably found in each of the repeats (also known as endonexin fold) (Swaijo and Seaton, 1994). The crystal structures of the N-terminal truncated annexin I, II, III, V and XII have been determined (Weng et al, 1993; Burger et al, 1996; Huber et al, 1992; Bewley et al 1993; Concha et al, 1993; Favier-Perron et al, 1996; Lueck et al, 1995). Studies of the above molecular structures have confirmed that the conserved C-terminal domains of annexins (at least that of annexin I and V) have a globular structure which consists almost exclusively of tightly packed α-helices (Huber et al; 1990; Weng et al, 1993). These studies also permitted the determination of the location of calcium binding sites in annexins. Taken together, the combination of biochemical data and X-ray crystographic studies, it has been demonstrated that the core domain of the annexins is resistant to limited proteolysis and contains the binding sites for Ca^{2+} and negatively charged phospholipids (Huber et al 1990, Huber et al 1990, Huber et al 1992). *In vitro* models indicate that the interactions between the polar head groups of the phospholipid and the annexins are possibly mediated by Ca^{2+} through the formation of phospholipid-calcium-annexin ternary complexes and that this combination leads to phospholipid-vesicle binding to annexins with high affinity in a bilayer or monolayer configuration (Creutz 1992, Tait et al, 1989; Yoshizaki et al, 1991). Thus, the function of the core domain of annexins is to bind to Ca^{2+} and phospholipids which can trigger several biological processes such as membrane aggregation and macromolecular trafficking.

Although annexins are collectively labelled as Ca^{2+} binding proteins, they differ from other well characterized calcium binding proteins, namely the EF-hand protein family (e.g., calmodulin), in terms of their structural architecture and their affinities for calcium ions. The EF-hand proteins contain typical Ca^{2+} binding sites spatially formed by helix-loop-helix motifs (Kretsinger 1980; Moncrief et al 1990; Nakayama et al 1992). The concentration of Ca^{2+} required for activation of these proteins is within the nanomolar range (Klee 1988). In contrast to EF-hand protein family, the crystal structure studies of annexins have shown that Ca^{2+} binding sites in annexins lack the typical helix -loop-helix motifs (EF hands) (Kretsinger and Creutz 1986). Furthermore, the dissociation constants of annexins bound Ca^{2+} are within the micromolar to millimolar range (Klee et al, 1988; Schlaepfer et al, 1987).

3.2. N-terminal domain of annexins.

In contrast to the C-terminal domains, the sequences of the N-terminal domains (tail) of annexins are highly variable. The N-terminal domains vary between 5 (e.g., annexin V) to more than 170 amino acid residues in length (e.g., annexin VII and XI) (Raynal and Pollard 1994). Moreover, the sequence of the N-terminal domains is thought to be extremely important in dictating and governing the functions of annexins (Drust and Creutz , 1988). Therefore, the N-termini of annexins are known as the regulatory region in that it contains sites for interactions with other proteins, sites for phosphorylation by PKC and Tyrosine-specific protein kinase (Varticovski et al, 1988, Wang and Creutz 1994). It has been demonstrated that members of the annexin family are able to bind proteins of the S100 family at their N-termini (William et al, 1996; Glenney et al, 1986; Towle et al, 1992; Naka et al, 1994; Watanabe et al, 1993). The S100 family of proteins are a subgroup of the EF hand calcium binding proteins thought to play an important role in cellular proliferation, differentiation and inflammation (Donato 1991). More recently, Mailliard et al (1996) identified another 10 kDa that belongs to the S100 family in extracts of cultured A431 cells that interacted with annexin I through the first 14 N-terminal amino acids. The role of the S100 proteins in annexin binding remains to be elucidated. In addition, the N-termini of annexins are very sensitive to proteolytic cleavage and cleavage at certain sites of the sequence results in an alteration in the requirement for Ca^{2+} (Ando et al, 1989; Drust and Creutz 1988).

4. Differential expression of annexins and their subcellular localization

Annexins are widely distributed in many cells from different tissues including adrenal medulla (Geisow and Burgone 1982; Creutz et al, 1983; Geisow et al, 1984), brain (Sudhof et al, 1984; Rhoads et al, 1985), lung (De et al, 1986; Fauvel et al, 1987), mammary and intestinal epithelial cells (Braslau et al, 1984; Gerke and Weber 1984; Shadle et al, 1985, Glenney 1986), lymphocytes (Sato et al, 1987) and neutrophils (Davies and Crumpton 1985; Owens et al, 1984). Differential expression of various members of the annexin family of proteins has been found during development. McKanna (1992) and McKanna and Cohen (1989) showed that annexin I was immunolocalized exclusively to the floor plate in rat CNS at embryonic stage and early postnatal development. In a more recent study, Hamre and coworkers (1995) examined the expression of five members of annexins (I, II, IV, V and VI) in the developing murine CNS. In that study, annexins I, II and IV

were shown to have distinct immunolocalization in developing and mature mice. However, annexin IV was the first annexin to be expressed in the embryonic stage (Day 9.5) with the widest midline distribution while annexin I was the last to be expressed (Day 11.5) with strict distribution. Based on the latter observations, it was proposed that the differential expression of annexins can be used as markers for different structures in the developing murine central nervous system and annexin IV may play an important role in the early development of central nervous system.

The differential expression of annexins has also been observed in malignant tumour cells (Masaki et al, 1996; Ahn et al, 1997; Sato-Matsumura et al, 1996). This aspect has been thoroughly studied in hepatocytes (Masaki et al, 1994; Masaki et al, 1996). Annexin I was not detected in normal and chronic lesions of liver tissue, whereas it was overexpressed at the transcriptional and the translational levels in human hepatocellular carcinoma, especially in poorly differentiated cancer cells (Masaki et al, 1996). Using an immunological staining approach, Ahn et al (1997) demonstrated that annexin I was not detectable in the ductal luminal cells of normal breast tissues and benign tumours but was generally expressed in various types of breast cancers, including noninvasive ductal carcinoma *in situ*. These results indicated that the expression of annexin I correlates with malignant tumour progression and that annexin I may be involved in the early stages of development of certain types of cancers.

Studies of subcellular localization of annexins have shown that the intracellular pools of annexins are tightly linked to Ca^{2+} levels. In the presence of high Ca^{2+}, annexins are able to bind negatively charged phospholipids at the inner leaflet of the plasma membrane and behave as peripheral plasma membrane proteins (Drust and Creutz 1991; Huber et al, 1992). However, in most cases, the membrane associated form was released by a calcium chelating agent (EDTA) with the exception of certain types of annexins whose dissociation from the plasma membrane required nonionic detergents (Sheets et al, 1987). The membrane bound form of annexin I can be converted to the soluble form after phosphorylation by the epidermal growth factor receptor at Tyr^{21} (Sheets et al, 1987). The translocation of annexin from the cytoplasm to the plasma membrane might be required by their biological functions.

5. Biological function of annexins

While much is known about the biochemical and structural properties of annexins, the cellular functions are largely unknown. *In vitro* studies have implicated annexins in numerous intracellular processes including vesicular trafficking (Creutz, 1992), membrane fusion during exocytosis(Creutz et al,1987; Drust and Creutz 1988) and endocytosis (Burgoyne and Clague 1994), mitogenic signal transduction (Hollenberg, et al 1988), ion channel formation (Polland and Rojas 1988), inhibition of phospholipase A2 and anticoagulation(Huang et al, 1986) as well as carcinogenesis (Reinhard et al, 1993; Cole et al, 1992; Masaki et al, 1996). More recent studies have also implicated certain members of the annexin family in programmed cell death or apoptosis (Reutelingsperger and Van Heerde, 1997).

5.1. Annexins and exocytosis and vesicular trafficking.

Exocytosis is a general mechanism by which large quantities of molecules enclosed within a cell vacuole are transferred to the cell surroundings following fusion of the vacuole with the plasma membrane. Membrane fusion is a key event in exocytosis and is often triggered by an intracellular messenger or an increase in the intracellular calcium concentration, generated , for example, in response to an external stimulus. Membrane fusion does not occur spontaneously because of the electrostatic forces produced by the high negative surface charge of the phosphate groups of the phospholipids. Cations such as Ca^{2+} and Mg^{2+} are thought to mediate membrane fusion by interrupting the electrostatic repulsion between two membrane surface (Francis et al, 1992; Doubell et al, 1991). These hypotheses have been confirmed *in vitro* in experiments using high concentrations of Ca^{2+} or Mg++ (above 1 mM) (Raynal and Pollard 1994). However, as the latter concentration of cations is much higher than that in the cytoplasm, other factors are likely to regulate membrane fusion in the intracellular environment. Despite the *in vitro* which support the hypothesis that some members of the annexin family promote exocytosis is supported by *in vitro* studies, the proposed function of annexins in intracellular vesicular trafficking is challenged by their requirement of "super-physiological" concentration of Ca^{2+}. The use of "Ca^{2+}sensitive dyes" to measure intracellular Ca^{2+} has led to the suggestion that the physiological concentrations of Ca^{2+} in the cytoplasm of secreting cells is in the nanomolar range (McConkey and Orrenius 1995; Ranyl and Pollard, 1994). This fact would tend to preclude a role for annexins in exocytosis. More recently, it was suggested that high concentration of Ca^{2+} in local areas (micro domain) can reach 200 to

300 μM as measured by a low-sensitive Ca^{2+}-dependent photo protein, n-Aeqnorin-J (Llinas et al, 1992). This fact suggested that the average Ca^{2+} concentration in the entire cytoplasm is not able to reflect the spatial and temporal local area of high Ca^{2+}. Thus, the role of annexin in exocytosis, *in vivo*, is under intense investigation.

Searching for mediators of membrane fusion in endocrine or exocrine cells , Creutz and his coworkers (1978) purified a protein from bovine adrenal medulla (now named annexin VII) that caused the aggregation of secretory vesicles in the presence of 10 μM Ca^{2+}. It was later found that this concentration of Ca^{2+} caused the self-association of annexin VII in the absence of phospholipids (Creutz et al 1979). With the exception of annexin V (Blackwood and Ernst , 1990), all annexins have been shown to promote membrane aggregation in a Ca^{2+}-dependent manner. Furthermore, using resonance energy transfer assay, Zaks and Creutz (1991) proposed that the self-association events enabled annexins to bind to membranes and reduced their requirement for high concentration of Ca^{2+} (from 1 mM to 10 μM).

Using synthetic membrane vesicles, it was shown that annexins can be subdivided into two categories, one promoting and the other inhibiting membrane aggregation and fusion (Blackwood and Ernest 1990, Tsao 1990). Annexins I , II, III ,VII and XI belong to the former group that stimulate membrane fusion events with different requirement for Ca^{2+} , while annexin V and VI are members of the latter and appeared devoid of such property (Blackwood and Ernst 1990; Creutz et al, 1987). Several studies have clearly shown that annexins differ greatly in their capacity to aggregate membranes and self-associate. Besides annexin VII, human annexin I is another family member that had been throughly studied in term of its membrane aggregation activity. These studies had focussed on the possible regions of peptide sequence that is related to phospholipid binding and the region that governed membrane aggregation events.

In the early 1990s, Blackwood and Ernst (1990) first demonstrated that annexin I mediated Ca^{2+} dependent fusion of phosphotidylserine liposomes. Subsequently, Oshry and his coworkers (1991) provided other evidence that annexin I also promoted fusion of phosphotidylserine/phosphotidylethanolamine liposomes with neutrophil membranes. At that time, whether a single annexin molecule could mediate membrane vesicle aggregation by interacting with two bilayer or whether annexin-annexin interaction were necessary, was not clear. Using native

annexin I purified from the cytosol of neutrophils, or recombinant annexin I, Meers and his coworkers (1991) showed that annexin I is a predominant mediator that enhances membrane aggregation in a Ca^{2+} dependent fashion. In the presence of cytosolic extracts, the fusion rate of liposomes was gradually increased with 2-20 μM of Ca^{2+}. A similar phenomenon was seen in the presence of increasing concentration of annexin I from different preparations with Ca^{2+} at 1 mM. They also showed that the monomers of annexin I could contact two membranes simultaneously at a point of intervesicular linkage based on the fact that covalent linkage of photoaffinity phospholipid derivatives with annexin I in vesicular aggregates did not allow complete disaggregation of vesicle by EDTA.

While it was quite certain that phospholipid binding sites are located in the consensus sequence of the core domain of annexin I (Glenney et al, 1986; Schlaepfer et al, 1987), the domain which promotes membrane fusion was unclear. A protein engineering approach was applied to form a chimeric protein between annexin I and annexin V by different groups mainly based on the fact that annexin V does not participate in membrane fusion event and annexins shared similar sequence of the core domain. Ernest and coworkers (1991) constructed a chimeric protein between 41-118 of annexin I and 93-320 of annexin V and expressed it in a bacterial expression system. In the presence of the first repeat of annexin I, the chimera promoted Ca^{2+}-dependent membrane fusion. This observation led to the conclusion that the first repeat of annexin I was critical for membrane fusion. However, in contrast to Eanest' studies, Hoekstra et al (1993) suggested that the N-terminal of annexin I governed membrane fusion using the same approach except that the chimera was generated through the entire tail of annexin I (1-45) and core domain of annexin V. Evidence from the three dimensional structure of annexin I indicated that all the Ca^{2+} and lipid binding sites are located on one side of the molecule while the tail domain of annexin I is located on the opposite side of the molecule (Weng et al 1993). Thus, membrane-membrane fusion may occur through protein-protein interactions between annexins bound to different membranes. Logically, the tail of annexin I is much more important in initiating membrane fusion. Consistent with this speculation is the observation that a monoclonal antibody to the N-terminus of annexin I inhibits vesicle aggregation and fusion but not lipid binding (Meers et al, 1992). The speculation that N-terminal of annexin I is important in membrane fusion is also supported by studies with annexin I constructs lacking parts

of the N-terminal sequence. By generating different truncated forms of annexin I through the N-terminal peptide sequence, Wang and Creutz (1994) showed that the truncated annexin I at Lys^{26} caused 4 times enhancement of Ca^{2+} sensitivity in the membrane aggregation assay. In contrast, cleavage at Trp^{12} resulted in 3 fold decrease in Ca^{2+} sensitivity. Thus, the ability of annexin I to aggregate membranes is highly sensitive to changes in the structure of its N-terminal domain.

Analysis of the amino acid sequence of N-terminal domain of annexin I revealed several phosphorylation sites including Tyr^{21}, Ser^{27}, Ser^{28} and Thr^{24}. Schldaepfer and Haiglerhad (1988) have shown that Ser and Thr sites could be equally phosphorylated by protein kinase C *in vitro*. In a later study, Wang and Creutz (1992) showed that phosphorylation of annexin I by protein kinase C strongly inhibits its ability to aggregate chromaffin granules. Mutagenesis studies of the phosphorylation sites of the human and bovine annexin I suggested that phosphorylation of Ser^{27} and Thr^{24} by protein kinase C led to an increase in the requirement of Ca^{2+} for annexin I to aggregate chromaffin granules and phosphatidylserine vesicles (Wang and Creutz 1994). It is very likely that phosphorylation of annexin I in these sites blocks the interactions between the tail and the core domains of annexin I, hence inhibition of the membrane aggregation and fusion event (Francoise et al 1996). Interestingly, phosphorylation of annexin I does not affect or mildly promotes its ability to bind to membranes (Wang and Creutz 1992; Francoise et al, 1996).Consistent with these results is the observation that a monoclonal antibody to the N-terminus of annexin I inhibits vesicular aggregation but not binding(Meers et al, 1992). This suggested that membrane binding and aggregation are sequential events that involve different regions of the annexin I molecule. Phosphorylation of Tyr^{21} *in vitro* could reduce the requirement for Ca^{2+} by five fold in membrane aggregation (Schlaepfer and Haiglerhad 1988) . This is somewhat controversial to the idea that the conformational changes due to phosphorylation of annexin I would inhibit membrane aggregation, since *in vivo* studies suggested that tyrosine phosphorylation was obscured because the phosphorylation of annexin I on Tyr^{21} is highly susceptible to proteolysis at Lys^{26}. The fact that truncated molecule minus the first 26 amino acids from N-terminal requires lower levels of Ca^{2+} to aggregate membrane vesicles led to the speculation that phosphorylation of annexin I at Tyr^{21} might inhibit its membrane aggregation function. Hence, proteolysis could restore this function (Wang and Creutz 1994).

5.2. Inhibition of phospholipase A 2.

Phospholipase A2 and possibly other phospholipases are important enzymes that are responsible for the release of arachidonic acid (AA), required for the synthesis of prostaglandins, in response to various stimuli. Enhanced phospholipase A2 activity could result in an accelerated release of arachidonic acid from phospholipid to cytosol. The consequences of this process are pathogenic inflammatory and necrotic events. The activity of phospholipases is a key factor in this process and negative regulatory events on these enzymes are able to decrease the substrate for prostaglandin synthesis. In the early 1980s, an effort to search for these regulatory factors of the enzyme led to the discovery of a protein (annexin I) expressed in rabbit neutrophils induced by glucocorticoids which inhibited phospholipase A2 activity (Schiffmann K et al, 1980). Following this study, the effects of annexin on the inhibition of phospholipase activity and inflammation had been extensively investigated due to its pharmacological significance. However, it was not clear if annexins directly interacted with phospholipase A2 in a classic enzyme/inhibitor manner. The evidence that phospholipase A2 activity could be abolished when the ratio of annexin/phospholipid in the reaction mixture was decreased suggested an indirect effect of annexins on the regulation of phospholipase A2 activity (Davidson et al, 1987; Aarsman et al, 1987). However, modulation of phospholipase A2 is likely indirect and the inhibitory effect may be due to the depletion of its substrate by annexin bound to phospholipid in the presence of Ca^{2+} (Lavidson et al, 1987; Aarsman et al, 1987). To investigate the substrate dependency in annexin/phospholipase A2 interactions, Bastian et al (1993) measured the activity of phospholipase A2 prepared from human epidermis and dermis homogenates in the presence of annexin I, II or V that were preincubated in separate series either with the substrate or with the enzyme. They found that a partial inhibition of both epidermal and dermal phospholipase A2 activities with all tested annexins. Moreover, the inhibitory effects were dependent on the annexin/phospholipid ratio which occurred only at very high annexin concentrations relative to the amount of the substrates. These results led to the current "substrate depletion model" and annexins block the access of phospholipase A2 to its substrate by binding to phospholipids.

5.3. Role of Annexin I in signal transduction

Growth factors are important molecules in the cascade of intracellular signal transduction.

This process begins when receptor tyrosine kinases on the cell surface bind to their cognate growth factors. Subsequently, activation of these receptors leads to a series of biochemical reactions through intracellular kinase cascade. The phosphorylation of intracellular molecules including nuclear transcription factors will regulate gene expression and cell proliferation. Earlier studies (Bargman et al, 1986; Berjcel 1989; Dellibovi et al, 1987) have demonstrated that many oncogenic proteins possess functions as growth factors. These proteins often lie at the center of an intracellular signal transduction pathway and actively accelerate such cellular events as proliferation and transformation through phosphorylation, and hence activate a variety of proteins. erb and $pp60^{v\text{-}src}$ are typical examples of tyrosine kinases encoded by their cognate oncogenes. A search for the substrate whose phosphorylation could regulate cell proliferation led to the identification of annexin I and II as major substrates for EGFR and $pp60^{v\text{-}src}$, respectively (Pepinsky et al, 1986; Glenney and Tack , 1985). By peptide mapping , sequencing and immunological analysis, Pepinsky and Sinclair (1986) first demonstrated that annexin I or p35 kD is a substrate for EGF receptor in A431 cells. Annexin II was shown to be phosphorylated at the N-termini by pp60v-src in Rous sarcoma virus transformed cells (Glenney and Tack , 1985). A later study by Karasik et al (1988) suggested that annexin I and II are also substrates for tyrosine kinases associated with the insulin receptor. Another study showed that tyrosine phosphorylation of annexin I could enhance its lipid binding activity and decrease its requirement for Ca^{2+} (Wang and Creutz 1994). It was also found that annexin I is involved in the internalization and degradation of EGF receptor since it was translocated to the plasma membrane during EGF induced membrane ruffling(Swairjo and Seaton 1994). Taking together, these observations strongly suggested that tyrosine phosphorylation of annexin I an II could modify their lipid binding and hence may be important for their functions as intermediate signalling molecules. With the exception of annexin V, all annexins are substrates for PKC (Dubois et al 1995; Rothut et al, 1995; Raynal et al, 1993; Schlaepfer et al, 1992; Dubois et al, 1996). The function of annexin phosphorylation by PKC has been extensively studied in various cell types. Interestingly, in several cancer cell lines, annexin I appears to be resistant to phosphorylation by PKC although it contains typical substrate site for PKC phosphorylation (Wijkander et al, 1989, Raynal et al 1993, Bienkowski et al 1989). It has been proposed that natural PKC inhibitors could be responsible for the lack of annexin I phosphorylation in the cells (Raynal and Pollard, 1994).

5.4. Annexin I and cell transformation.

Although studies have implicated the overexpression of annexin I and cell transformation in certain type of malignancies (Ahn et al, 1997, Masaki et al 1996, Col et al 1993), the actual effect of annexin I in mal-regulation of cell proliferation is poorly understood. Based on the fact that tyrosine phosphorylation is an important event in carcinogenesis and annexin I is an excellent substrate for epidermal receptor tyrosine kinase, the association between overexpression of tyrosine phosphorylated annexin I and cell transformation had been intensely investigated by different approaches. Using immunohistochemical staining, *in situ* hybridization and anti-phosphotyrosine monoclonal antibodies, Masaki et al (1996) observed that the overexpression of annexin I was detected in human hepatocellular carcinoma and in damaged liver induced with CCL4. However, a cytosolic form of annexin I was highly phosphorylated only in hepatocellular carcinoma cells. This observation led to the speculation that annexin I might be one of the factors contributing to cell transformation. Tyrosine phosphorylation of annexin I is likely essential for annexin I to exert its effect on tumourogenesis.

5.5. Other functions of annexins.

As an important protein family, it is believed that annexins exhibit diverse biological functions in the intracellular milieu. In particular, the hypothesis that annexins fulfill their effects by interacting with other proteins in a Ca^{2+} dependent fashion has received much attention. Annexins are able to bind to cytoskeletal proteins and this interaction suggested that annexins may be involved in the process of cell migration, adhesion, growth and differentiation (Raynal and Pollard 1994). The interactions between annexin I and S100 protein, or annexin II and P11 (proteins belonging to the EF-hand family of Ca^{2+} binding proteins) suggests that annexins may be important in cell proliferation and differentiation (Naka et al, 1994; Donato 1991; Glenney et al, 1986; William et al, 1996). Although the association of annexins with biological membranes via their Ca^{2+}-dependent membrane aggregation and fusion properties have been extensively investigated for years, the precise interactions between annexins and the lipid bilayer has not been well established yet. The ability of annexins (annexin I , VII) to form voltage sensitive Ca^{2+} channels has been demonstrated by using patch clamp with liposome or planar lipid bilayer (Pollard et al, 1988; Rojas et al, 1990). In those *in vitro* studies, annexin attachment to the targeted membrane in the presence of Ca^{2+} and

its integration in the lipid bilayer are thought to be important steps for Ca^{2+} (and possibly other cations) conductance in response to the stimuli from extracellular environment.

REFERENCES

Aarsman, A.J., Mynbeek, G., van den Bosch, H., Rothhut. B., Prieur, B., Comera, C., Jordan, L., and Russo-Marie F. Lipocortin inhibition of extracellular and intracellular phospholipases A2 is substrate concentration dependent FEBS Letters.219(1):176-80,1987

Abe, T., Hasegawa, S., Taniguchi, K., Yokomizo, A., Kuwano, T., Ono, M., Mori, T., Hori S. Kohno, K., and Kuwano, M. Possible involvement of multidrug-resistance-associated protein (MRP) gene expression in spontaneous drug resistance to vincristine, etoposide and adriamycin in human glioma cells. International Journal of Cancer. 58(6):860-4, 1994 .

Abolhoda, A., Wilson, A., Ross, H., Scotto, K., and Burt, M., Transcription of the MDR 1 gene following transcient in vivo exposure of human metastatic sarcoma to doxrubicin. Abstract 1685 (p251). The 88th Annual Meeting of American Association for Cancer Research 1997.

Abraham, I., Chin, K.V., Gottesman, M.M., Mayo, J.K., and Sampson, K.E. Transfection of a mutant regulatory subunit gene of cAMP-dependent protein kinase causes increased drug sensitivity and decreased expression of P-glycoprotein. Experimental Cell Research. 189(1):133-41, 1990

Ahn, S.H., Sawada, H., Ro, J.Y., and Nicolson,G.L. Differential expression of annexin I in human mammary ductal epithelial cells in normal and benign and malignant breast tissues. Clinical & Experimental Metastasis. 15(2):151-6, 1997

Ando, Y., Imamura .S., Hong, Y.M., Owada, M.K., Kakunaga, T., and Kannagi, R. Enhancement of calcium sensitivity of lipocortin I in phospholipid binding induced by limited proteolysis and phosphorylation at the amino terminus as analyzed by phospholipid affinity column chromatography. Journal of Biological Chemistry. 264(12):6948-55, 1989

Awasthi, S., Singhal ,S.S., Srivastava, S.K., Zimniak, P., Bajpai, K.K., Saxena, M., Sharma, R., Ziller, S.A. 3rd., Frenkel, E.P., and Singh, S.V., et al. Adenosine triphosphate-dependent transport of doxorubicin, daunomycin, and vinblastine in human tissues by a mechanism distinct from the Journal of Clinical Investigation. 93(3):958-65, 1994

Azzaria, M., Schurr, E., and Gros ,P. Discrete mutations introduced in the predicted nucleotide-binding sites of the mdr1 gene abolish its ability to confer multidrug resistance. Molecular & Cellular Biology. 9(12):5289-97, 1989

Bakos, E., Hegedus. T., Hollo, Z., Welker, E., Tusnady, G.E., Zaman,G.J., Flens, M.J., Varadi A., and Sarkadi, B. Membrane topology and glycosylation of the human multidrug resistance-associated protein. Journal of Biological Chemistry. 271(21):12322-6, 1996

Bargmann, C.I., Hung, M.C., and Weinberg, R.A., The neu oncogene encodes an epidermal growth factor receptor-related protein. Nature. 319(6050):226-30, 1986

Bartek, J., Bartkova, J., Vojtesek, B., Staskova ,Z., Rejthar, A., Kovarik ,J., and Lane, D.P. Patterns of expression of the p53 tumour suppressor in human breast tissues and tumours in situ and in vitro. International Journal of Cancer. 46(5):839-44, 1990

Barton, G.J., Newman, R.H., Freemont, P.S., and Crumpton, M.J. Amino acid sequence analysis of the annexin super-gene family of proteins. European Journal of Biochemistry. 198(3):749-60, 1991

Barrand, M.A., Heppell-Parton, A.C., Wright, K.A., Rabbitts, P.H., and Twentyman, P.R. A 190 Kda protein overexpressed in non-P-glycoprotein-containing multidrug resistant cells and its relationship to MRP gene. Journal of the National Cancer Institute. 86(2):110-7, 1994

Baslau, D.L., Ringo, D.L., and Rocha, V. Synthesis of novel calcium-dependent proteins associated with mammary epithelial cell migration and differentiation. Experimental Cell Research. 155(1):213-21, 1984

Bastian, B.C., Sellert, C., Seekamp, A., Romisch, J., Paques, E.P., and Braker, E.B. Inhibition of human skin phospholapse A2 by licoportins is undirect effect of substrate/licorpotin interaction. Journal of investigative dermatology. 10(3)359-363, 1993

Bates, S.E., Currier, S.J., Alvarez. M., and Fojo, A.T. Modulation of P-glycoprotein phosphorylation and drug transport by sodium butyrate. Biochemistry. 31(28):6366-72, 1992

Batist, G., Behrens, B.C., Makuch. R., Hamilton, T.C., Katki, A.G., Louie, K.G., Myers, C.E.and Ozols, R.F., Serial determinations of glutathione levels and glutathione-related enzyme activities in human tumor cells in vitro . Biochemical Pharmacology. 35(13):2257-9, 1986

Bech-Hansen, N.T., Till, J.E., and Ling, V. :Pleiotropic phenotype of colchicine-resistant CHO cells cross-resistance and collateral sensitivity. Journal of Cellular Physiology. 88(1):23-31, 1976

Beck, W.T. and Cirtain, M.C. Continued expression of vinca alkaloid resistance by CCRF-CEM cells after treatment with tunicamycin or pronase. Cancer Research. 42(1):184-9, 1982

Beja, O., and Bibi, E. Multidrug resistance protein (Mdr)-alkaline phosphatase hybrids in Escherichia coli suggest a major revision in the topology of the C-terminal half of Mdr. Journal of Biological Chemistry. 270(21):12351-4, 1995

Bejcek, B.E., Li, D.Y.and Deuel, T.F. Transformation by v-sis occurs by an internal autoactivation mechanism. Science. 245(4925):1496-9, 1989

Bell, D.R., Gerlach, J.H., Kartner. N., Buick ,R.N., and Ling, V. Detection of P-glycoprotein in ovarian cancer: a molecular marker associated with multidrug resistance. Journal of Clinical Oncology. 3(3):311-5, 1985

Bewley, M.C., Boustead, C.M.,. Walker, J.H., Waller, D.A., and Huber, R. Structure of chicken annexin V at 2.25-A resolution. Biochemistry. 32(15):3923-9, 1993

Bhardwaj, G., Gerlach, J.H., Mackie, J.E., Grant, C.E., Almquist, K.C., Stewart ,A.J., Kurz, E.U., Duncan, A.M., and Deeley, R.G. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line [see comments].Comment in: Science 1993 May 14;260(5110):879 1992

Bibi, E., and Beja, O. Membrane topology of multidrug resistance protein expressed in Escherichia coli. N-terminal domain. Journal of Biological Chemistry. 269(31):19910-5, 1994

Biedler,L., and Riehm, H. Cellular resistance to actinomycin D in Chinese hamster cells in vitro: cross-resistance, radioautographic, and cytogenetic studies.Cancer Research. 30(4):1174-84, 1970

Biedler, J.L , and Peterson, R.H.F. Althed plasma membrane glycoconjugates of Chinese hamster cells with aquired resistance to actinomycin D, daunorubicin and vincristine, In A.C. Sartorelli, J.S.

Lazo, J.R., and Bertino (eds), Molecular Actions and targets for cancer chemotherapeutical agents. Bristol-Myers Cancer Symposia Vol. 2,pp453-458. New York Academic Press. 1981

Bienkowski, M.J., Petro, M.A., and Robinson, L.J. Inhibition of thromboxane A synthesis in U937 cells by glucocorticoids. Lack of evidence for lipocortin 1 as the second messenger. Journal of Biological Chemistry. 264(11):6536-44, 1989

Blackwood, R.A., and Ernst, J.D. Characterization of Ca2(+)-dependent phospholipid binding, vesicle aggregation and membrane fusion by annexins. Biochemical Journal. 266(1):195-200, 1990

Boesch,D., Muller, K., Pourtier-Manzanedo, A., and Loor, F. Restoration of daunomycin retention in multidrug-resistant P388 cells by submicromolar concentrations of SDZ PSC 833, a nonimmunosuppressive cyclosporin derivative. Experimental Cell Research. 196(1):26-32, 1991

Bordow, S.B., Haber, M., Madafiglio, J., Cheung, B., Marshall, G.M., and Norris, M.D. Expression of the multidrug resistance-associated protein (MRP) gene correlates with amplification and overexpression of the N-myc oncogene in childhood neuroblastoma. Cancer Research. 54(19):5036-40, 1994

Borst, P., and Schinkel,A.H. Genetic dissection of the function of mammalian P-glycoproteins. [Review] [46 refs] Trends in Genetics. 13(6):217-22, 1997

Bradley, G., Georges , E., and Ling, V. Sex-dependent and independent expression of the P-glycoprotein isoforms in Chinese hamster. Journal of Cellular Physiology. 145(3):398-408, 1990

Braslau, D.L., Ringo, D.L., and Rocha, V. Synthesis of novel calcium-dependent proteins

associated with mammary epithelial cell migration and differentiation. Experimental Cell Research. 155(1):213-21, 1984

Breuninger, L.M., Paul, S., Gaughan, K., Miki, T., Chan, A., Aaronson, S.A., and Kruh, G.D. Expression of multidrug resistance-associated protein in NIH/3T3 cells confers multidrug resistance associated with increased drug efflux and altered intracellular drug distribution. Cancer Research. 55(22):5342-7, 1995

Burger, A., Berendes, R., Liemann, S., Benz, J., Hofmann, A., Gottig, P., Huber, R., Gerke, V. Thiel C. Romisch, J., and Weber, K. The crystal structure and ion channel activity of human annexin II, a peripheral membrane protein. Journal of Molecular Biology. 257(4):839-47, 1996

Burger, H., Nooter, K., Sonneveld, P., Van Wingerden, K.E., Zaman, G.J., and Stoter.G.High expression of the multidrug resistance-associated protein (MRP) in chronic and prolymphocytic leukaemia. British Journal of Haematology. 88(2):348-56, 1994

Burgoyne, R.D. and Geisow, M.J. The annexin family of calcium-binding proteins. Review article. [Review] Cell Calcium. 10(1):1-10, 1989

Burgoyne, R.D., and Clague, M.J. Annexins in the endocytic pathway. [Review] Trends in Biochemical Sciences. 19(6):231-2, 1994

Buschman, E., and Gros, P. Functional analysis of chimeric genes obtained by exchanging homologous domains of the mouse mdr1 and mdr2 genes. Molecular & Cellular Biology. 11(2):595-603, 1991

Buschman, E., Arceci, R.J., Croop, J.M., Che, M., Arias, I.M. Housman D.E., and Gros, P. mdr2 encodes P-glycoprotein expressed in the bile canalicular membrane as determined by isoform-specific antibodies. Journal of Biological Chemistry. 267(25):18093-9, 1992

Cabral. F., Abraham, I., and Gottesman, M.M. Isolation of a taxol-resistant Chinese hamster ovary cell mutant that has an alteration in alpha-tubulin. Proceedings of the National Academy of Sciences of the United States of America. 78(7):4388-91, 1981

Campos. L., Rouault, J,P., Sabido, O., Oriol, P., Roubi, N., and Vasselon, C. High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. Blood. 81(11):3091-6, 1993

Carlsen, S.A., Till, J.E., and Ling V. Modulation of membrane drug permeability in Chinese hamster ovary cells. Biochimica et Biophysica Acta. 455(3):900-12, 1976

Cartwright, P.H., Ilderton. E., Sowden, J.M., and Yardley, H.J. Inhibition of normal and psoriatic epidermal phospholipase A2 by picomolar concentrations of recombinant human lipocortin I. British

Journal of Dermatology. 121(2):155-60, 1989

Center, M.S. Mechanisms regulating cell resistance to adriamycin. Evidence that drug accumulation in resistant cells is modulated by phosphorylation of a plasma membrane glycoprotein. Biochemical Pharmacology. 34(9):1471-6, 1985

Chambers, T.C., Chalikonda, I., and Eilon G. Correlation of protein kinase C translocation, P-glycoprotein phosphorylation and reduced drug accumulation in multidrug resistant human KB cells. Biochemical & Biophysical Research Communications. 169(1):253-9, 1990

Chambers, T.C., Pohl, J., Raynor, R.L., and Kuo, J.F.Identification of specific sites in human P-glycoprotein phosphorylated by protein kinase C. Journal of Biological Chemistry. 268(7):4592-5, 1993

Chambers, T.C., Pohl, J., Glass, D.B., and Kuo, J.F. Phosphorylation by protein kinase C and cyclic AMP-dependent protein kinase of synthetic peptides derived from the linker region of human P-glycoprotein. Biochemical Journal. 299 (Pt 1):309-15, 1994

Chan, H.S., Haddad, G., Thorner, P.S., DeBoer. G., Lin, Y.P., Ondrusek. N., Yeger, H., and Ling, V. P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma [see comments]. Comments Comment in: N Engl J Med 1992 Apr 23;326(17):1162-3 New England Journal of Medicine. 325(23):1608-14, 1991.

Chan, H.S. Thorner, P.S., Haddad. G., and Ling, V. Immunohistochemical detection of P-glycoprotein: prognostic correlation in soft tissue sarcoma of childhood. Journal of Clinical Oncology. 8(4):689-704, 1990

Chen, C.J., Chin J.E., Ueda, K., Clark, D.P., Pastan, I., and Gottesman, M.M. Roninson IB. Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. Cell. 47(3):381-9, 1986

Cianfriglia, M. Willingham, M.C., Tombesi, M., Scagliotti, GV., Frasca, G., and Chersi A. P-glycoprotein epitope mapping. I. Identification of a linear human-specific epitope in the fourth loop of the P-glycoprotein extracellular domain by MM4.17 murine monoclonal antibody to human multi-drug-resistant cells. International Journal of Cancer. 56(1):153-60, 1994

Cole, S.P. The 1991 Merck Frosst Award. Multidrug resistance in small cell lung cancer. [Review] Canadian Journal of Physiology & Pharmacology. 70(3):313-29, 1992

Cole, S.P., Bhardwaj, G., Gerlach ,J.H., Mackie, J.E., Grant, C.E., Almquist, K.C., Stewart, A.J., Kurz, E.U., Duncan, A.M. and Deeley, R.G. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 1993 May 14;260(5110):879

Cole, S.P. Sparks, K.E., Fraser, K. Loe, D.W., Grant, C.E., Wilson, G.M., and Deeley RG. Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. Cancer Research. 54(22):5902-10, 1994

Concha, N.O., Head, J.F., Kaetzel, M.A., Dedman, J.R., and Seaton, B.A. Rat annexin V crystal structure: Ca(2+)-induced conformational changes.Science. 261(5126):1321-4, 1993

Colombel, M., Symmans, F., Gil, S., O'Toole, K.M., Chopin, D., Benson, M., Olsson, C.A. Korsmeyer S. and Buttyan, R. Detection of the apoptosis-suppressing oncoprotein bc1-2 in hormone-refractory human prostate cancers. American Journal of Pathology. 143(2):390-400, 1993

Connor, T.M., Knudson, C.M., Korsmeyer, S.J., and Lowe, S.W. bax-deficiency promotes drug resistance and oncogenic transformation by attenuating p53-dependent apoptosis. Proceedings of the National Academy of Sciences of the United States of America. 94(6):2345-9, 1997

Cornwell, M.M., Pastan, I., and Gottesman, M.M., Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein.Journal of Biological Chemistry. 262(5):2166-70, 1987

Creutz, C.E., Pazoles, C.J., and Pollard, H.B. Identification and purification of an adrenal medullary protein (synexin) that causes calcium-dependent aggregation of isolated chromaffin granules. Journal of Biological Chemistry. 253(8):2858-66, 1978

Creutz. C.E., and Sterner, D.C. Calcium dependence of the binding of synexin to isolated chromaffin granules. Biochemical & Biophysical Research Communications. 114(1):355-64, 1983

Creutz, C.E., Dowling, L.G., Sando, J.J., Villar-Palasi, C., Whipple, J.H., and Zaks,W.J. Characterization of the chromobindins. Soluble proteins that bind to the chromaffin granule membrane in the presence of Ca2+. Journal of Biological Chemistry. 258(23):14664-74, 1983

Creutz, C.E. The annexins and exocytosis. Science. 258(5084):924-31, 1992

Creutz, C.E., Snyder, S.L., Daigle, S.N., and Redick, J. Identification, localization, and functional implications of an abundant nematode annexin. Journal of Cell Biology. 132(6):1079-92, 1996

Croop, J.M., Raymond, M., Haber, D., Devault. A., Arceci, R.J., Gros, P., and Housman DE. The three mouse multidrug resistance (mdr) genes are expressed in a tissue-specific manner in normal mouse tissues. Molecular & Cellular Biology. 9(3):1346-50, 1989

Crumpton, M.J., and Dedman. J.R. Protein terminology tangle . Nature. 345(6272):212, 1990

Dalton, W.S., Cress, A.E., Alberts, D.S., and Trent JM . Cytogenetic and phenotypic analysis of a human colon carcinoma cell line resistant to mitoxantrone. Cancer Research. 48(7):1882-8, 1988

Danks MK. Yalowich JC. Beck WT. Atypical multiple drug resistance in a human leukemic cel line selected for resistance to teniposide (VM-26). Cancer Research. 47(5):1297-301, 1987

Davidson, FF., Dennis, EA., Powell, M., and Glenney, J.R Jr. Inhibition of phospholipase A2 by "lipocortins" and calpactins. An effect of binding to substrate phospholipids. Journal of Biological Chemistry. 262(4):1698-705, 1987

Davies AA., and Crumpton, M.J. Identification of calcium-binding proteins associated with the lymphocyte plasma membrane. Biochemical & Biophysical Research Communications. 128(2):571-7, 1985

De, B.K., Misono, K.S. Lukas, T.J., Mroczkowski, B., and Cohen, S. A calcium-dependent 35-kilodalton substrate for epidermal growth factor receptor/kinase isolated from normal tissue. Journal of Biological Chemistry. 261(29):13784-92, 1986

Demange, P., Voges, D., Benz. J., Liemann. S., Gottig, P., Berendes, R., Burger, A., and Huber R. AnnexinV: the key to understanding ion selectivity and voltage regulation?. Trends in Biochemical Sciences. 19(7):272-6, 1994

Descoteaux, S. Shen, P.S. Ayala, P., and Orozco, E. Samuelson J. P-glycoprotein genes of Entamoeba histolytica. Archives of Medical Research. 23(2):23-5, 1992.

Devault, A. and Gros, P. Two members of mouse mdr gene family confer multidrug resistance with overlapping but distinct drug specificities. Molecular Cell Biology. 10:1652-1663, 1990

Devine, S.E. Ling, V., and Melera, P.W. Amino acid substitutions in the sixth transmembrane domain of P-glycoprotein alter multidrug resistance. Proceedings of the National Academy of Sciences of the United States of America. 89(10):4564-8, 1992

Doubell, A.F., Bester, A.J., and Thibault, G. Annexins V and VI: major calcium-dependent atrial secretory granule-binding proteins. Hypertension. 18(5):648-56, 1991

Doige, C.A., and Ames, G.F. ATP-dependent transport systems in bacteria and humans: relevance to cystic fibrosis and multidrug resistance. Annual Review of Microbiology. 47:291-319, 1993.

Donato, R. Perspectives in S-100 protein biology. Review article. Cell Calcium. 12(10):713-26, 1991

Doyle, L.A. Mechanisms of drug resistance in human lung cancer cells. Seminars in Oncology. 20(4):326-37, 1993

Drust, D.S. and Creutz, C.E. Aggregation of chromaffin granules by calpactin at micromolar levels of calcium. Nature. 331(6151):88-91, 1988

Dusre, L., Mimnaugh, E.G., Myers, C.E., and Sinha, B.K. Potentiation of doxorubicin cytotoxicity by buthionine sulfoximine in multidrug-resistant human breast tumor cells. Cancer Research. 49(3):511-5, 1989

Eastman, A. Mechanisms of resistance to cisplatin..Cancer Treatment & Research. 57:233-49, 1991.

Eastman, A., and Schulte, N. Enhanced DNA repair as a mechanism of resistance to cis-diamminedichloroplatinum(II). Biochemistry. 27(13):4730-4, 1988

Eliopoulos, A.G., Kerr, D.J., Herod, J. Hodgkins, L. Krajewski, S., Reed .J.C., and Young LS. The control of apoptosis and drug resistance in ovarian cancer: influence of p53 and Bcl-2. Oncogene. 11(7):1217-28, 1995

Emans, N., Gorvel, J.P., Walter, C., Gerke, V., Kellner, R., Griffiths, G., and Gruenberg, J. Annexin II is a major component of fusogenic endosomal vesicles. Journal of Cell Biology. 120(6):1357-69, 1993

Endicott, J.A., and Ling, V. The biochemistry of P-glycoprotein-mediated multidrug resistance. [Review] Annual Review of Biochemistry. 58:137-71, 1989.

Evans, C,D., Mirski, S.E., Danks, M.K., and Cole SP. Reduced levels of topoisomerase II alpha and II beta in a multidrug-resistant lung-cancer cell line. Cancer Chemotherapy & Pharmacology. 34(3):242-8, 1994.

Fauvel. J., Vicendo, P., Roques, V., Ragab-Thomas, J., Granier, C., Vilgrain I. Chambaz E. Rochat, H., Chap, H., and Douste-Blazy, L. Isolation of two 67 kDa calcium-binding proteins from pig lung differing in affinity for phospholipids and in anti-phospholipase A2 activity. FEBS Letters. 221(2):397-402, 1987

Favier-Perron, B. , Lewit-Bentley, A., and Russo-Marie, F. The high-resolution crystal structure of human annexin III shows subtle differences with annexin V. Biochemistry. 35(6):1740-4, 1996

Fearnhead, H.O., McCurrach, M.E., O'Neill, J., Zhang, K., Lowe, S.W., and Lazebnik, Y.A. Oncogene-dependent apoptosis in extracts from drug-resistant cells. Genes & Development. 11(10):1266-76, 1997

Fine, R.L., Patel, J., and Chabner, B.A. Phorbol esters induce multidrug resistance in human breast cancer cell. Proceedings of the National Academy of Sciences of the United States of America. 85(2):582-6, 1988

Fisher, DE. Apoptosis in cancer therapy: crossing the threshold. [Review] [30 refs] Cell.

78(4):539-42, 1994

Fisher, G.A., and Sikic, B.I. Clinical studies with modulators of multidrug resistance. [Review] Hematology - Oncology Clinics of North America. 9(2):363-82, 1995

Flens, M.J. Izquierdo, M.A., Scheffer, G.L., Fritz, J.M., Meijer, C.J. Scheper, R.J., and Zaman. G.J. Immunochemical detection of the multidrug resistance-associated protein MRP in human multidrug-resistant tumor cells by monoclonal antibodies. Cancer Research. 54(17):4557-63, 1994

Flower, R.J.and Blackwell, G.J. Anti-inflammatory steroids induce biosynthesis of a phospholipase A2 inhibitor which prevents prostaglandin generation. Nature. 278(5703):456-9, 1979

Flower, R.J. The lipocortins and their role in controlling defense reactions. Advances in Prostaglandin, Thromboxane, & Leukotriene Research. 15:201-3, 1985.

Flower, R.J. Eleventh Gaddum memorial lecture. Lipocortin and the mechanism of action of the glucocorticoids. [Review]. British Journal of Pharmacology. 94(4):987-1015, 1988

Fojo, A.T. Shen, D.W., Mickley, L.A. Pastan, I., and Gottesman, M.M. Intrinsic drug resistance in human kidney cancer is associated with expression of a human multidrug-resistance gene. Journal of Clinical Oncology. 5(12):1922-7, 1987 .

Fojo, A.T., Ueda, K., Slamon, D.J., Poplack, D.G., Gottesman, M.M. and Pastan, I. Expression of a multidrug-resistance gene in human tumors and tissues. Proceedings of the National Academy of Sciences of the United States of America. 84(1):265-9, 1987

Ford, J.M., and Hait, W.N., Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacological Reviews. 42(3):155-99, 1990

Ford, J.M., and Hait, W.N . Pharmacologic circumvention of multidrug resistance. Cytotechnology. 12(1-3):171-212, 1993

Ford, J.M. Modulators of multidrug resistance. Preclinical studies. Hematology - Oncology Clinics of North America. 9(2):337-61, 1995

Francis, J.W., Balazovich, K.J., Smolen. J.E., Margolis, D.I., and Boxer, L.A. Human neutrophil annexin I promotes granule aggregation and modulates Ca(2+)-dependent membrane fusion. Journal of Clinical Investigation. 90(2):537-44, 1992

Gazdar, A.F., Carney, D.N., Russell, E.K., Sims. H.L., Baylin, S.B., Bunn, P.A Jr.,Guccion, J.G., and Minna, J.D. Establishment of continuous, clonable cultures of small-cell carcinoma of lung which have amine precursor uptake and decarboxylation cell properties. Cancer Research.

40(10):3502-7, 1980 .

Geisow, M.J. and Burgoyne, R.D., Calcium-dependent binding of cytosolic proteins by chromaffin granules from adrenal medulla. Journal of Neurochemistry. 38(6):1735-41, 1982

Geisow, M.J., Walker, J.H., Boustead,C., and Taylor,W., Annexins--new family of Ca2+-regulated-phospholipid binding protein. Bioscience Reports. 7(4):289-98, 1987

Gekeler, V., Ise, W., Sanders, K.H., Ulrich, W.R., and Beck, J. The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. Biochemical & Biophysical Research Communications. 208(1):345-52, 1995

Gerke, V., and Weber, K. Identity of p36K phosphorylated upon Rous sarcoma virus transformation with a protein purified from brush borders; calcium-dependent binding tonon-erythroid spectrin and F-actin. EMBO Journal. 3(1):227-33, 1984

Georges E., Bradley, G., Gariepy, J., and Ling, V. Detection of P-glycoprotein isoforms by gene-specific monoclonal antibodies. Proceedings of the National Academy of Sciences of the United States of America. 87(1):152-6, 1990

Georges and E., Zhang, J.T., and Ling, V. Modulation of ATP and drug binding by monoclonal antibodies against P-glycoprotein. Journal of Cellular Physiology. 148(3):479-84, 1991 Sep.

Georges, E., Tsuruo, T., and Ling, V. Topology of P-glycoprotein as determined by epitope mapping of MRK-16 monoclonal antibody. Journal of Biological Chemistry. 268(3):1792-8, 1993

Georges, E., Sharom, F.L., and Ling, V. Multidrug resistance and chemosensitization: therapeutic implications for cancer chemotherapy. Advances in pharmacology. 21: 185-220, 1990

Gerlach. J.H., Kartner, N., Bell, D.R. amd Ling, V. Multidrug resistance. Cancer Surveys. 5(1):25-46, 1986.

Gerlach, J.H., Endicott, J.A., Juranka, P.F., Henderson, G., Sarangi, F., Deuchars, L., and Ling, V. Homology between P-glycoprotein and a bacterial haemolysin transport protein suggests a model for multidrug resistance. Nature. 324(6096):485-9, 1986

Gerlach, J.H., Bell. D.R., Karakousis, C., Slocum, H.K., Kartner, N., Rustum, Y.M., Ling, V., and Baker RM. P-glycoprotein in human sarcoma: evidence for multidrug resistance. Journal of Clinical Oncology. 5(9):1452-60, 1987

Gerlach, J.H. Structure and function of P-glycoprotein.Cancer Treatment & Research. 48:37-53, 1989.

Germann, U.A., Willingham, M.C., and Pastan I. Gottesman MM. Expression of the human multidrug transporter in insect cells by a recombinant baculovirus. Biochemistry. 29(9):2295-303, 1990

Germann, U.A., Chambers, T.C., Ambudkar, S.V., Licht, T., Cardarelli, C.O. Pastan, I., and Gottesman MM. Characterization of phosphorylation-defective mutants of human P-glycoprotein expressed in mammalian cells. Journal of Biological Chemistry. 271(3):1708-16, 1996

Giannakakou, P., Sackett, D.L. Kang, Y.K., Zhan, Z., Buters, J.T., and Fojo T. Poruchynsky MS. Paclitaxel-resistant human ovarian cancer cells have mutant beta-tubulins that exhibit impaired paclitaxel-driven polymerization. Journal of Biological Chemistry. 272(27):17118-25, 1997

Glavy, J.S., Horwitz, S.B., and Orr, G. Identification of the in vivo phosphorylation sites for acidic-directed kinases in murine mdr1b P-glycoprotein. Journal of Biological Chemistry. 272(9):5909-14, 1997

Glenney, J.R Jr., Boudreau, M., Galyean ,R., Hunter, T., and Tack, B. Association of the S-100-related calpactin I light chain with the NH2-terminal tail of the 36-kDa heavy chain. Journal of Biological Chemistry. 261(23):10485-8, 1986

Glenney, J.R Jr., and Tack, B.F. Amino-terminal sequence of p36 and associated p10: identification of the site of tyrosine phosphorylation and homology with S-100. Proceedings of the National Academy of Sciences of the United States of America. 82(23):7884-8, 1985

Godwin, A.K., Meister, A., O'Dwyer, P.J., Huang, C.S., Hamilton, T.C., and Anderson, M.E. High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. Proceedings of the National Academy of Sciences of the United States of America. 89(7):3070-4, 1992

Goldstein, L.J., Galski, H., Fojo, A., Willingham .M., Lai, SL., Gazdar, A., Pirker, R., Green, A., Crist W., and Brodeur G.M. et al. Expression of a multidrug resistance gene in human cancers. Journal of the National Cancer Institute. 81(2):116-24, 1989

Goodfellow,H.R., Sardini, A., Ruetz, S., Callaghan, R., Gros, P., McNaughton, P.A., and Higgins, C.F. Protein kinase C-mediated phosphorylation does not regulate drug transport by the human multidrug resistance P-glycoprotein. Journal of Biological Chemistry. 271(23):13668-74, 1996

Gottesman, M.M., and Pastan, I. Biochemistry of multidrug resistance mediated by the multidrug transporter. [Review] Annual Review of Biochemistry. 62:385-427, 1993.

Gottesman, M.M. How cancer cells evade chemotherapy: sixteenth Richard and Hinda Rosenthal | Foundation Award Lecture. [Review] [74 refs] Cancer Research. 53(4):747-54, 1993

Grant, C.E., Valdimarsson, G., Hipfner, D.R., Almquist, K.C., Cole, S.P., and Deeley, DG. Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. Cancer Research. 54(2):357-61, 1994

Gros, P., Ben, Neriah, Y.B., Croop, J.M., and Housman, D.E. Isolation and expression of a complementary DNA that confers multidrug resistance. Nature. 323(6090):728-31, 1986

Gros, P., Croop, J., and Housman, D. Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. Cell. 47(3):371-80, 1986

Gros, P., Dhir, R., Croop, J., and Talbot F. A single amino acid substitution strongly modulates the activity and substrate specificity of the mouse mdr1 and mdr3 drug efflux pumps. Proceedings of the National Academy of Sciences of the United States of America. 88(16):7289-93, 1991

Gruenberg, J., and Maxfield, F.R. Membrane transport in the endocytic pathway. [Review] Current Opinion in Cell Biology. 7(4):552-63, 1995

Hamada, H., and Tsuruo, T. Functional role for the 170- to 180-kDa glycoprotein specific to drug-resistant tumor cells as revealed by monoclonal antibodies. Proceedings of the National Academy of Sciences of the United States of America. 83(20):7785-9, 1986

Hamada, H., Hagiwara, K., Nakajima, T., and Tsuruo, T. Phosphorylation of the Mr 170,000 to 180,000 glycoprotein specific to multidrug-resistant tumor cells: effects of verapamil, trifluoperazine, and phorbol esters. Cancer Research. 47(11):2860-5, 1987

Hamilton, T.C., Winker. M.A., Louie. K..G., Batist, G., Behrens, B.C., Tsuruo, T., Grotzinger KR. McKoy W.M., Young, R.C., and Ozols, R.F. Augmentation of adriamycin, melphalan, and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. Biochemical Pharmacology. 34(14):2583-6, 1985

Hamre, K.M., Chepenik, K.P., and Goldowitz, D. The annexins: specific markers of midline structures and sensory neurons in the developing murine central nervous system. Journal of Comparative Neurology. 352(3):421-35, 1995

Hartwell, L.H., and Kastan, M.B., Cell cycle control and cancer. Science. 266(5192):1821-8, 1994

Hartwell, L.H., Szankasi, P., Roberts, C.J., Murray, A.W., and Friend S. Integrating genetic approaches into the discovery of anticancer drugs. Science. 278(5340):1064-8, 1997

Hawkins, D.S., Demers, G.W., and Galloway, D.A. Inactivation of p53 enhances sensitivity to multiple chemotherapeutic agents. Cancer Research. 56(4):892-8, 1996

Heijn, M., Oude, Elferink, R.P., and Jansen, P.L.M. ATP-dependent multispecific organic anion transport system in rat erythrocyte membrane vesicles. American Journal of Physiology. 262(1 Pt 1):C104-10, 1992

Hendrickson, N., Sifri, C.D., Henderson, D.M., Allen, T., Wirth, D.F., and Ullman, B. Molecular characterization of the ldmdr1 multidrug resistance gene from Leishmania donovani. Molecular & Biochemical Parasitology. 60(1):53-64, 1993

Higgins, C.F.The multidrug resistance P-glycoprotein. [Review].Current Opinion in Cell Biology. 5(4):684-7, 1993

Hipfner, D.R., Gauldie, S.D., Deeley, R.G., and Cole, S.P. Detection of the M(r) 190,000 multidrug resistance protein, MRP, with monoclonal antibodies. Cancer Research. 54(22):5788-92, 1994

Hirata, F., del Carmine, R., Nelson, C.A., Axelrod, J., Schiffmann. E., Warabi. A., De Blas, A.L. Nirenberg, M., Manganiello, V., Vaughan, M., Kumagai, S., Green I. Decker JL.and Steinberg, A.D. Presence of autoantibody for phospholipase inhibitory protein, lipomodulin, in patients with rheumatic diseases. Proceedings of the National Academy of Sciences of the United States of America. 78(5):3190-4, 1981

Hofmann C., Gropp. R., and von der Mark, K. Expression of anchorin CII, a collagen-binding protein of the annexin family, in the developing chick embry Developmental Biology. 151(2):391-400, 1992

Hoekstra, D., Buist-Arkema, R., Klappe, K., and Reutelingsperger, C.P. Interaction of annexins with membranes: the N-terminus as a governing parameter as revealed with a chimeric annexin.Biochemistry. 32(51):14194-202, 1993

Hollstein, M., Sidransky, D., Vogelstein, B., Harris, C.C. p53 mutations in human cancers. [Review][96 refs] Science. 253(5015):49-53, 1991

Huang, K.S., Wallner, B.P., Mattaliano, R.J., Tizard, R., Burne, C., Frey, A., Hession, C. McGray, P. Sinclair, L.K. and Chow, E.P. et al. Two human 35 kd inhibitors of phospholipase A2 are related to substrates of pp60v-src and of the epidermal growth factor receptor/kinase. Cell. 46(2):191-9, 1986

Huang, Y. Ibrado, A.M., Reed, J.C., Bullock, G., Ray, S., Tang, C., and Bhalla, K. Co-expression of several molecular mechanisms of multidrug resistance and their significance for paclitaxel cytotoxicity in human AML HL-60 cells. Leukemia. 11(2):253-7, 1997

Huber. R., Schneider, M., Mayr, I., Romisch, J., and Paques, E.P. The calcium binding sites in human annexin V by crystal structure analysis at 2.0 A resolution. Implications for membrane binding

and calcium channel activity. FEBS Letters. 275(1-2):15-21, 1990

Huber, R., Berendes, R., Burger, A., Schneider, M., Karshikov, A., Luecke, H., Romisch, J., and Paques E. Crystal and molecular structure of human annexin V after refinement. Implications for structure, membrane binding and ion channel formation of the annexin family of proteins. Journal of Molecular Biology. 223(3):683-704, 1992

Huber, R. Berendes, R., Burger, A., Schneider, M. , Karshikov, A., Luecke, H., Romisch, J., and Paques E. Crystal and molecular structure of human annexin V after refinement. Implications for structure, membrane binding and ion channel formation of the annexin family of proteins. Journal of Molecular Biology. 223(3):683-704, 1992

Ichikawa, M., Yoshimura ,A., Furukawa, T., Sumizawa, T., Nakazima, Y., and Akiyama Glycosylation of P-glycoprotein in a multidrug-resistant KB cell line, and in the human tissues. Biochimica et Biophysica Acta. 1073(2):309-15, 1991

Inaba, M., Fukui, M., Yoshida, N., Tsukagoshi, S., and Sakurai Y. Collateral sensitivity of 6-mercaptopurine-resistant sublines of P388 and L1210 leukemia to the new purine antagonists, 5-carbamoyl-1H-imidazol-4-yl piperonylate and 4-carbamoylimidazolium 5-olate. Cancer Research. 42(3):1103. 1982

Ishikawa, T. ATP/Mg2+-dependent cardiac transport system for glutathione S-conjugates. A study using rat heart sarcolemma vesicles. Journal of Biological Chemistry. 264(29):17343-8, 1989

Ishikawa, T. The ATP-dependent glutathione S-conjugate export pump [see comments]. [Review] Trends in Biochemical Sciences. 17(11):463-8, 1992

Izquierdo, M.A., Scheffer, G.L., Flens, M.J., Giaccone, G., Broxterman, H.J., Meijer, C.J., and van der Valk P. Scheper RJ. Broad distribution of the multidrug resistance-related vault lung resistance protein in normal human tissues and tumors. American Journal of Pathology. 148(3):877-87, 1996

Izquierdo, M.A., Scheffer, GL., Flens, M.J., Shoemaker, R.H., Rome, L.H., and Scheper, R.J. Relationship of LRP-human major vault protein to in vitro and clinical resistance to anticancer drugs. [Review] [26 refs] Cytotechnology. 19(3):191-7, 1996.

Izquierdo, M.A., Scheffer, G.L., Flens, M.J., Schroeijers, A.B., van der Valk, P., and Scheper, R.J. Major vault protein LRP-related multidrug resistance. European Journal of Cancer. 32A(6):979-84, 1996

Jachez, B.and Nordmann, R. Loor F.Restoration of taxol sensitivity of multidrug-resistant cells by the cyclosporine SDZ PSC 833 and the cyclopeptolide SDZ 280-446. Journal of the National Cancer Institute. 85(6):478-83, 1993

Johnsson, N., Marriott, G., and Weber, K. p36, the major cytoplasmic substrate of src tyrosine protein kinase, binds to its p11 regulatory subunit via a short amino-terminal amphiphatic helix. EMBO Journal. 7(8):2435-42, 1988

Juranka, P.F., Zastawny, R.L., and Ling, V. P-glycoprotein: multidrug-resistance and a superfamily of membrane-associated transport proteins. [Review] [66 refs] FASEB Journal. 3(14):2583-92, 1989

Kaetzel, M.A., Hazarika ,P., and Dedman, J.R. Differential tissue expression of three 35-kDa annexin calcium-dependent phospholipid-binding proteins. Journal of Biological Chemistry. 264(24):14463-70, 1989

Kamesaki, S., Kamesaki, H., Jorgensen, T.J., Tanizawa, A., Pommier, Y., and Cossman, J. bcl-2 protein inhibits etoposide-induced apoptosis through its effects on events subsequent to topoisomerase II-induced DNA strand breaks and their repair [published erratum appears in Cancer Res 1994 Jun 1;54(11):3074]. Cancer Research. 53(18):4251-6, 1993

Kartner, N., Shales, M., Riordan, J.R.,and Ling, V. Daunorubicin-resistant Chinese hamster ovary cells expressing multidrug resistance and a cell-surface P-glycoprotein. Cancer Research. 43(9):4413-9, 1983 Sep.

Kartner. N., Riordan, J.R., and Ling, V. Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. Science. 221(4617):1285-8, 1983

Kartner, N., Evernden-Porelle, D., Bradley, G., and Ling V. Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. Nature. 316(6031):820-3, 1985

Kavallaris, M., Kuo, D.YS., Burkhart. C.A., Regl. D.L., Norris, M.D., Haber, M., and Horwitz S.B.Taxol-resistant epithelial ovarian tumors are associated with altered expression of specific beta-tubulin isotypes. Journal of Clinical Investigation. 100(5):1282-93, 1997

Kaye, S.B. Clinical drug resistance: the role of factors other than P-glycoprotein. American Journal of Medicine. 99(6A):40S-44S, 1995

Kennelly, P.J., and Krebs, E.G. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. Journal of Biological Chemistry. 266(24):15555-8, 1991

Kerr, J.F. Winterford, C.M., and Harmon, B.V. Apoptosis. Its significance in cancer and cancer therapy Cancer. 73(8):2013-26, 1994

Kessel. D., Botterill, V., and Wodinsky, I. Uptake and retention of daunomycin by mouse leukemic cells as factors in drug response. Cancer Research. 28(5):938-41, 1968

Kim R., Hirabayashi, N., Nishiyama, M., Saeki, S., Toge, T., and Okada, K., Expression of MDR1, GST-pi and topoisomerase II as an indicator of clinical response to adriamycin. Anticancer Research. 11(1):429-31, 1991

Kitada, S., Takayama, S., De Riel, K., Tanaka, S., and Reed J.C. Reversal of chemoresistance of lymphoma cells by antisense-mediated reduction of bcl-2 gene expression. Antisense Research & Development. 4(2):71-9, 1994

Klee, C.B., Ca2+-dependent phospholipid- (and membrane-) binding proteins. Biochemistry. 27(18):6645-53, 1988

Kloke, O., and Osieka, R, Interaction of cyclosporin A with antineoplastic agents. Klinische Wochenschrift. 63(20):1081-2, 1985

Kondo, S., Barnett, G.H., Hara, H., Morimura, T., and Takeuchi, J., MDM2 protein confers the resistance of a human glioblastoma cell line to cisplatin-induced apoptosis. Oncogene. 10(10):2001-6, 1995

Kool, M., de Haas, M., Scheffer, G.L., Scheper, R.J., van Eijk, M.J., Juijn, J.A., Baas, F., and Borst, P. Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. Cancer Research. 57(16):3537-47, 1997.

Kramer, R.A., Zakher, J., and Kim, G. Role of the glutathione redox cycle in acquired and de novo multidrug resistance. Science. 241(4866):694-7, 1988

Kretsinger, R.H. Structure and evolution of calcium-modulated proteins. Critical Reviews in Biochemistry. 8(2):119-74, 1980.

Kretsinger, R.H. Creutz CE. Cell biology. Consensus in exocytosis . Nature. 320(6063):573, 1986

Krishnamachary, N., and Center, M.S. The MRP gene associated with a non-P-glycoprotein multidrug resistance encodes a 190-kDa membrane bound glycoprotein. Cancer Research. 53(16):3658-61, 1993 Aug 15.

Kruh, G.D., Chan, A., Myers, K., Gaughan, K., Miki ,T., and Aaronson, S.A. Expression complementary DNA library transfer establishes mrp as a multidrug resistance gene. Cancer Research. 54(7):1649-52, 1994

Leier, I.. Jedlitschky G. Buchholz U. Cole SP. Deeley RG. Keppler D. The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. Journal of Biological Chemistry. 269(45):27807-10, 1994

Ling ,V. and Baker, R.M. Dominance of colchicine resistance in hybrid CHO cells. Somatic Cell Genetics. 4(2):193-200, 1978 Mar.

Ling, V. Kartner, N., Sudo, T., Siminovitch, L., and Riordan, J.R, Multidrug-resistance phenotype in Chinese hamster ovary cells. Cancer Treatment Reports. 67(10):869-74, 1983

Linn, S.C., Giaccone, G., van Diest, P.J., Blokhuis, W.M., van der, Valk. P. van Kalken, C.K. Kuiper CM. Pinedo, H.M., Baak, J.P. Prognostic relevance of P-glycoprotein expression in breast cancer. Annals of Oncology. 6(7):679-85, 1995

Liu, Z., Lheureux. F., Pouliot, J.F., Heckel, A, Bamberger, U., and Georges, E. BIB W22, potent multidrug resisctance-reversing agent, binds directly to P-gp and accumulates in drug resistance cells. Molecular Pharmacology. 50(3):482-492, 1996

Llinas, R., Sugimori, M., and Silver, R.B. Microdomains of high calcium concentration in a presynaptic terminal. Science. 256(5057):677-9, 1992

Loo, T.W., and Clarke, D.M. Functional consequences of phenylalanine mutations in the predicted transmembrane domain of P-glycoprotein. Journal of Biological Chemistry. 268(27):19965-72, 1993

Loo ,T.W., and Clarke, D.M. Mutations to amino acids located in predicted transmembrane segment 6 (TM6) modulate the activity and substrate specificity of human P-glycoprotein. Biochemistry. 33(47):14049-57, 1994

Loe, D.W., Deeley, R.G.,. and Cole, S.P. Biology of the multidrug resistance-associated protein, MRP. [Review].European Journal of Cancer. 32A(6):945-57, 1996

Loe, D.W., Stewart, R.K., Massey,T.E., Deeley, RG., and Cole ,S.P. ATP-dependent transport of aflatoxin B1 and its glutathione conjugates by the product of the multidrug resistance protein (MRP) gene. Molecular Pharmacology. 51(6):1034-41, 1997

Loe, D.W., Almquist, K.C. Deeley, R.G., and Cole SP. Multidrug resistance protein (MRP)-mediated transport of leukotriene C4 and chemotherapeutic agents in membrane vesicles. Demonstration of glutathione-dependent vincristine transport. Journal of Biological Chemistry. 271(16):9675-82, 1996

Lowe, S.W., Ruley, H.E., Jacks, T., and Housman, D.E. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell. 74(6):957-67, 1993

Luecke, H., Chang, B.T., Mailliard, WS., Schlaepfer, D.D., and Haigler ,H.T. Crystal structure of the annexin XII hexamer and implications for bilayer insertion. Nature. 378(6556):512-5, 1995

Lum, B.L., Fisher, G.A., Brophy, N.A., Yahanda, A.M., Adler, K.M., Kaubisch, S., Halsey, J.

and Sikic, B.I. Clinical trials of modulation of multidrug resistance. Pharmacokinetic and pharmacodynamic considerations. Cancer. 72(11 Suppl):3502-14, 1993

Ma ,D.D., Scurr, R.D., Davey, R.A., Mackertich, S.M., Harman, D,H., Dowden, G., Isbister, J,P.and Bell, D.R. Detection of a multidrug resistant phenotype in acute non-lymphoblastic leukaemia. Lancet. 1(8525):135-7, 1987

Ma, L.. Krishnamachary, N.. and Center, M.S. Phosphorylation of the multidrug resistance associated protein gene encoded protein P190. Biochemistry. 34(10):3338-43, 1995

Mailliard, W.S., Haigler, H.T., and Schlaepfer, D.D. Calcium-dependent binding of S100C to the N-terminal domain of annexin Journal of Biological Chemistry. 271(2):719-25, 1996

Masuda, H., Ozols, R.F., Lai, G.M., Fojo, A., Rothenberg, M., and Hamilton TC. Increased DNA repair as a mechanism of acquired resistance to cis-diamminedichloroplatinum (II) in human ovarian cancer cell lines. Cancer Research. 48(20):5713-6, 1988

Marquardt, D., McCrone, S., and Center, M.S., Mechanisms of multidrug resistance in HL60 cells: detection of resistance-associated proteins with antibodies against synthetic peptides that correspond to the deduced sequence of P-glycoprotein. Cancer Research. 50(5):1426-30, 1990

Masaki, T., Tokuda,,M., Fujimura, T., Ohnishi, M., Tai, Y., Miyamoto, K., Itano, T., Matsui ,H., Watanabe, S., and Sogawa, K. et al. Involvement of annexin I and annexin II in hepatocyte proliferation: can annexins I and II be markers for proliferative hepatocytes?. Hepatology. 20(2):425-35, 1994

Masaki, T., Tokuda, M., Ohnishi, M., Watanabe, S., Fujimura ,T., Miyamoto, K., Itano, T. Matsui, H., Arima, K., Shirai, M., Maeba, T., Sogawa, K., Konishi, R,. Taniguchi, K., Hatanaka, Y., Hatase, O. and Nishioka M. Enhanced expression of the protein kinase substrate annexin in human hepatocellular carcinoma. Hepatology. 24(1):72-81, 1996

Matsumoto, Y., Takan ,H., and Fojo, T. Cellular adaptation to drug exposure: Evolution of the drug resistant phenotype. 88th annunal meeting of American Association For Cancer Research. Abstract 1684, 1997

McGrath,T., and Center, M.S. Adriamycin resistance in HL60 cells in the absence of detectable P-glycoprotein. Biochemical & Biophysical Research Communications. 145(3):1171-6, 1987

McGrath, T., Center, M.S. Mechanisms of multidrug resistance in HL60 cells: evidence that a surface membrane protein distinct from P-glycoprotein contributes to reduced cellular accumulation of drug. Cancer Research. 48(14):3959-63, 1988

McGrath, J.P., and Varshavsky, A., The yeast STE6 gene encodes a homologue of the mammalian

multidrug resistance P-glycoprotein. Nature. 340(6232):400-4, 1989

McKanna, J.A. and Cohen, S. The EGF receptor kinase substrate p35 in the floor plate of the embryonic rat CNS. Science. 243(4897):1477-9, 1989

McKanna ,J.A. Optic chiasm and infundibular decussation sites in the developing rat diencephalon are defined by glial raphes expressing p35 (lipocortin 1, annexin I). Developmental Dynamics. 195(2):75-86, 1992

McConkey, D.J ,and Orrenius, S. The role of Calcium in the regulation of Apoptosis. Journal of Leulocyte biology. 59:775-783, 1995

Mellado, W., and Horwitz, S.B. Phosphorylation of the multidrug resistance associated glycoprotein. Biochemistry. 26(22):6900-4, 1987

Minderman, H., Vanhoefer, U., Toth, K., Minderman, M.D., and Rustum, Y.M. A unique human ovarian carcinoma cell line expressing CD34 in association with selection for multidrug resistance. Cancer. 78(11):2427-36, 1996

Mirski, S.E., Gerlach, J.H., and Cole, S.P. Multidrug resistance in a human small cell lung cancer cell line selected in adriamycin. Cancer Research. 47(10):2594-8, 1987

Withoff, S., de Vries, E.G., Keith, W.N., Nienhuis, E.F., van der Graaf, W.T., Uges. D.R., and Mulder, N.H. Differential expression of DNA topoisomerase II alpha and -beta in P-gp and MRP-negative VM26, mAMSA and mitoxantrone-resistant sublines of the human SCLC cell line GLC4. British Journal of Cancer. 74(12):1869-76, 1996

Miyashita, T. Reed JC.bcl-2 gene transfer increases relative resistance of S49.1 and WEHI7.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticoids and multiple chemotherapeutic drugs. Cancer Research. 52(19):5407-11, 1992

Moncrief, N.D. Kretsinger, R.H., and Goodman, M. Evolution of EF-hand calcium-modulated proteins. I. Relationships based on amino acid sequences. Journal of Molecular Evolution. 30(6):522-62, 1990

Monzo, M., Martinez, M., De, Anta., Gonzalezlarriba, J.L., Aberola, V., Benito, D., Abrad, A., and Rosell, R. B-tubulin gene mutations induce taxol chemoresistance in non small cell lung cancer. 88th annual meeting Of American Association For Cancer Research. 1997

Moore ,P.B., and Dedman, J.R. Calcium binding proteins and cellular regulation. [Review] [58 refs] Life Sciences. 31(26):2937-46, 1982

Moore, P.B., Kraus-Friedmann, N., and Dedman, J,R. Unique calcium-dependent hydrophobic

binding proteins: possible independent mediators of intracellular calcium distinct from calmodulin. Journal of Cell Science. 72:121-33, 1984

Moss, S.E (ed) Annexins. 1992, Portland Press Ltd., London

Muller, M., Meijer, C., Zaman, G.J., Borst, P., Scheper, R.J., Mulder, N.H., Vries, E.G., and Jansen, P.L. Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport. Proceedings of the National Academy of Sciences of the United States of America. 91(26):13033-7, 1994

Mukhopadhyay, T., Batsakis, J.G., Kuo, M.T., Expression of the mdr (P-glycoprotein) gene in Chinese hamster digestive tracts. Journal of the National Cancer Institute. 80(4):269-75, 1988

Naka, M., Qing, Z.X., Sasaki, T., Kise, H., Tawara ,I., Hamaguchi. S. and Tanaka, T. Purification and characterization of a novel calcium-binding protein, S100C, from porcine heart. Biochimica et Biophysica Acta. 1223(3):348-53, 1994

Nakayama, S., Moncrief, N.D.,and Kretsinger, R.H. Evolution of EF-hand calcium-modulated proteins. II. Domains of several subfamilies have diverse evolutionary histories. [Review] Journal of Molecular Evolution. 34(5):416-48, 1992

Nare,B., Prichard, R.K., and Georges E. Characterization of rhodamin 123 bind to P-gp in human multidrug resistant cells. Molecular Pharmacology. 45:1145-1152, 1994

Nebert, D.W., Nelson, D.R., Coon, M.J., Estabrook, R.W., Feyereisen, R., Fujii-Kuriyama, Y. Gonzalez, F.J., Guengerich, F.P., Gunsalus, I.C., and Johnson, E.F. et al. The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature [published erratum appears in DNA. Cell Biology 1991 Jun;10(5):397-8]. [Review] [110 refs] DNA & Cell Biology. 10(1):1-14, 1991

Ng, W.F., Sarangi, F., Zastawny, R.L., Veinot-Drebot, L., and Ling. V, Identification of members of the P-glycoprotein multigene family. Molecular & Cellular Biology. 9(3):1224-32, 1989

Nogae, I., Kohno, K., Kikuchi, J., Kuwano, M., Akiyama, S., Kiue, A., Suzuki, K., Yoshida, Y., Cornwell, M,M., and Pastan I., et al. Analysis of structural features of dihydropyridine analogs needed to reverse multidrug resistance and to inhibit photoaffinity labeling of P-glycoprotein.Biochemical Pharmacology. 38(3):519-27, 1989

Nooter, K., Bosman, F.T., Burger, H., van Wingerden, K.E., Flens, M.J., Scheper, R.J., Oostrum, R,G., Boersma. A.W., van der Gaast, A.,and Stoter, G. Expression of the multidrug resistance-associated protein (MRP) gene in primary non-small-cell lung cancer. Annals of Oncology. 7(1):75-81, 1996

Nooter, K., Brutel de la Riviere, G., Look, M.P., van Wingerden, K.E., Henzen-Logmans, S.C. Scheper, R.J., Flens, M.J., Klijn, J.G., Stoter, G., and Foekens ,J.A. The prognostic significance of expression of the multidrug resistance-associated protein (MRP) in primary breast cancer. British Journal of Cancer. 76(4):486-93, 1997.

Nunez, G., London, L., Hockenbery, D., Alexander, M., McKearn, J.P. and Korsmeyer, S.J. Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. Journal of Immunology. 144(9):3602-10, 1990

Ohnishi, M., Tokuda ,M., Masaki, T., Fujimura, T., Tai, Y., Matsui, H., Itano, T., Ishida, T., Takahara, J. and Konishi, R. et al. Changes in annexin I and II levels during the postnatal development of rat pancreatic islets. Journal of Cell Science. 107 (Pt 8):2117-25, 1994

Orr, G.A., Han, E.K., Browne, P.C., Nieves, E., O'Connor, B.M., Yang ,C.P., and Horwitz, S.B. Identification of the major phosphorylation domain of murine mdr1b . P-glycoprotein. Analysis of the protein kinase A and protein kinase C phosphorylation sites. Journal of Biological Chemistry. 268(33):25054-62, 1993

Paul, S., Breuninger, L.M., and Kruh, G.D. ATP-dependent transport of lipophilic cytotoxic drugs by membrane vesicles prepared from MRP-overexpressing HL60/ADRcells. Biochemistry. 35(44):14003-11, 1996

Paulusma, C.C., Bosma, P.J., Zaman, G.J., Bakker, C.T., Otter, M., Scheffer ,G.L., Scheper, R.J. and Borst P. Oude Elferink RP. Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. Science. 271(5252):1126-8, 1996

Paul, S., Belinsky, M.G., Shen, H., and Kruh. G.D.Structure and in vitro substrate specificity of the murine multidrug resistance-associated protein. Biochemistry. 35(42):13647-55, 1996

Pearce, H.L., Safa, A.R., Bach, N.J., Winter, M.A., Cirtain, M.C., and Beck, W,T. Essential features of the P-glycoprotein pharmacophore as defined by a series of reserpine analogs that modulate multidrug resistance. Proceedings of the National Academy of Sciences of the United States of America. 86(13):5128-32, 1989

Perego, P., Giarola, M., Righetti, S.C., Supino, R., Caserini C. Deli., D., Pierotti, M.A., Miyashita, T. Reed J. and C. Zunino F. Association between cisplatin resistance and mutation of p53 gene and reduced bax expression in ovarian carcinoma cell systems. Cancer Research. 56(3):556-62, 1996

Perez RP. Hamilton TC. Ozols RF. Resistance to alkylating agents and cisplatin: insights from ovarian carcinoma model systems. Pharmacology & Therapeutics. 48(1):19-27, 1990.

Pepinsky, R.B., Sinclair, L.K., Browning, J.L., Mattaliano, R.J., Smart, J.E., Chow, E.P., Falbel

T. Ribolini A., Garwin, J.L., and Wallner, B.P. Purification and partial sequence analysis of a 37-kDa protein that inhibits phospholipase A2 activity from rat peritoneal exudates. Journal of Biological Chemistry. 261(9):4239-46, 1986

Pepinsky RB. Sinclair LK. Epidermal growth factor-dependent phosphorylation of lipocortin. Nature. 321(6065):81-4, 1986

Pepinsky, R.B., Tizard, R., Mattaliano, R.J., Sinclair, L.K., Miller, G.T., Browning ,J.L. Chow EP. Burne C., Huang, K.S. and Pratt, D. et al, Five distinct calcium and phospholipid binding proteins share homology with lipocortin I. Journal of Biological Chemistry. 263(22):10799-811, 1988

Pinkoski, M.J., Bhardwaj, G., and Deeley, R.G. Elevated expression of annexin II (lipocortin II, p36) in a multidrug resistant small cell lung cancer cell line. British Journal of Cancer. 65(4):498-502, 1992

Pollard, H.B., and Rojas, E. Ca2+-activated synexin forms highly selective, voltage-gated Ca2+ channels in phosphatidylserine bilayer membranes. Proceedings of the National Academy of Sciences of the United States of America. 85(9):2974-8, 1988

Pollard, H.B., Guy, H.R., Arispe, N., de la Fuente, M., Lee, G., adn Rojas EM. Pollard JR. Srivastava M. Zhang-Keck ZY. Merezhinskaya N. et al. Calcium channel and membrane fusion activity of synexin and other members of the Annexin gene family. Biophysical Journal. 62(1):15-8, 1992

Pommier, Y., Kerrigan, D., Schwartz, R.E., Swack, J.A., and McCurdy, A. Altered DNA topoisomerase II activity in Chinese hamster cells resistant to topoisomerase II inhibitors. Cancer Research. 46(6):3075-81, 1986

Powis, G., and Prough, R..A Metablism of anticancer drugs. Philadelphia: Taylor, 1987

Priebe, W., Krawczyk, M., Kuo, M.T., Yamane, Y., Savaraj, N., and Ishikawa T. Chemical syntheses of doxrobucin-glutathione conjugates and demonstration of their inhibitions in LTC4 transport by MRP/GSX pump. Abstrate 2945, 88th Annual Meeting of American Association of Cancer Research. 1997.

Ramu A., Glaubiger, D., and Weintraub, H. Differences in lipid composition of doxorubicin-sensitive and -resistant P388 cells. Cancer Treatment Reports. 68(4):637-41, 1984

Rao, U.S., and Scarborough, G.A.Direct demonstration of high affinity interactions of immunosuppressant drugs with the drug binding site of the human P-glycoprotein. Molecular Pharmacology. 45(4):773-6, 1994

Raynal, P., Hullin, F., Ragab-Thomas, J.M., Fauvel, J., and Chap, H., Annexin 5 as a potential regulator of annexin 1 phosphorylation by protein kinase C. In vitro inhibition compared with quantitative data on annexin distribution in human endothelial cells. Biochemical Journal. 292 (Pt 3):759-65, 1993

Raynal, P.and Pollard, H.B. Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. Biochimica et Biophysica Acta. 1197(1):63-93, 1994

Reutelingsperger, C.P. and van Heerde, W.L. Annexin V, the regulator of phosphatidylserine-catalyzed inflammation and coagulation during apoptosis. Cellular & Molecular Life Sciences. 53(6):527-32, 1997.

Reed, J.C. Bcl-2: prevention of apoptosis as a mechanism of drug resistance. Hematology - Oncology Clinics of North America. 9(2):451-73, 1995

Reed, J.C. Bcl-2 family proteins: strategies for overcoming chemoresistance in cancer. [Review] [190 refs] Advances in Pharmacology. 41:501-32, 1997.

Rhoads, AR., Lulla, M.., Moore, PB., and Jackson ,C.E. Characterization of calcium-dependent membrane binding proteins of brain cortex. Biochemical Journal. 229(3):587-93, 1985

Riordan, J.R., Deuchars, K., Kartner, N., Alon ,N., Trent, J., and Ling , V. Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. Nature. 316(6031):817-9, 1985

Riordan, J.R., Rommens, J.M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J. Lok S. Plavsic, N., and Chou, J.L., et al. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA Science. 245(4922):1066-73, 1989

Rojas, E., Pollard, HB., Haigler, H.T., Parra, C., and Burns, A.L. Calcium-activated endonexin II forms calcium channels across acidic phospholipid bilayer membranes. Journal of Biological Chemistry. 265(34):21207-15, 1990

Rosenberg, M.F., Callaghan, R., Ford, R.C. and Higgins, C.F. Structure of the multidrug resistance P-glycoprotein to 2.5 nm resolution determined by electron microscopy and image analysis. Journal of Biological Chemistry. 272(16):10685-94, 1997

Ruetz, S. and Gros, P. Phosphatidylcholine translocase: a physiological role for the mdr2 gene. Cell. 77(7):1071-81, 1994

Safa, A.R., Glover, C.J., Sewell, J.L., Meyers, M.B., Biedler, J.L,. and Felsted, R.L. Identification of the multidrug resistance-related membrane glycoprotein as an acceptor for calcium channel

blockers. Journal of Biological Chemistry. 262(16):7884-8, 1987

Safa, A.R., Mehta, N.D., and Agresti, M., Photoaffinity labeling of P-glycoprotein in multidrug resistant cells with photoactive analogs of colchicine. Biochemical & Biophysical Research Communications. 162(3):1402-8, 1989

Salmon, S.E., Grogan ,T.M., Miller, T., Scheper, R., and Dalton, W.S. Prediction of doxorubicin resistance in vitro in myeloma, lymphoma, and breast cancer by P-glycoprotein staining. Journal of the National Cancer Institute. 81(9):696-701, 1989

Sato, E.F., Morimoto, Y.M., Matsuno, T.,Miyahara, M., and Utsumi, K., Neutrophil specific 33 kDa protein: its Ca2+- and phospholipid-dependent intracellular translocation. FEBS Letters. 214(1):181-6, 1987

Sato, H., Gottesman, M,M., Goldstein, L,J., and Pastan, I. Block AM. Sandberg AA. Preisler HD. Expression of the multidrug resistance gene in myeloid leukemias. Leukemia Research. 14(1):11-21, 1990.

Sato-Matsumura, K.C., Koizumi, H., Matsumura, T., Ohkawara, A., Takasu, T., Furuta, Y., Sawa H. and Nagashima K. Localization of annexin I (lipocortin I, p35) mRNA in normal and diseased human skin by in situ hybridization. Archives of Dermatological Research. 288(10):565-9, 1996

Scheffer, G.L., Wijngaard ,P.L., Flens, M.J., Izquierdo, M.A., Slovak, M.L,. Pinedo, H.M., Meijer, C.J., Clevers, H.C., and Scheper, R.J. The drug resistance-related protein LRP is the human major vault protein Comment in: Nat Med 1995 Nat Med Jun;1(6):527

Schinkel, A.H., Roelofs, E.M., and Borst, P.Characterization of the human MDR3 P-glycoprotein and its recognition by P-glycoprotein-specific monoclonal antibodies. Cancer Research. 51(10):2628-35, 1991

Schinkel, A.H., Smit, J.J., van Tellingen, O., Beijnen, J,H., Wagenaar ,E., van Deemter, L., Mol C.A., van der Valk, M.A., Robanus-Maandag, E.C.,and te Riele HP. Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. Cell. 77(4):491-502, 1994

Schinkel, A.H., Kemp, S., Dolle, M., Rudenko, G., and Wagenaar, E. N-glycosylation and deletion mutants of the human MDR1 P-glycoprotein.Journal of Biological Chemistry. 268(10):7474-81, 1993

Singh SV. Nair S. Ahmad H. Awasthi YC. Krishan A.Glutathione S-transferases and glutathione peroxidases in doxorubicin-resistant murine leukemic P388 cells. Biochemical Pharmacology. 38(20):3505-10, 1989

Schlaepfer, D.D., Mehlman, T., Burgess, W,H., and Haigler, H.T. Structural and functional

characterization of endonexin II, a calcium- and phospholipid-binding protein. Proceedings of the National Academy of Sciences of the United States of America. 84(17):6078-82, 1987

Schlaifer, D., Brousset, P, Attal, M., Voigt, J.J., Laurent, G., and Delsol, G. Immunohistochemical detection of multidrug resistance associated P-glycoprotein in stromal cells of malignant lymphomas. Nouvelle Revue Francaise d Hematologie. 32(5):365-7, 1990

Scheper, R.J. Broxterman, H.J., Scheffer, G.L., Kaaijk, P.. Dalton, W.S., van Heijningen, T.H. van Kalken, C.K., Slovak, M.L., de Vries, E.G. and van der Valk, P. Overexpression of a M(r) 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. Cancer Research. 53(7):1475-9, 1993

Shapiro, A.B. and Ling, V. Using purified P-glycoprotein to understand multidrug resistance. Journal of Bioenergetics & Biomembranes. 27(1):7-13, 1995

Schurr, E., Raymond, M., Bell, J.C., and Gros, P. Characterization of the multidrug resistance protein expressed in cell clones stably transfected with the mouse mdr1 cDNA. Cancer Research. 49(10):2729-33, 1989

Sheets, E.E., Giugni, T.D., Coates, G.G., Schlaepfer,D., and Haigler, H,T. Epidermal growth factor dependent phosphorylation of a 35-kilodalton protein in placental membranes. Biochemistry. 26(4):1164-72, 1987

Siegfried. J.A., Kennedy, K.A., Sartorelli, A.C., andTritton, T.R. The role of membranes in the mechanism of action of the antineoplastic agent adriamycin. Spin-labeling studies with chronically hypoxic and drug-resistant tumor cells.Journal of Biological Chemistry. 258(1):339-43, 1983

Sikic ,B.I., Fisher, G.A., Lum, B.L., Halsey, J., and Beketic-Oreskovic, L. Modulation and prevention of multidrug resistance by inhibitors of P-glycoprotein. Cancer Chemotherapy & Pharmacology. 40 Suppl:S13-9, 1997.

Skach, W.R., and Lingappa, V.R., Transmembrane orientation and topogenesis of the third and fourth membrane-spanning regions of human P-glycoprotein (MDR1). Cancer Research. 54(12):3202-9, 1994 .

Skach, W.R., Calayag, M.C., and Lingappa, V.R., Evidence for an alternate model of human P-glycoprotein structure and biogenesis. Journal of Biological Chemistry. 268(10):6903-8, 1993

Smit, J.J., Schinkel, A.H., Oude, Elferink, R.P., Groen, A.K., Wagenaar, E., van Deemter, L. Mol, C.A, Ottenhoff, R., van der Lugt ,N.M. and van Roon, M.A. et al. Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell. 75(3):451-62, 1993

Sonneveld, P., Durie, B.G., Lokhorst, H.M., Marie, J.P., Solbu, G., Suciu, S., Zittoun, R. Lowenberg B. and Nooter K. Modulation of multidrug-resistant multiple myeloma by cyclosporin. The Leukaemia Group of the EORTC and the HOVON [see comments].Comment in: Lancet 1993

Feb 27;341(8844):558-9

Sonveaux, N., Shapiro, A.B. Goormaghtigh, E., and Ling, V. Ruysschaert JM. Secondary and tertiary structure changes of reconstituted P-glycoprotein. A Fourier transform attenuated total reflection infrared spectroscopy analysis. Journal of Biological Chemistry. 271(40):24617-24, 1996 Oct 4.

Spies, T., Bresnahan, M., Bahram, S., Arnold, D., Blanck, G., Mellins, E., Pious, D., and DeMars R. A gene in the human major histocompatibility complex class II region controlling the class I antigen presentation pathway [see comments]. Comment in: Nature 1990 Dec 20-27;348(6303):674-5 Nature. 348(6303):744-7, 1990

Spiers, A.S. Multiple drug resistance, the MDR gene, and the law of maximum perversity as it applies to oncology: an hypothesis. [Review] [25 refs] Hematological Oncology. 12(4):155-61, 1994
Staats, J., Marquardt, D., and Center, M.S. Characterization of a membrane-associated protein kinase of multidrug-resistant HL60 cells which phosphorylates P-glycoprotein. Journal of Biological Chemistry. 265(7):4084-90, 1990

Stewart, A.J., Canitrot, Y., Baracchini, E., Dean, N.M., Deeley, R.G., and Cole, S, P. Reduction of expression of the multidrug resistance protein (MRP) in human tumor cells by antisense phosphorothioate oligonucleotides. Biochemical Pharmacology. 51(4):461-9, 1996 Feb 23

Stride, B.D. Valdimarsson, G., Gerlach, J.H., Wilson, G.M., Cole, S.P., and Deeley RG.Structure and expression of the messenger RNA encoding the murine multidrug resistance protein, an ATP-binding cassette transporter.Molecular Pharmacology. 49(6):962-71, 1996

Sudhof, T,C., Ebbecke, M., Walker, J,H., Fritsche U., and Boustead C. Isolation of mammalian calelectrins: a new class of ubiquitous Ca2+-regulated proteins. Biochemistry. 23(6):1103-9, 1984

Sugawara, I., Arai, T., Yamashita, T., Yoshida, A., Masunaga, A., and Itoyama, S. Expression of multidrug resistance-associated protein (MRP) in anaplastic carcinoma of the thyroid.Cancer Letters. 82(2):185-8, 1994 .

Swairjo, M.A., and Seaton, B.A. Annexin structure and membrane interactions: a molecular perspective. [Review]Annual Review of Biophysics & Biomolecular Structure. 23:193-213, 1994.

Sugawara, I., Nakahama, M., Hamada, H., Tsuruo, T., and Morris ,S. Apparent stronger expression in the human adrenal cortex than in the human adrenal medulla of Mr 170,000-180,000 P-glycoprotein. Cancer Research. 48(16):4611-4, 1988

Tait JF., Gibson, D., and Fujikawa, K. Phospholipid binding properties of human placental anticoagulan protein-I, a member of the lipocortin family. Journal of Biological Chemistry. 264(14):7944-9, 1989

Takano, H., Kohno, K., Ono, M., Uchida ,Y., and Kuwano, M Increased phosphorylation of DNA topoisomerase II in etoposide-resistant mutants of human cancer KB cells. Cancer Research. 51(15):3951-7, 1991.
Tew, KD. Glutathione-associated enzymes in anticancer drug resistance. ,Cancer Research. 54(16):4313-20, 1994

Yoshizaki, H., Hashimoto, Y., Arai, K., Ohkuchi, M., Shiratsuchi, M., Shidara, Y., and Maki, M. Phospholipid-binding properties of calphobindin-II(annexin VI), anticoagulant protein from human placenta. Chemical & Pharmaceutical Bulletin. 39(10):2617-21, 1991

Tidefelt, U., Elmhorn-Rosenborg, A. , Paul, C., Hao, X.Y., Mannervik ,B., and Eriksson LC. Expression of glutathione transferase pi as a predictor for treatment results at different stages of acute nonlymphoblastic leukemia. Cancer Research. 52(12):3281-5, 1992
Trambas, C.M., Muller, K., and Woods, G.M. P-glycoprotein mediated multidrug resistance and its implications for pathology. Pathology 29:122--30

Tsujimoto, Y., and Croce, C.M. Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. Proceedings of the National Academy of Sciences of the United States of America. 83(14):5214-8, 1986

Tsujimoto Y., and Croce, C.M. Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. Proceedings of the National Academy of Sciences of the United States of America. 83(14):5214-8, 1986 J

Twentyman, P.R., Fox, N.E., Wright, K.A., and Bleehen, N.M., Derivation and preliminary characterisation of adriamycin resistant lines of human lung cancer cells. British Journal of Cancer. 53(4):529-37, 1986

Ueda,K., Pastan I., and Gottesman,M.M.Isolation and sequence of the promoter region of the human multidrug-resistance (P-glycoprotein) gene.Journal of Biological Chemistry. 262(36):17432-6, 1987

Van der Bliek, A.M., Meyers, M.B., Biedler, J.L., Hes, E., and Borst, P. A 22-kd protein (sorcin/V19) encoded by an amplified gene in multidrug-resistant cells, is homologous to the calcium-binding light chain of calpain. EMBO Journal. 5(12):3201-8, 1986

Van der Bliek, A.M., Baas, F., Ten Houte de Lange, T. , Kooiman, P.M., Van der, Velde-Koerts T. and Borst P. The human mdr3 gene encodes a novel P-glycoprotein homologue and gives rise to alternatively spliced mRNAs in liver. Cancer Research. 48(21):5927-32, 1988

Van Helvoort, A., Smith, A.J., Sprong, H., Fritzsche, I., Schinkel, A.H., Borst, P., and van Meer, G. MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. Cell. 87(3):507-17, 1996

Varticovski, L., Chahwala, S,B., Whitman, M., Cantley, L., Schindler, D., Chow , E.P., Sinclair LK.and Pepinsky RB. Location of sites in human lipocortin I that are phosphorylated by protein tyrosine kinases and protein kinases A and C. Biochemistry. 27(10):3682-90, 1988

Versantvoort, C.H.,Broxterman, H.J., Pinedo, H.M., de, Vries, E.G., Feller, N., Kuiper, C.M.and Lankelma J. Energy-dependent processes involved in reduced drug accumulation in multidrug-resistant human lung cancer cell lines without P-glycoprotein expression. Cancer Research. 52(1):17-23, 1992

Versantvoort ,C.H., Bagrij, T., Wright, K.A.,and Twentyman, P.R. On the relationship between the probenecid-sensitive transport of daunorubicin or calcein and the glutathione status of cells overexpressing the multidrug resistance-associated protein (MRP). International Journal of Cancer. 63(6):855-62, 1995 a

Versantvoort, C.H., Broxterman, H.J., Bagrij, T., Scheper, R.J., and Twentyman PR. Regulation by glutathione of drug transport in multidrug-resistant human lung tumour cell lines overexpressing multidrug resistance-associated protein. British Journal of Cancer. 72(1):82-9, 1995b

Verweij, J., Herweijer, H., Oosterom, R., van der Burg, M.E., Planting, A.S., Seynaeve, C., Stoter G. and Nooter, K. A phase II study of epidoxorubicin in colorectal cancer and the use of cyclosporin-A in an attempt to reverse multidrug resistance. British Journal of Cancer. 64(2):3 61-4, 1991

Vickers, P,J., Dufresne, M.J., and Cowan, K.H, Relation between cytochrome P450IA1 expression and estrogen receptor content of human breast cancer cells. Molecular Endocrinology. 3(1):15 7-64, 1989

Wallner, B.P., Mattaliano, R.J., Hession, C., Cate, R.L., Tizard, R., Sinclair, L,K., Foeller, C. Chow EP. Browing, J.L., andRamachandran, K.L. et al. Cloning and expression of human lipocortin, a phospholipase A2 inhibitor with potential anti-inflammatory activity. Nature. 320(6057):77-81, 1986

Wang ,W., and Creutz, C.E. Role of the amino-terminal domain in regulating interactions of annexin I with membranes: effects of amino-terminal truncation and mutagenesis of the phosphorylation sites.Biochemistry. 33(1):275-82, 1994

Wang, Y., Pan, X.Q., Lheureux, F., and Georges E. Overexpression of a 40-kDa protein in human multidrug resistant cells. Biochemical & Biophysical Research Communications. 236(2):483-8, 1997

Weng, X., Luecke, H., Song ,I.S., Kang, D.S., Kim, S.H. and Huber, R., Crystal structure of human annexin I at 2.5 A resolution. Protein Science. 2(3):448-58, 1993

Weinstein, et al, Multidrug resistance gene product (pgp) in normal tissue and tumours. Adv. Patholgy and Lab. Medicine, PP 207-234, 1991

Wheeler, C., Rader, R., and Kessel, D. Membrane alterations associated with progressive adriamycin resistance. Biochemical Pharmacology. 31(16):2691-3, 1982

Wijkander J. Sundler R. A role for protein kinase C-mediated phosphorylation in the mobilization of arachidonic acid in mouse macrophages. Biochimica et Biophysica Acta. 1010(1):78-87, 1989

William, F., Haigler, H.T., and Kraft, A.S. Lack of phosphorylation of lipocortin I in A431 epidermoid carcinoma cells treated with phorbol esters. Biochemical & Biophysical Research Communications. 160(2):474-9, 1989

Willingham, M.C., Cornwell, M.M., Cardarelli, C.O., Gottesman, M.M., and Pastan I. Single cell analysis of daunomycin uptake and efflux in multidrug-resistant and -sensitive KB cells: effects of verapamil and other drugs. Cancer Research. 46(11):5941-6, 1986

Wirl, G., and Schwartz-Albiez, R. Collagen-binding proteins of mammary epithelial cells are related to Ca2(+)- and phospholipid-binding annexins. Journal of Cellular Physiology. 144(3):511-22, 1990

Wilson. C.M., Serrano, A.E.. Wasley, A., Bogenschutz, M.P., Shankar, A.H., and Wirth, D.F. Amplification of a gene related to mammalian mdr genes in drug-resistant Plasmodium falciparum. Science. 244(4909):1184-6, 1989 Jun 9.

Wu, G.S., and El-Diery, W.S. p53 and chemosensitivity . Nature Medicine. 2(3):255-6, 1996

Yoshizaki, H., Hashimoto, Y., Arai, K., Ohkuchi, M., Shiratsuchi, M., Shidara, Y., and Maki, M. Phospholipid-binding properties of calphobindin-II(annexin VI), anticoagulant protein from human placenta. Chemical & Pharmaceutical Bulletin. 39(10):2617-21, 1991

Zaks, W.J. and Creutz, C.E. Ca(2+)-dependent annexin self-association on membrane surfaces Biochemistry. 30(40):9607-15, 1991

Zaman, G.J., Flens, M.J., van Leusden, M.R. de Haas, M., and Mulder, H.S. Lankelma J.Pinedo HM. Scheper RJ. Baas F. Broxterman HJ. et al.The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump.Proceedings of the National Academy of Sciences of the United States of America. 91(19):8822-6, 1994

Zhang, J.T.and Ling, V, Study of membrane orientation and glycosylated extracellular loops of mouse P-glycoprotein by in vitro translation. Journal of Biological Chemistry. 266(27):18224-32, 1991

Zhang, J.T., Duthie, M., and Ling V. Membrane topology of the N-terminal half of the hamster P-glycoprotein molecule. Journal of Biological Chemistry. 268(20):15101-10, 1993.

CHAPTER II.

MANUSCRIPT I.

RATIONALE FOR STUDY

Multidrug resistance (MDR) in chemotherapeutic treatment of cancer patients has been associated with the overexpression of P-glycoprotein (P-gp). Biochemical and molecular characterization of P-gp has revealed that P-gp is a member of a large family of membrane ATP trafficking proteins, the ATP binding cassette (or ABC-transport proteins) (Higgins, 1995). P-gp-ATPase activity has been demonstrated in both P-gp expressing MDR cell lines and in purified P-gp reconstituted into defined liposomes (Gottestman and Pastan, 1993; Shapiro and Ling, 1994). The mode of action of P-gp as a drug efflux pump in the transport of structurally and functionally dissimilar drugs has been established. Studies using photoactive drug analogues have suggested that P-gp drug binding domains resides in hydrophobic sequences localized to the lipid bilayer (Greenberger, 1993; Raviv et al, 1990). In addition, we and others have shown that P-gp-drug interactions are inhibited with low concentration of non-ionic detergents at the levels that do not affect the integrity of the lipid bilayer. Mutations in P-gp transmembrane domains were shown to affect P-gp MDR function and substrate specificity (Zordan-Nudo et al. 1993; Low and Sharom 1993). However, it is not known how P-gp ATPase is coupled to the drug binding and transport. To further understand the correlation between P-gp ATPase activity and drug binding, we pursued this study using chemical modification of P-gp with N-ethylmaleimide.

This work has been published in Anticancer Research. (17:353-360, 1997)

N-ethylmaleimide Increases P-glycoprotein Photoaffinity Labeling With Iodoaryl-Azidoprazosin in Multidrug Resistant Cells*

YING WANG AND ELIAS GEORGES

Institute of Parasitology, McGill University, Macdonald Campus, Ste-Anne de Bellevue, Quebec, CANADA.

*Corresponding author: Elias Georges, Institute of Parasitology, McGill University, 21,111 Lakeshore Road, Ste-Anne de Bellevue, Quebec, CANADA, H9X 1C0.
Telephone 514-398 8137, Fax 514-398 7857.

Key Words: P-glycoprotein, Phosphorylation, Multidrug-resistance, N-ethylmaleimide, Photoaffinity labeling.

ABBREVIATIONS: MDR, Multidrug-resistance; P-gp, P-glycoprotein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NEM, N-ethylmaleimide.

ABSTRACT

P-glycoprotein (P-gp) mediates a multidrug resistance (MDR) phenotype in tumour cell lines selected with lipophilic cytotoxic drugs. Transport studies using purified P-glycoprotein reconstituted into defined liposomes have shown an energy-dependent drug efflux of structurally dissimilar drugs. In this report, we have examined the effects of N-ethylmaleimide, a potent inhibitor of P-gp ATPase, on P-gp drug binding in intact MDR cells and in plasma membranes. Our results show that short term treatment of MDR cells with 1-50 μM N-ethylmaleimide led to a concentration-dependent increase in P-gp photoaffinity labelling with iodoaryl-azidoparazosin (IAAP). In addition, N-ethylmaleimide potentiates [^{3}H] vinblastine accumulation in drug-resistant but not in sensitive cells. Comparison of IAAP photolabeled P-gp from intact cells with or without N-ethylmaleimide treatment did not show differences in the pattern of IAAP photolabeled peptides. Thus, the observed increase in P-gp photolabeling with IAAP in N-ethylmaleimide treated cells is not due to photolabeling at different sites. Incubation of MDR cells with [^{14}C] N-ethylmaleimide showed that P-gp is directly modified at several cysteine residues as found from a complete proteolytic digestion of [^{14}C] N-ethylmaleimide labeled P-gp. The comparison of V8 *staphylococcus aureus* peptides from [^{14}C] N-ethylmaleimide or IAAP modified P-gp showed some peptides to co-migrate on SDS PAGE. However, modification of plasma membranes from drug resistant cells with N-ethylmaleimide did not show a dose-dependent increase in P-gp photolabeling with IAAP as seen with intact MDR cells. Interestingly, N-ethylmaleimide appears to increase P-gp phosphorylation by inhibiting the turnover of P-gp phosphates. However, inhibition of P-gp phosphorylation with calyculin A did not show an increase in P-gp photolabeling in MDR cells. Taken together, the results of this study suggest that N-ethylmaleimide potentiates P-gp photolabeling with IAAP by inhibiting P-gp ATPase thereby increasing the local concentration of IAAP in intact MDR cells. Furthermore, inhibition of P-gp ATPase by N-ethylmaleimide does not lead to a conformational change that affects P-gp drug binding.

INTRODUCTION

P-glycoprotein is a 170 kDa transmembrane surface ATPase that is a member of a large family of membrane transport proteins, the ATP Binding Cassette (or ABC-transport proteins) [1-3]. Members of the ABC-transport proteins that are homologous to P-gp include the multidrug resistance-associated protein (MRP) [4], the cystic fibrosis transmembrane regulator (CFTR) [5], and the a-mating factor transporter in yeast (STE6) [6]. In rodents, the P-gp gene family is made up of three structurally similar but functionally distinct isoforms (classes I, II, and III) [7,8]. Overexpression of P-gp classes I or II but not III in tumour cells confers MDR [9-11]. More recently, purified P-gp reconstituted into defined lipid vesicles was shown to mediate an energy-dependent transport of structurally dissimilar drugs [12-14].

The broad substrate specificity of P-gp is not well understood. P-gp has been shown to mediate the transport of many dissimilar compounds that include lipophilic drugs, ATP, and small hydrophobic peptides [15-18]. Studies using photoactive drug analogues have suggested that the P-gp drug binding domain resides within hydrophobic sequences localized to the lipid bilayer [19,20]. In addition, we and others have shown that P-gp-drug interactions are inhibited by low concentrations of non-ionic detergents at levels that do not affect the integrity of the lipid bilayer [21,22]. Mutations in P-gp transmembrane sequences were also shown to affect P-gp MDR function and substrate specificity [23-26]. Taken together, these observations led to the suggestion that P-gp drug binding domains are localized in transmembrane sequences and that P-gp may be a "flipase" [27]. However, little is known about P-gp drug binding domain(s) and how its ATPase is coupled to drug binding and transport. In one study [28], mutation of lysine residues in P-gp ATP-binding cassettes inhibited its ATPase activity but not drug binding, while in another study [29], inhibition of ATP binding to P-gp with epitope-specific monoclonal antibodies inhibited P-gp-drug binding. Furthermore, not all drugs that are transported by P-gp modulate its ATPase activity. For example, both verapamil or vinblastine were shown to increase P-gp ATPase, while colchicine (also substrate for P-gp drug efflux pump) did not [12-14].

N-ethylmaleimide (NEM) and vanadate at low concentrations were shown to inhibit P-gp ATPase in MDR cells [30] and in liposomes containing P-gp [12-14]. Though the mechanism by which NEM inhibits P-gp ATPase is not clear, NEM has been shown to modify Cysteine residues

in the ATP binding domains of V-type ATPases [31]. Further, NEM was shown recently to modify a single cysteine residue in a recombinant N-terminal ATP-binding domain of P-gp and to inhibit its ATPase activity [32]. In this study it was of interest to determine how the inhibition of P-gp ATPase by NEM affects P-gp drug binding and transport in intact MDR cells. The results of this study show that NEM causes a dose-dependent increase in P-gp drug binding in MDR cells but not in plasma membranes. The increase in P-gp photoaffinity labelling with IAAP in intact cells is investigated further with respect to other effects of NEM, for example, the direct modification of P-gp with NEM and modulation of P-gp phosphorylation.

MATERIALS AND METHODS

Materials - Carrier-free [^{32}P] inorganic phosphate (8 mCi/ml), [^{125}I] Iodoarylazidoprazosin (2200 Ci/mmol), [^{14}C]N-ethylmaleimide (5.1 mCi/mmol), and [^{3}H]vinblastine (25 Ci/mmol) were purchased from Amersham Biochemical Inc. (Mississauga, Ontario, Canada). Protein A-coupled sepharose was purchased from Pharmacia Inc., Quebec, Canada. Drug-sensitive human lymphoma cells (CEM) were a gift from Dr. W. Beck, St. Jude Children's Research Hospital, Memphis, TN. The monoclonal antibody C219 and the Chinese hamster drug-sensitive (Auxb1) and colchicine-resistant cells ($CH^{R}C5$), and vinblastine resistant CEM cells ($CEM/VLB^{1.0}$) were generous gifts from Dr. V. Ling at British Columbia Cancer Centre, Vancouver, Canada. All other chemicals were of the highest commercial grade available.

Cell Culture and Plasma Membrane Preparation - Drug-sensitive (CEM or Auxb1) and their resistant mutants ($CEM/VLB^{1.0}$ or $CH^{R}C5$) were grown in α-MEM medium containing 10% fetal calf serum (Hyclone, Inc.). Plasma membranes of CEM or $CEM/VLB^{1.0}$ cells were prepared essentially as described by Lin et al. [33]. Briefly, cells were washed three times in ice-cold phosphate-buffered saline (PBS) and resuspended in a hypotonic lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.4) containing protease inhibitors (2 mM PMSF, 30 μM leupeptin and pepstatin). Cells were homogenized using a Dunce glass homogenizer and the cell lysate was centrifuged at low speed (3000 xg) to remove unbroken cells and nuclei. The resultant supernatant was made up to 10 mM $CaCl_2$ final concentration and mixed on ice. The calcium-induced membrane aggregates were precipitated by high speed centrifugation at 100,000 xg for 1 hr at 4°C using a Beckman SW50 rotor. The enriched plasma membrane pellet was washed with 10 mM Tris-HCl, pH 7.4, and 250 mM sucrose and stored at -80°C until needed. Protein concentration was determined by the method of Lowry et al [34] using bovine serum albumin as a standard.

Photoaffinity Labelling - Intact cells (1 X 10^5 cells/sample) or plasma membrane fractions (~25 μg protein) were preincubated for 30 min in the absence or presence of 1, 10, or 50 μM of NEM, or Calyculin A. Cells or plasma membranes were then incubated with 20 nM IAAP in α-MEM or

phosphate-buffered saline for 60 min at room temperature in the dark. Samples were transferred to ice for 10 min and UV irradiated at 254 nm (Stratagene UV crosslinker; Stratagene, La Jolla, CA).

[^{32}P] Metabolic Labelling of Cells and Immunoprecipitation - Cells were incubated with carrier-free ^{32}P inorganic phosphate (250 μCi/ml) in a-MEM minus phosphate for 3 hr. Metabolically radiolabeled cells were incubated in the presence of NEM before or following ^{32}P labelling (see Results). For immunoprecipitation, cells were washed in ice cold PBS and lysed in buffer A containing 50 mM Tris-HCl, pH 7.4, 1% sodium dodecyl sulphate (SDS), 150 mM NaCl, 50 mM of mixture of mono- and dibasic sodium phosphate, 50 mM sodium fluoride, and protease inhibitors (30 μM leupeptin, 30 μM pepstatin, and 2 mM PMSF). Samples were vortexed and passed several times through a 26 gauge needle to shear chromosomal DNA. Immunoprecipitation was started following clarification of the cell lysate by centrifugation. Equal amounts of cell lysate proteins were separately incubated with 10 μg of C219 monoclonal antibody [35] and allowed to incubate overnight at 4°C with rotation. The cell lysate containing P-gp-C219 mAb complex was further incubated with protein-A coupled sepharose (Pharmacia, Inc.) for 1 hr at room temperature and washed several times as previously described [29]. P-gp-C219 mAb complex was eluted from the protein-A beads in SDS-sample buffer and resolved on SDS-PAGE.

Drug Accumulation - For drug accumulation studies, drug sensitive and resistant cells were washed three times in PBS plus glucose and incubated without or with 1 to 50 μM of NEM for 30 minutes. Following NEM pre-treatment of cells, [^{3}H]vinblastine (1 μM) was added to all cells and samples removed after 15, 30, or 60 min of incubation at 37°C. Cells were washed three times with 5 ml of ice-cold PBS. The washed cell pellets were solubilized in 1 N NaOH and the resultant cell lysates were mixed with an equal volume of 1 N NCl. Samples (100 μl) of cell lysates were spotted onto a Whatman filter discs (GF/B glass fiber) and the radioactivity in each sample was measured by liquid scintillation spectrometry using a Beckman LKB1219 Rackbeta counter. The results for each time point were the averages from three separate samples and are expressed as pmol drug /mg cell lysate protein, (±S.D.)

NEM Modification of Proteins and Protease Cleavage - For NEM modification, intact cells or plasma membranes were incubated for 30 minutes at room temperature with 1 mM NEM containing 1 μM [^{14}C] NEM. The reaction was stopped with excess beta-mercaptoethanol and cells or plasma membranes were washed several time with PBS and pelleted by centrifugation. P-gp modified with [^{14}C] NEM or IAAP was immunoprecipitated with C219 mAb and resolved on SDS-PAGE. Immunoprecipitated P-gp was visualized by a short exposure of a wet unstained polyacrylamide gel to Kodak film. [^{14}C]NEM or IAAP modified P-gp in gel slices was digested with *Staphylococcus aureus* V8 protease (0.1 - 10 μg) in the wells of 15% acrylamide as previously described by Cleveland et al. [36].

Gel Electrophoresis and Western Blotting - Plasma membrane proteins (~25 μg) were resolved on SDS-PAGE using the Fair-banks gel system with some modifications [37]. Briefly, proteins were dissolved in 1/5 vol. of 5X solubilization buffer I (2% SDS, 50 mM dithiothreitol (DTT), 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0), and mixed with an equal volume of buffer II (2X buffer I, and 9 M urea). Gel slabs containing the resolved membrane proteins were either fixed in 40% methanol, 10% acetic acid or transferred onto nitrocellulose membrane according to the method by Towbin et al [38].The nitrocellulose membrane was probed with P-gp specific monoclonal antibody (0.5 μg/ml). Dried gels were exposed to Kodak X-ray film at -70 °C.

RESULTS and DISCUSSION

Earlier reports have shown that NEM is a potent inhibitor of P-gp ATPase in MDR cell lines [30] and in a reconstituted transport system containing purified P-gp [12-14]. However, it is not clear how inhibition of P-gp ATPase with NEM affect P-gp drug-interaction or drug binding domain (s). Thus, it was of interest to examine the effects of NEM on P-gp drug binding and transport in MDR cells and plasma membranes. In this study, P-gp-drug interactions were examined using the photoactive-radioactive drug Iodoarylazidoprasozin (IAAP) previously shown to bind specifically to P-gp[19]. Drug-sensitive (CEM) and -resistant (CEM/VLB$^{1.0}$) cells were photoaffinity labeled with IAAP in the absence or presence of increasing concentrations of NEM (1-50 μM). The results in figure 1a show the photolabeling of a 170 kDa protein (P-gp) with IAAP in intact cells without and with NEM. The addition of increasing concentrations of NEM (1, 10 , or 50 μM) to intact drug-resistant cells showed a concentration-dependent increase in the photolabeling of P-gp (figure 1a, lanes 6-8). Although small increase in P-gp photolabeling was seen in the presence of 1 μM NEM, the presence of 10 or 50 μM NEM led to a greater (2 - 5 fold) increase. Also, the increase in P-gp photolabeling with NEM was specific since no similar increase in the photolabeling of other proteins in drug-resistant cells was observed (figure 1a). Similar concentrations of NEM (1-50 μM) added to intact drug-sensitive cells did not affect the photolabeling of cellular proteins with IAAP (figure 1a, lanes 2-4). To show that the observed increase in the photolabeling of P-gp with IAAP in the presence of NEM is not due to modulations of P-gp levels, identical samples to those in figure 1a were resolved on SDS PAGE and probed with P-gp-specific monoclonal antibody (C219). Figure 1b shows a 170 kDa protein of equal intensity in cell extracts from drug-resistant cells. It should be mentioned that NEM at the concentration and incubation conditions used in this study did not lead to a significant decrease of cell viability as determined by Trypan dye exclusion assay (data not shown).

Following the above increase in P-gp photolabeling with IAAP in the presence of NEM, it was of interest to examine the effect of NEM on the accumulation of drugs in MDR cells. Figure 2a shows the accumulation of [^{3}H] vinblastine in drug-sensitive cells in the absence or presence of 1, 10, or 50 μM NEM. The accumulation of [^{3}H] vinblastine in CEM cells was not significantly altered in the presence of increasing concentrations of NEM (figure 2a). However, when similar drug accumulation experiments were conducted using CEM/VLB1.0 resistant cells (figure 2b), the presence of NEM in

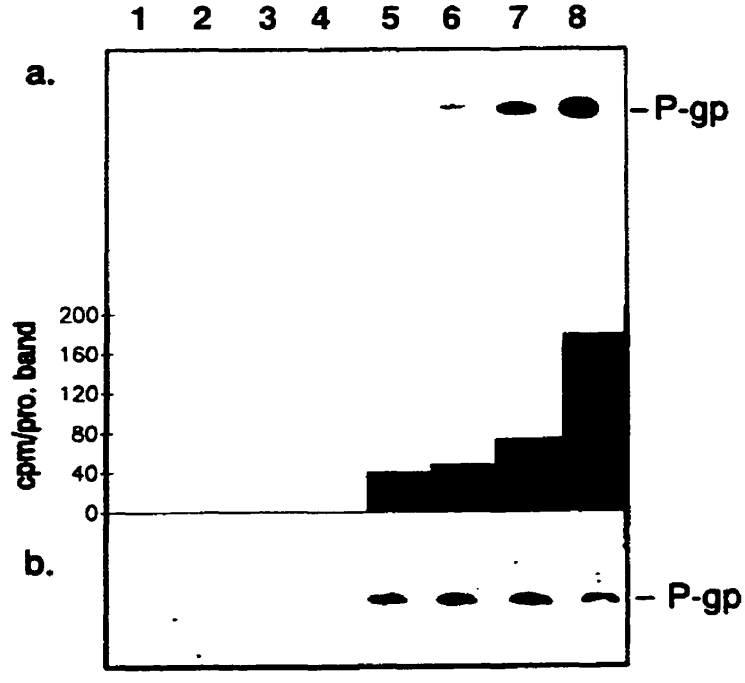


Figure 1. Effect of NEM on the photolabeling of P-gp in intact cells. CEM drug-sensitive or CEM/VLB$^{1.0}$ - resistant cells were photoaffinity labeled with IAAP in the absence or presence of 1-50 μM of NEM. Following photolabeling, cells were lysed and the proteins were resolved on SDS-PAGE. Lanes 1 - 8 of figure 1a show photoaffinity labeled proteins from drug-sensitive (lane 1-4) or resistant cells (lane 5-8) incubated in the absence (lane 1 or 5, respectively) or presence of 1, 10, or 50 μM of NEM (lanes 2-4 or 6-8, respectively). The pre-incubation of drug-resistant cells with NEM resulted in a dramatic increase in the photolabeling of the 170 kDa protein as indicated in the bar graph shown below in figure 1a. Figure 1b shows a Western blot probed with C219 mAb of identical samples as in figure 5a. Note a single 170 kDa protein of equal intensity was detected in each of the four lanes containing total cell lysate from drug-resistant cells incubated in the absence (lane 5, figure 1b) or presence of 1-50 μM of NEM (lanes 6-8).

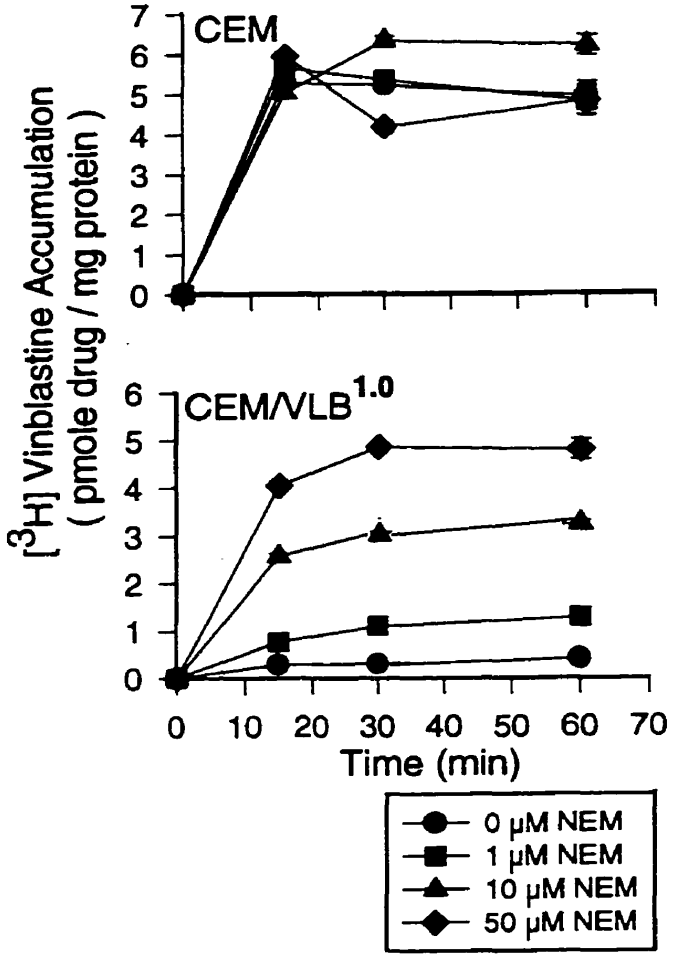


Figure 2. NEM potentiates the accumulation of [^{3}H] vinblastine in MDR cells. Drug-sensitive and -resistant cells were pre-incubated with 1, 10, or 50 μM of NEM prior to the addition of [^{3}H] vinblastine (1 μM final). In the absence of NEM, [^{3}H] vinblastine does not accumulate in drug-resistant cells. Preincubation of drug-resistant cells with increasing concentrations of NEM (1, 10, or 50 μM) results in a dose dependent increase in [^{3}H] vinblastine accumulation in drug-resistant; while similar concentrations of NEM did not significantly increase [^{3}H] vinblastine accumulation in drug sensitive cells. Each time point is expressed as an average of triplicate samples (± S.D.).

the incubation media led to a concentration-dependent increase in the accumulation of [^{3}H] vinblastine. It should be mentioned that NEM caused a similar increase in P-gp photoaffinity labelling and drug accumulation in other MDR cell lines (including the Chinese hamster ovary cells, Auxb1 and other Colchicine-resistant mutant CHRC5, data not shown). Taken together, These results suggest that NEM treatment of MDR cells increases P-gp binding to IAAP likely by inhibiting its ATPase activity. Thus, in the absence of an active drug efflux pump, IAAP accumulates in the lipid bilayer and saturates P-gp drug binding site (s). Moreover, the above results also indicate that inhibition of P-gp ATPase with NEM does not affect its ability to bind IAAP. Alternatively, the increase in P-gp photolabeling may be due to changes in IAAP binding site(s) in P-gp in NEM treated MDR cells. To address the latter possibility, IAAP photolabeled P-gp from MDR cells with or without NEM treatment were subjected to Staphylococcus aureus V8 protease and the resultant photoaffinity labeled peptides were analysed by SDS-PAGE. Figure 3 shows partial cleavage of IAAP photolabeled P-gp digested with 0.1 μg - 10 μg of V8 protease. The results in figure 3 show no difference in the pattern of IAAP photolabeled peptides of P-gp from cells incubated with or without NEM. Therefore, the observed increase in P-gp photolabeling in intact MDR cells in the presence of NEM is not due to the photolabeling of other sites in P-gp but due to increased labelling of the same domains. However, given the presence of cysteine residues in P-gp sequences other than those found in the ATP binding cassettes, the effect of NEM on IAAP photolabeling of P-gp maybe due to direct modification of such cysteine residues. Previous studies with another transporter, the Lactose permease, have shown that NEM modification of a single cysteine (Cys^{148}) in transmembrane domain 5 inhibited the binding and the translocation of by the lactose permease [review in 43]. Thus it is conceivable that modification of cysteine residues in P-gp other than those in ATP-binding cassettes could alter P-gp-drug binding and photolabeling with IAAP.

The primary structure of P-gp encodes for seven highly conserved cysteine residues localized to several domains thought to be important for P-gp MDR function [figure 4C]. For example, transmembranes 2,7 and 11 which contain one cysteine each have been implicated in P-gp MDR function [24,25,41,42]. Furthermore, earlier domain mapping studies [41,44] of P-gp drug binding sites(s) have identified short IAAP-labeled tryptic peptides (7kDa) to encode sequences adjacent to transmembrane 12. Interestingly, mutations of all seven cysteine residues to alanine did not affect

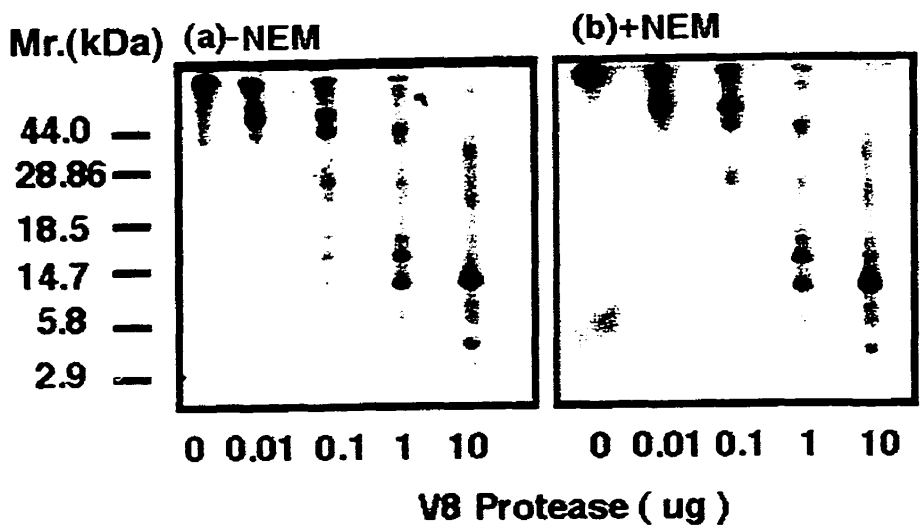


Figure 3. Cleveland maps of IAAP photolabeled P-gp in with or without NEM treatment. Intact MDR cells incubated without or with 50 μM NEM were photolabeled with IAAP and proteins resolved on SDS PAGE. Gel slices containing IAAP photolabeled P-gp from cells incubated without or with NEM were digested with 0.01-10 μg of V8 protease in the wells of a second gel. The molecular weight markers are indicated to the left of the gel.

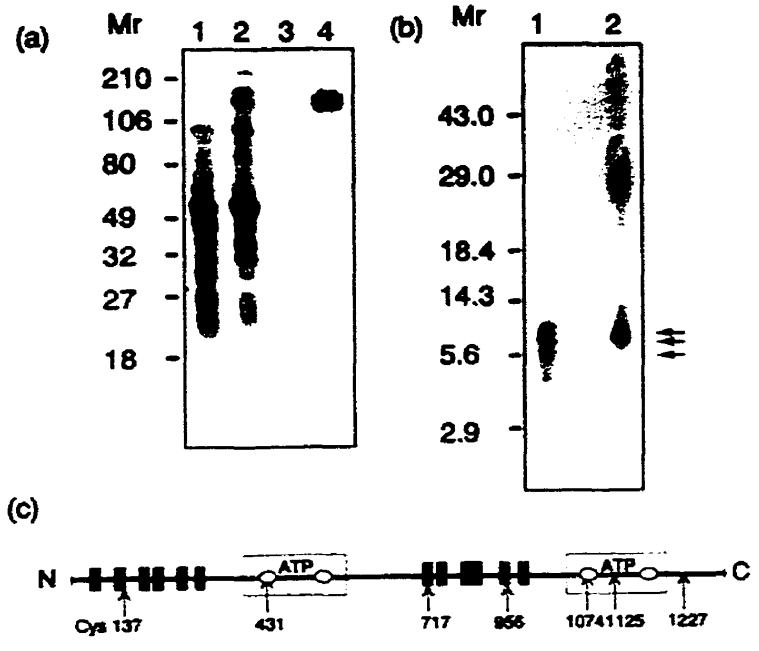


Figure 4. Covalent modification of P-gp with [14C] NEM or IAAP. Drug-sensitive or -resistant cells were incubated with 10 μM of [^{14}C] NEM at physiological pH for 30 minutes. Figure 4a shows total cell lysate from drug-sensitive (lane 1) and -resistant (lane 2) cells following [^{14}C] NEM modification. Lanes 3 and 4 show the immuno-precipitation with IgG2a or C219 mAb from [^{14}C] NEM modified resistant cells. Figure 4b show V8 protease Cleveland peptide maps of [^{14}C] NEM (lane 1) or IAAP (lane 2) modified P-gp. The positions of the various [^{14}C] NEM or [^{125}I] IAAP modified peptides are indicated by arrow heads. Figure 4c shows a schematic of P-gp predicated secondary structure with the positions of the Cysteine residues indicated by arrow heads.

P-gp MDR function [40]. However, modification of a single cysteine residue in recombinant N-terminal ATP-binding domain of P-gp with NEM inhibited its photolabeling with 8-azido-ATP and ATPase activity [32]. Thus, the addition of the maleimide moiety onto cysteine residues, in contrast to mutations of cysteine residues, could affect P-gp ATPase activity and drug binding. To determine whether NEM modifies P-gp in MDR cells, CEM and CEM/VLB$^{1.0}$ cells were incubated with [^{14}C] NEM and equal amounts of total cell proteins were resolved on SDS-PAGE (figure 4a; lanes 1 and 2, respectively). Several proteins were differentially modified with [^{14}C] NEM in drug sensitive and resistant cells. To determine if P-gp is among the proteins modified with [^{14}C] NEM, lanes 3 and 4 show [^{14}C] NEM radiolabeled proteins immunoprecipitated from CEM/VLB$^{1.0}$ cell lysate with an irrelevant IgG2a or C219 mAb, respectively. Lane 4 shows a single 170 kDa protein labeled with [^{14}C] NEM. These results demonstrate that P-gp is directly modified with [^{14}C] NEM in MDR cells. Moreover, given the localization of Cys956 near transmembrane 12 of P-gp, it was of interest to determine if similar domains of P-gp are labeled with [^{14}C] NEM and the photoactive drug IAAP. P-gp labeled with [^{14}C] NEM or IAAP was purified, digested in a gel slice with V8 protease and the radiolabeled peptides resolved on SDS-PAGE (see Experimental Procedures). The results in figure 4b show a peptide map of [^{14}C] NEM or IAAP labeled peptides (lanes 1 and 2, respectively). Digestion of [^{14}C] NEM labeled P-gp with V8 protease resulted in three labeled peptides (8.2 kDa, 6.5 kDa, and 5.7 kDa); while digestion of IAAP labeled P-gp resulted in a major peptide with an apparent molecular mass of ~7.2 kDa. The latter peptide labeled with IAAP was previously [41] shown to encode sequences in P-gp immediately adjacent to transmembrane 12. Thus, although some of the NEM labeled peptides co-migrate with the IAAP labeled peptide, it is not clear if co-migrating peptides encode for the same amino acid sequence. Direct amino acid sequencing should reveal the identity of the IAAP and NEM modified peptides. However, if the increase in IAAP photolabeling of P-gp in intact cells by NEM is due to modification of sequences in P-gp drug binding domain, then similar increase in P-gp IAAP photolabeling should be observed when membranes from NEM treated MDR cells are photolabeled with IAAP. Figure 5a shows the photolabeling of plasma membranes from CEM drug-sensitive and CEM/VLB$^{1.0}$ drug-resistant cells treated with NEM (1 - 50 μM). The results in figure 5a show a 170 kDa photoaffinity labeled protein with IAAP in the absence (lane 5) or presence of 1 -50μM of NEM. The photolabeling of P-gp with IAAP in plasma membranes was not significantly

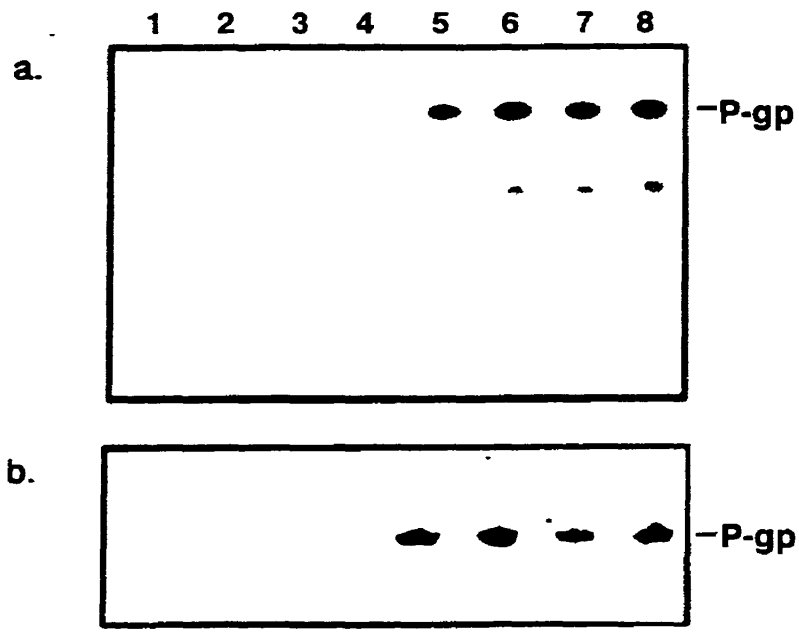


Figure 5. Photolabeling of P-gp in NEM treated plasma membranes. Membrane fractions from drug-sensitive (CEM) or -resistant (CEM/VLB$^{1.0}$) cells were photoaffinity labeled in the absence or the presence of increasing concentrations of NEM (1-50 μM). Lanes 1-8 show IAAP photoaffinity labeled plasma membrane fractions from drug-sensitive (lane1-4) and -resistant cells (lane 5-8) incubated in the absence (lane 1 or 5, respectively) and in the presence of 1, 10, and 50 μM of NEM (lanes 2-4 or 6-8, respectively). Figure 5b shows a Western blot, of identical samples as in figure 5a, probed with C219 mAb. A 170 kDa protein (or P-gp) was detected in plasma membranes from drug-resistant (lanes 5-8) but not from drug-sensitive cells (lanes 1-4).

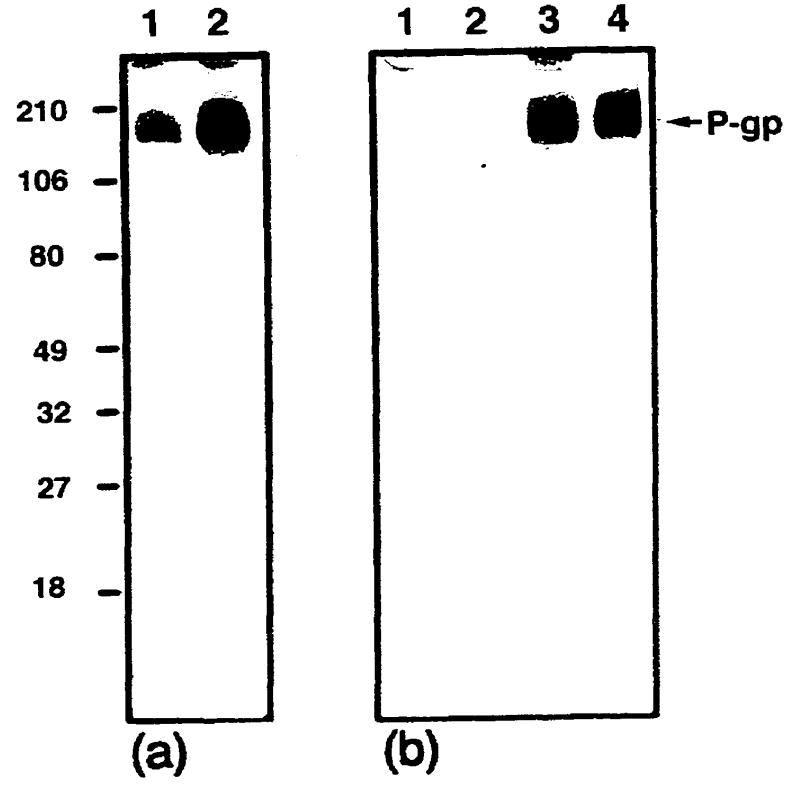


Figure 6. P-gp in vivo phosphorylation in the presence of NEM. Drug-resistant cells were metabolically labeled with ^{32}P in the absence or presence of NEM under three different conditions. Figure 6a shows an immuno-precipitation of ^{32}P radiolabeled P-gp from cells incubated in the absence (lane 1) or in the presence of 50 μM NEM (lane 2) where ^{32}P radiolabel was added to cells 30 minutes prior to the addition of NEM. To determine the effect of NEM on the turn-over of P-gp phosphorylation, P-gp was immuno-precipitated from cells that were metabolically labeled with ^{32}P for 3 hr (pulse) and then the 32P label was chased with excess cold phosphate in the absence (lane 1) or in the presence of 1, 10, or 50 μM NEM (lanes 2-4, respectively; see Experimental Procedures).

increased in the presence of NEM. Western blot analysis of the same plasma membrane samples as in figure 5a probed with C219 mAb shows equal amount of proteins were loaded per well. Taken together, these results suggest that the increase in P-gp photolabeling in intact cells is not due to the direct modification of P-gp drug binding domain.

P-gp was shown to be phosphorylated at several serine residues in the linker domain [45]. Moreover, modulation of P-gp phosphorylation with various drugs has been suggested by some to affect P-gp MDR function [46,47]. Given the results of an earlier report [48] which demonstrated a large increase in P-gp phosphorylation in NEM treated Chinese hamster lung cells, it was of interest to determine if the observed increase in P-gp photolabeling with IAAP following NEM treatment of MDR cells was due to changes in P-gp phosphorylation state. However, it was not clear from that study how NEM modulates P-gp phosphorylation. The results in figure 6a show P-gp phosphorylation in CEM/VLB$^{1.0}$ cells is increased dramatically when the ^{32}P label was added 30 min before NEM. Lanes 1 and 2 show the immunoprecipitation of P-gp from CEM/VLB$^{1.0}$ cells incubated in the absence or in the presence of 50 μM NEM, respectively. To determine if the increase in P-gp [^{32}P] labelling is due to the inhibition of ^{32}P turn-over, CEM/VLB$^{1.0}$ cells were "pulsed" with [^{32}P] for 3 hr without NEM followed by a "chase period" in the presence of excess cold phosphate in the absence or in the presence of 1 - 50μM of NEM. Figure 6b shows the turn-over of [^{32}P] radiolabel on P-gp in the absence and in the presence of 1, 10 or 50 μM NEM. The results in figure 6b suggest that the presence of 10 and 50μM NEM inhibit the turn-over of [^{32}P] on P-gp. By contrast, the [^{32}P] label on P-gp was completely turned-over in the absence of NEM (figure 6b). Taken together, the results in figure 6 suggest that the increase in P-gp phosphorylation in response to NEM treatment is due to inhibition of P-gp phosphate turn-over. Thus, P-gp is more phosphorylated in the presence of NEM.. A similar effect on P-gp phosphorylation was previously observed using the phosphatase inhibitors, okadaic acid and Calyculin A [49, unpublished results]. To determine if higher phosphorylation state of P-gp is responsible for its increased photoaffinity labelling with IAAP, CEM/VLB$^{1.0}$ cells were treated with calyculin A and then photolabeled with IAAP. The results in figure 7a show IAAP photolabeled in CEM/VLB$^{1.0}$ cells in the absence and presence of increasing concentrations of calyculin A. calyculin A at 10-50 nM, which inhibits P-gp phosphorylation (results not shown), did not alter P-gp photolabeling with IAAP. These results are consistent with results from a recent study that showed mutation of several serine residues

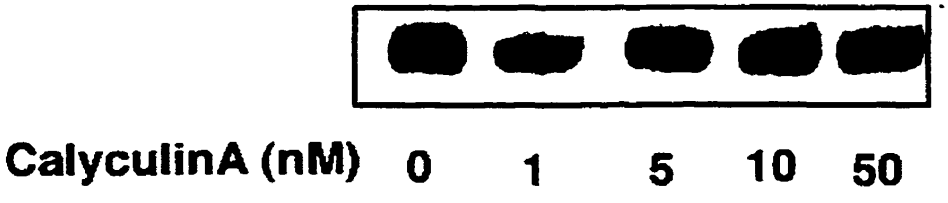


Figure 7. P-gp photolabeling with IAAP in the presence of Calyculin A. CEM/VLB1.0 cells were pre-incubated for 30 mins without or with 1-50 nM of Calyculin A. Cells were then photoaffinity labeled with IAAP are described in the Experimental Procedures.

in P-gp linker domain to alanine prevented P-gp in vivo phosphorylation but not its MDR function [50].

In conclusion, the effects of NEM on the photolabeling of P-gp by IAAP is not entirely clear. However, it is likely that NEM modulates P-gp photolabeling by modifying cysteine residues in P-gp ATP binding domains. We suggest that the inhibition of P-gp ATPase may contribute to the observed increase in P-gp photolabeling by allowing drugs to persist within the drug binding domain or to increase the local concentration of IAAP in the surrounding lipid bilayer. Furthermore, these results show that inhibition of P-gp ATPase does not inhibit the capacity of P-gp to bind drugs.

ACKNOWLEDGMENTS

The authors would like to thank their colleagues at the Institute of Parasitology for their critical reading of this manuscript and many thanks to Mrs. Zhi Liu for her technical assistance. This work was supported by funds from National Cancer Institute of Canada. Research at the Institute of Parasitology is partially supported by NSERC and Fonds FCAR pour laide la recherch.

REFERENCES

1. Gottesman, M. M. and Pastan. (1993). Annu. Rev. Biochem. 62, 385-427.

2. Endicott, J. A. and Ling, V.(1989). Annu. Rev. Biochem. 58, 137-171.

3. Higgins, C. F.(1992). Annu. Rev. Cell. Biol. 8, 67-113.

4. Cole, S. P., Sparks, K. E., Fraser, K., Loe, D. W., Grant, C. E., Wilson, G. M., and Deeley, R. G. (1994). Cancer Res. 54, 5902-5910.

5. Riordan, J. R.(1994). Annu. Rev. Physio. 55, 609-630.

6. McGrath,J.P. and Varshavsky, A (1989). Nature. 340, 400-404.

7. Ng, W. F., Sarangi, F., Zastawny, R. L., Veinot-Drebot, L. and Ling, V. (1989). Mol. Cell. Biol. 9,1224-1232.

8. Dhir.R, Buschman, E, and Gros, P.(1990). Bull.Cancer. 77, 1125-1129.

9. Devault, A and Gros, P.(1990). Mol.Cell.Biol. 10, 1652-1663.

10. Buschman, E and Gros, P.(1994). Cancer Res. 54, 4892-4893.

11. Schinkel , A. H., Roelofs, MEM and Borst, P.(1991). Cancer Res. 51,2628 2635.

12 .Shapiro, A., and Ling, V. (1994). J. Biol. Chem. 269:3745-3754.

13. Doige, C.A., Yu, X and Sharom, F. J.(1992). Biochem. Biophys.Acta. 1109, 149-160.

14. Ambudkar S.V., Lelong, I.H.,Zhang, J., Cardarelli, C. O., Gottesman, M. M. And Pastan, I. (1992). Proc. Natl. Acad. Sci.USA.89, 8472-8476.

15. Georges, E., Sharom.F.J., and Ling, V. (1990). Adv. Pharmacol. 21,185-220.

16. Nielson, D and Svovsgaard, T. (1992). Biochem. Biophys. Acta. 1139, 169-183.

17. Sarkadi. B., Muller, M.,Homolya, L., Hollo, Z., Seprodi, J., Germann, U.A., Gottesman M.M., Price, E.M and Boucher, R.C. (1994). FASEB J. 8, 766 770.

18. Abraham E.H., Prat,A.G.,Gerweck, L., Seneveratne, T., Arceci, R. J., Kramer, R., Guidotti G.and Cantiello, H.F.(1993). Proc. Natl. Acad. Sci. USA. 90, 312-316

19. Greenberger, L.M. (1993) J. Biol. Chem., 268:11417-11425.

20. Raviv V., Pollard, H.B., Bruggemann, E. P., Pastan, I. and Gottesman, M. M. (1990). J. Biol.Chem. 265, 3975-3980.

21. Zordan-Nudo.T, Ling, V , Liu, Z and Georges E. (1993). Cancer Res. 53, 5994 6000.

22. Loe, D.W. and Sharom, F.J. (1993). British J.Cancer. 68, 342-351.

23. Dhir, R., Grizzuti, K. and Gros, P. (1993). Biochemistry. 32, 9492-9499.

24 Loo,T. W., and Clarke,D.M. (1993). J. Biol. Chem. 268, 19965-19972.

25. Loo, T.W., and Clarke,D.M. (1994). Biochemistry. 33, 14049-14057.

26. Devine, S.E., Ling, V., and Melera, P.W. (1992) Proc. Natl. Acad. Sci., USA, 89:4564-4568.

27. Higgins, C.F.(1994). Cell 79:393-395.

28. Azzaria, M., Schurr, E., and Gros P. (1989) Mol. Cell. Biol, 9, 5289-5297.

29. Georges, E., Zhang, J-T. and Ling, V. (1991) J. Cell. Physiol., 148:479-484.

30. Al-Shawi, M.K., and Senior, A.E. (1993) J. Biol. Chem. 268, 4197-4206.

31. Feng, Y., and Forgac, M. (1994) J. Biol. Chem., 269:13224-13230.

32. Dayan, G., Baubichon-Cortay, H., Jault, J-M., Cortay, J-C., Deleage, G., Di Pietro, A. (1996) J. Biol. Chem. 271, 11652-11658.

33. Lin, P. H., Selinfreund, R., Wakshull, E., and Wharton, W., (1087) Biochemistry 26, 731-736.

34. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

35. Kartner, N., Evernden-Porelle, D., Bradely, G., and Ling, V. (1985) Nature (Lond.), 316, 820-823.

36. Cleveland, D.W., Fischer, S.G., Kirschner, M.W., and Laemmli, U.K. (1977) J. Biol. Chem. 252,1102-1106.

37. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971). Biochemistry, 10, 2606-2617.

38. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA. 76, 4350-4354.

39. Beck, W.T. (1983) Cancer Treat. Rep. 67:875-882.

40. Loo, T.W. and Clark, D, M. (1995) J. Biol. Chem., 270, 843-848.

41. Greenberger, L.M. (1993) J. Biol. Chem., 268, 11417-11425.

42. Gros, P. Dhir, R., Croop, J., and Talbot, F. (1991) Proc. Natl. Acad. Sci. USA. 88, 7289-7293.

43. Wright, K.J. Dormiair K. Mitaku S, Moroy T, Neuhaus JM, Seckler R, Vogel H, Weigel U, Jahnig F, Overath P (1985) Annals of the New York Academy of Science 456, 326-341.

44. Morris, D.I., Greenberger, L.M., Bruggemann, E.P., Cardarelli, C., Gottesman, M.M., Pastan, I., Seamon, K.B. (1994) Mol Pharmacol 46, 329-337.

45. Chambers, T.C., Pohl, J., Raynor, R.L., and Kuo, J.F.(1994) J Biol Chem. 268, 4592-4595

46. Fine, R.L, Patel. J., and Chabner, B.A (1988) Proc. Natl.Acad.Sci (USA) 85, 582-586.

47. Bates, S.E., Currier, S.J., Alcarez, M ,and Fojo, T (1992), Biochemistry 31,6366-6372.

48. Marsh, W., and Center, M.S (1985) Cancer Research. 45, 6088-6092.

49. Chambers TC. Zheng B. Kuo JF (1992) . Mol. Phamacol 41,1008-1015.

50. Germann UA. Chambers, T.C. Ambudkar, S.V., Licht, T., Cardarelli, C.O., Pastan,I., and Gottesman, M.M.(1996). J. Biol.Chem 271,1708-1716.

CHAPTER III.

MANUSCRIPT II.

CONNECTING STATEMENT 1

The discovery of P-gp has revealed a fundamental mechanism by which cancer cells evade chemotherapy. A correlation between the overexpression of P-gp and failure of chemotherapy has been established in haemetopoetic tumours and childhood malignancies (Chan et al,1990). Unfortunately, a similar correlation between P-gp expression and clinical MDR has not been observed in other cancers. The recent finding that some tumour cell lines overexpress another membrane transporter (multidrug resistance protein, MRP) suggested that other proteins or cellular alterations, alone or together with P-gp, could explain the MDR phenotype in some clinical tumours. Given the fact that the levels of drug resistance seen in P-gp or MRP transfectants are much lower than those observed in *in vitro* selected cell lines that express similar levels of these proteins suggests that other mechanisms may exist in MDR cell lines (Cole et al, 1994; Gros et al, 1986). In this study, we described the isolation and characterization of a 40 kDa protein (P-40) in several MDR tumour cell lines.

This work has been published in Biochemical and Biophysical Research Comunications (236:483-488, 1997).

Over-expression of a 40 kDa Protein in Human Multi-drug Resistant Cells

Ying Wang, Xing-Qing Pan, Francoise Lheureux and Elias Georges§

Institute of Parasitology, McGill University, Macdonald Campus,

Ste-Anne de Bellevue, Quebec, CANADA

§Corresponding author: Elias Georges, Institute of Parasitology, McGill University,21,111 Lakeshore Road, Ste Anne de Bellevue, Quebec, CANADA, H9X 3V9. Tel: 514 398 8137; FAX: 514 398 7857. Email: Elias_Georges@maclan.McGill.CA

Running Title: Multidrug resistant cells express high levels of a 40 kDa protein.

Key words: Multidrug resistance; P-glycoprotein; Multidrug Resistance protein (MRP); and P-40.

Abbreviations: mAb, monoclonal antibody; *MDR*, multidrug resistance; P-gp, P-glycoprotein; Multidrug Resistance-associated Protein, MRP; CHAPS, 3 [(3-cholaidopropyl) dimethylammonio] -1-propanesulfonate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

ABSTRACT

The use of anticancer drugs in the chemotherapeutic treatment of cancer patients frequently results in the emergence of drug resistant tumours. Selection of tumour cell lines *in vitro* has led to the identification of several proteins that mediate drug resistance to anticancer drugs. In this study, an immuno-dot blot method was used to isolate a monoclonal antibody (IPM96) which recognized a 40 kDa protein (or P-40) co-expressed with P-glycoprotein and MRP in several multidrug resistant cell lines (MCF-7/Adr, SKOV/VLB$^{1.0}$, H69/Adr, and HL60/AR). Furthermore, P-40 levels dropped significantly in one revertant cell line (H69/PR) derived from H69/AR cells. Interestingly, the expression of P-40 was also higher in two tumour cell lines (SKTax6a and A2780^{CP}) that were selected with paclitaxel or cisplatin but do not express P-gp or MRP. Immuno-fluorescence staining of cells with IPM96 showed both membrane and cytoplasmic staining. These results were confirmed by Western blot analysis of different subcellular fractions from MCF-7/Adr cells. The membrane bound P-40 was resistant to extraction with high salt, chelating agents and denaturing agents; but was solubilized with 10 mM CHAPS. Taken together, the overexpression of P-40 in multidrug resistant cells has not been previously determined and therefore could be important in the expression of the drug resistance phenotype.

INTRODUCTION

The use of anti-cancer drugs in appropriate combinations has lead to major improvements in the treatment of malignant tumours such as Hodgkin's disease and childhood leukemia (1, 2). Unfortunately, resistance to cytotoxic drugs in other cancers occurs frequently and is a major obstacle in the chemotherapeutic treatment of cancer patients (3). Several drug resistance mechanisms have been identified in tumour cell lines selected *in vitro* with anti-cancer drugs (4, 5). At least four proteins have been shown to mediate a multidrug resistance (*MDR*) phenotype to structurally and functionally dissimilar compounds. These proteins include the glutathione-S-transferase (6), topoisomerase II (7), and two ATP-binding transmembrane proteins (P-glycoprotein, encoded by *mdr1* and the *m*ultidrug *r*esistant-associated *p*rotein encoded by MRP genes) (8, 9). Recently (10), a *l*ung-*r*esistance *p*rotein (LRP) which is a major component of human vaults (cytoplasmic organelles thought to mediate intracellular transport processes) has been implicated in the expression of *MDR* in tumour cells.

The mechanism of P-gp or MRP-mediated *MDR* describes tumour cell lines that are defective in drug accumulation (11, 12). Both P-gp and MRP were shown to function as energy-dependent drug efflux pumps of broad substrate specificity (13-15). Transfection studies using cDNAs encoding *mdr1* or *mrp1* genes have clearly demonstrated that P-gp or MRP is sufficient to confer an *MDR* phenotype onto otherwise drug sensitive cells (15-18). However, the levels of drug resistance seen in P-gp or MRP transfectants are much lower than those observed in *in vitro* selected cell lines that express similar levels of these proteins (15-18). Therefore, it has been speculated that other cellular changes may be responsible for the observed high levels of drug resistance seen in *MDR* cells selected *in vitro* versus transfectant cells (19). Such cellular changes could amplify, or act independently of, P-gp and MRP functions. In this study, we report on the isolation of a monoclonal antibody that binds specifically to a 40 kDa protein overexpressed alone or together with P-gp or MRP in several *MDR* selected cells.

MATERIALS AND METHODS

Materials- [^{35}S] methionine (1000 Ci/mmol; Amersham Life Sciences, Inc.) and [^{125}I] goat anti-mouse antibody were purchased from Amersham Biochemical Inc. Protein-A Sepharose-4B was purchased from Bio-Rad Life Science Research. P-glycoprotein specific antibody (C219) and the SKOV-3, SKOV/VLB$^{1.0}$ cells were kindly provided by Dr. V. Ling at the BC. Cancer agency in Vancouver, BC. The MRP-specific monoclonal antibody (QCRL-1) and the H69, H69/AR and H69/PR cells were kindly provided by Drs. S. Cole and R. Deely at Queens University, Kingston, ON. Canada. The HL60 and HL60/AR cells were gifts from Dr. M.S. Center at Kansas State University, Manhattan, KS. MCF-7, MCF-7/Adr and MCF-7/Mitox cells were a generous gift from Dr. G. Batist at the Lady Davis Research Institute, Sir Mortimer B. Davis-Jewish General Hospital, in Quebec, Canada. The A2780 and A2780^{CP} cells were kindly provided by Dr. S.B. Howell at UCSD Cancer Center in La Jolla California. Nitrocellulose membranes containing cell lysates from SKOV-3 and SKTax6a cells were a gift from Dr. D. Rischin at the Peter MacCallum Cancer Institute in Melbourne, Australia. All other chemicals used were of the highest grade available.

Tissue culture and Cell Fractionation - Drug sensitive (MCF-7, H69, SKOV-3 and HL60) and resistant (MCF-7/Adr, MCF-7/Mitrox, H69/AR, SKOV/VLB$^{1.0}$ and HL60/AR) cells were cultured in α-MEM or RPMI-1640 media supplemented with 5% to 15% fetal calf serum (Hyclon. Inc.) as previously described (20-25). All cells were examined for Mycoplasma contamination every three months using the Mycoplasma PCR kit from Stratagene Inc. San Diego, CA. For cell fractionation, MCF-7/Adr cells (1X10^{9}) were washed three times with ice-cold PBS containing protease inhibitors (2 mM PMSF, 3μg/ml Leupeptin, 4 μg/ml pepstatin A and 1 μg/ml aprotinin) and homogenized in a hypotonic buffer (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) using the Danuce Homogenizer. The cell lysate was sequentially centrifuged at 1000 x g for 10 minutes, at 3000 xg for 15 minutes (P1), at 10,000 x g 15 minutes (P2) and at 100,000 x g for 60 minutes (P3) in a Beckman ultracentrifuge, using the SW 28 rotor. The pellet from the last centrifugation (P3) was resuspended in 5 mM Tris-HCl, pH 7.4 containing 8.6% sucrose and loaded onto a discontinuous sucrose gradient consisting of 16%, 31%, 45% and 60% sucrose in 5 mM Tris-HCl, pH 7.4. Samples

were centrifuged at 100,000 x g for four hours in a Beckman ultracentrifuge using SW 55 rotor. Fractions at the 16/31 and 31/45 interfaces were collected and washed with 5 mM Tris-HCl, pH 7.4 by centrifugation at 100,000g for 60 minutes. The amount of protein in the above samples was determined by the method of Lowry et al (26). Equal amounts of protein from the different pellets and the soluble fractions were fractionated on SDS PAGE, transferred to nitrocellulose membrane and analysed by Western blotting according to the method of Towbin et al. (27). Immunoreactive proteins were visualized by chemiluminescence using the ECL method (Amersham Inc.).

Selection of monoclonal Antibodies and hybridoma supernatant screening- BALB/c female mice (8 to 10 weeks old) were immunized with total cell lysate from MCF-7/Adr cells. Mice were immunized i.p. every 3-4 weeks for a period of 6 months. Three days prior to the fusion of SP2/O-Ag 14 myeloma (from ATTC type culture) with spleen cells, mice were boosted with 250 μg of total cell lysate injected i.v. into the tail vein. Cell fusion was done according to standard protocols (28) using polyethylene glycol 4000 (Boehringer Mannheim, Inc. Laval, QC.). Cells were then cultured in RPMI media containing 15% FCS, 100 mM hypoxanthine, 0.4 mM aminopterin and 16 mM thymidine. Ten days after fusion, 0.2 ml of hybridoma supernatants were mixed with 0.8 ml of 5% skim milk in PBS. Nitrocellulose strips dotted with 4 μg of total cell lysates from drug sensitive (H69 and MCF-7) and resistant (H69/AR and MCF-7/Adr) cells were incubated with the hybridoma supernatants. A total of 345 hybridoma clones were screened and hybridoma supernatants that recognized proteins in both drug resistant (H69/AR and MCF-7/Adr) cells but not drug sensitive cells (H69 or MCF-7) were expanded and cloned two times by limiting dilution. One antibody secreting hybridoma clone, IPM96, was characterized further in this study. The subtype of the monoclonal antibody IPM96 was determined by indirect immuno-enzymatic assay using the "mouse-Typer" from Bio-Rad Inc. according to the manufactures procedure. The isotype of IPM96 was determined to be IgG2b.

Metabolic Radiolabeling and Immunoprecipitation- MCF-7 and MCF-7/Adr cells at 70-80% confluence were metabolically labeled with [^{35}S] methionine (100 μCi/ml) for 3-4 hours at 37° C in methionine-free MEM. Cells were lysed and the cell lysates were immunoprecipitated with IgG_{2b},

IPM96 or C219 mAbs as previously described (29). The immunoprecipitated proteins were separated by SDS-PAGE using the Laemmli gel system (30). Polyacrylamide gels containing [^{35}S] methionine proteins were exposed to Kodak X-ray film following 30 minute incubation in an Amplify solution (Amersham Inc.).

Immunofluorescence Staining of Cells- Drug sensitive and resistant cells were washed with PBS and smeared onto glass slides by brief centrifugation at 1,500 rpm. The cytospins were air-dried and fixed for 10 minutes in ice-cold acetone. Cells were rinsed twice with PBS and incubated in 1% bovine serum albumin (BSA)/PBS for 30 minutes at room temperature. Slides were incubated with the first antibody (5 μg/ml 1% BSA/PBS) for 30 minutes followed by three two minutes rinses with PBS. FITC-conjugated goat-anti-mouse IgG (1:50 dilution) was added to slides and allowed to incubate for 30 minutes. After several washes, slides were mounted in PBS containing 50% glycerol and examined with a Nikon UFX-DX fluorescent microscope fitted with a 60X oil immersion objective. Photographs were taken with Kodak Tri-X pan film (400 ASA) at 800X magnification.

RESULTS

Isolation and characterization of IPM96 monoclonal antibody- To identify cellular changes common to *MDR* cell lines that overexpress P-gp or MRP, an immuno-dot blot method was used to isolate a monoclonal antibody, IPM96, that binds to proteins from drug resistant (MCF-7/Adr and H69/AR) but not drug sensitive (MCF-7 and H69) cells. Figure 1a shows the results of a Western blot containing total cell lysates from MCF-7 and MCF-7/Adr probed with IPM96 mAb. IPM96 bound specifically to a 40 kDa protein (P-40) in MCF-7/Adr but not in MCF-7 cells. Figure 1b shows the immunoprecipited proteins from [^{35}S] methionine metabolically labeled MCF-7/Adr cells using IPM96, C219 or an irrelevant IgG_{2b}. The results in figure 1b show that IPM96 immunoprecipitated a 40 kDa protein while a 170 kDa protein (or P-gp) was immunoprecipitated with C219 mAb. No [^{35}S] methionine labeled proteins were immunoprecipitated with an irrelevant IgG_{2b} (Figure 1b), nor did IPM96 and C219 mAbs precipitate a 40 kDa and a 170 kDa proteins from MCF-7 cells (data not shown).

Expression of 40 kDa protein in MDR cells - To determine if P-40 is overexpressed in other *MDR* cell lines, a Western blot of total cell lysates from MCF-7/Adr and SKOV/VLB$^{1.0}$ cells (P-gp positive *MDR* cells) and from H69/AR and HL60/AR (MRP-positive *MDR* cells) was probed with IPM96 mAb (Figure 2a). P-40 was detected at much higher levels in *MDR* cells than in parental drug sensitive cell lines (figure 2a). Furthermore, P-40 levels were much lower in one revertant cell line (e.g., H69/PR; (31)) derived from H69/Adr cells (figure 2a). With the exception of one parental cell line (SKOV3), which expressed low levels of P-40, no signal was detected in MCF-7, H69 and HL60 drug sensitive cells. Figure 2a also shows similar Western blots probed with P-gp-specific mAb (C219 mAb), MRP-specific mAb (QCRL-1 mAb) and tubulin antiserum. The latter results confirm the expression of P-gp or MRP in the different *MDR* cell lines.

In addition to P-gp and MRP-mediated *MDR* cells, P-40 expression was examined in other *MDR* cell lines (MCF-7/Mitox, SKTax6a and A2780^{CP}) selected for resistance to mitoxantrone, paclitaxel or cisplatin, but showed no detectable levels of P-gp or MRP (32-34, 25). Interestingly, while MCF-7/Mitox resistant cells did not express P-40 (figure 2b), a significant increase in P-40 levels was seen

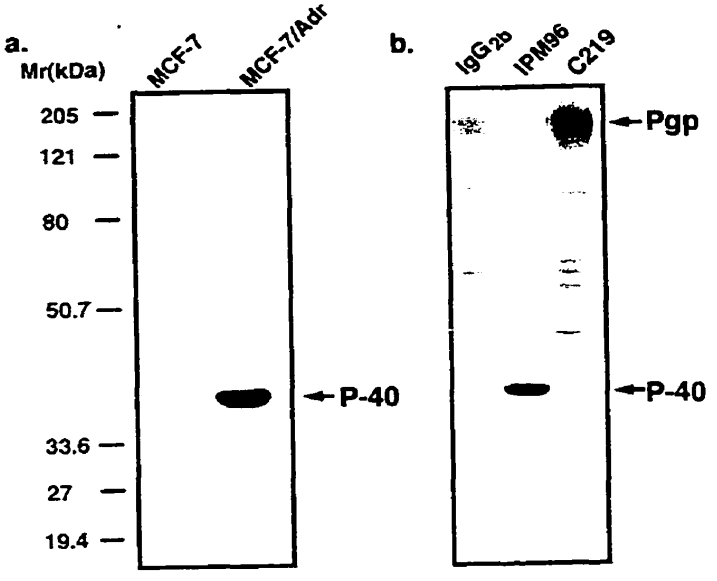


Figure 1. Binding of IPM96 mAb to a 40 kDa protein (P-40) in MCF-7/Adr cells. Western blot analysis of cell lysates from drug sensitive (MCF-7) and resistant (MCF-7/Adr) cells probed with IPM96 mAb show high expression of a 40 kDa protein in MCF-7/Adr cells **(a)**. Figure 1b shows an immunoprecipitation of [^{35}S] methionine labeled MCF-7 or MCF-7/Adr cells with an irrelevant IgG_{2b}, IPM96, or C219 mAbs. A 40 kDa and 170 kDa [^{35}S] methionine labeled polypeptides were immunoprecipitated with IPM96 and C219 mAbs, respectively **(b)**.

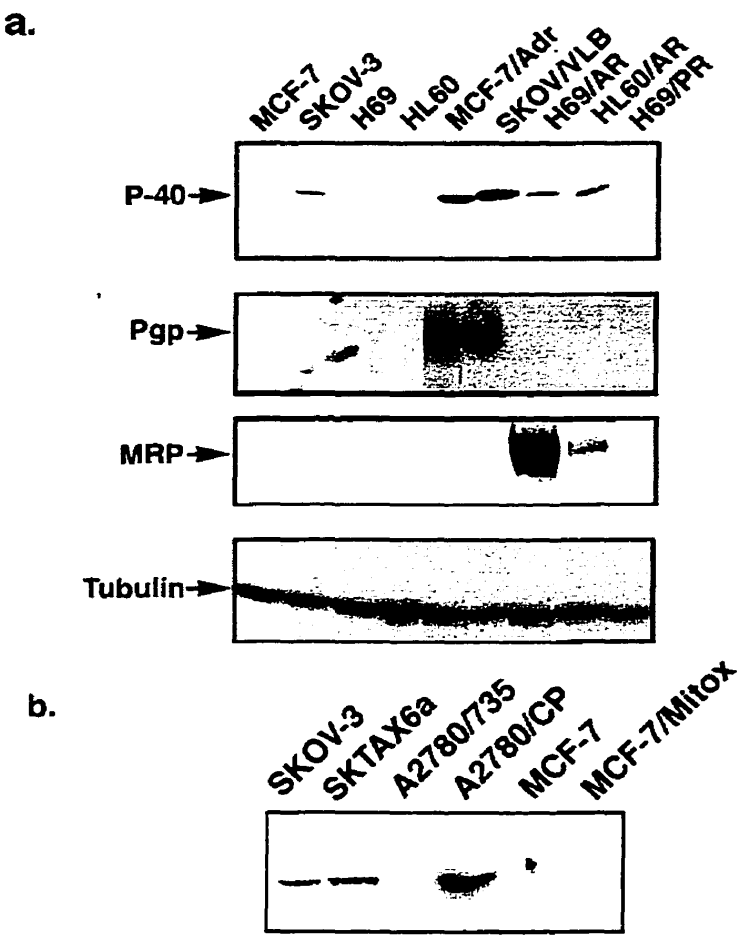


Figure 2. Expression of P-40 in human *MDR* cells. Total cell lysates from drug sensitive (MCF-7, SKOV-3, and HL60), resistant (MCF-7/Adr, SKOV/VLB$^{1.0}$, H69/AR and HL60/AR) and a revertant cell (H69/PR) were fractionated on SDS PAGE and probed for P-40 with IPM96 mAb, P-gp with C219 mAb, MRP with QCRL-1 mAb, and tubulin with an antiserum for tubulin **(a)**. Similarly, Figure 2b shows a Western blot of total cell lysates from drug sensitive (SKOV-3, A2780 and MCF-7) and resistant (SKTax6a, A2780^{CP} and MCF-7/Mitox) cells probed with IPM96 mAb.

in SKTax6a and A2780^{CP} cells (figure 2b). Table I summarizes the results from the Western blot analyses for P-40, P-gp and MRP expression in all the cell lines examined in this study.

Membrane and Soluble Pools of P-40- Given the above results, it was of interest to determine the subcellular localization of P-40 in *MDR* cells. Figure 3a shows an indirect immunofluorescence staining of MCF-7 and MCF-7/Adr cells with IPM96 mAb. MCF-7/Adr cells showed strong fluorescence staining at the cell membrane and a more diffused staining in the cytoplasm. By contrast, MCF-7 drug sensitive cells did not show any significant fluorescence when stained for P-40 with IPM96 mAb (Figure 3a).

To further characterize the subcellular expression of P-40, cell fractions from MCF-7/Adr cells enriched for mitochondrial (P1), Golgi membranes (P2), microsomes and plasma membranes (P3) and soluble proteins (S5) were prepared by differential centrifugation. The P3 fraction was further purified on a discontinuous sucrose gradient. A highly enriched plasma membrane and microsomal fractions at 16/31 and 31/47 interfaces ($P_{6/31}$ and $P_{31/47}$) were collected and examined for P-40 levels. The purity of the above subcellular fractions was established using marker enzymes as previously described (35) (data not shown). Figure 3b shows a Western blot of the different cellular fractions probed with IPM96 and C219 mAbs. P-40 was found in all different fractions including the soluble fraction (figure 3b). However, higher levels of P-40 were found in the plasma membrane enriched fraction (16/31) than in the other fractions (figure 3b). Similarly, P-gp was found in all pellet fractions but was not detectable in the soluble fraction (figure 3b). Of interest is the relative amount of P-40 and P-gp in P16/31 fraction compared to the other fractions.

To examine the nature of interactions between P-40 and the plasma membrane, the effects of agents that disrupt weak forces on P-40 association with the plasma membrane are shown in figure 4. The results in figure 4 shows that high salt (1.5 M NaCl) and chelating agents (5 mM EDTA) did not cause the release of P-40 from the plasma membrane. The denaturing agent urea did cause the release of some P-40 from the membrane; while the presence of a detergent (10 mM CHAPS) led to the complete release of P-40 into the soluble fraction (figure 4). In addition, treatment of $P_{16/31}$ membrane fractions with phosphatidylinositol (PI)-specific phospholipase C (36) did not affect the

association of P-40 with the plasma membrane (data not shown). Hence, P-40 is not a PI anchored protein.

Table 1. Relative Expression of MDR Related Protein

Cell line	Selecting drug	P-gp	MRP	P-40	Ref
MCF-7		-	-	-	
MCF-7/Adr	Adriamycin	++++	-	+++	(20)
MCF-7/Mitox	Mitoxantron	-	-	-	(32,33)
SKOV3		-	-	+	
SKOV3/VLB$^{1.0}$	Vinblastine	+++++	-	++++	(21)
SKTax6a	Taxol	-	-	++	(34)
H69		-	-	-	
H69/AR	Adriamycin	-	+++++	++	(22,31)
H69/PR		-	-	+/-	(31)
HL60		-	-	-	
HL60/AR	Adriamycin	-	+	++	(24)
A2780		-	-	-	
A2780^{CP}	Cisplatin	-	-	+++	(25)

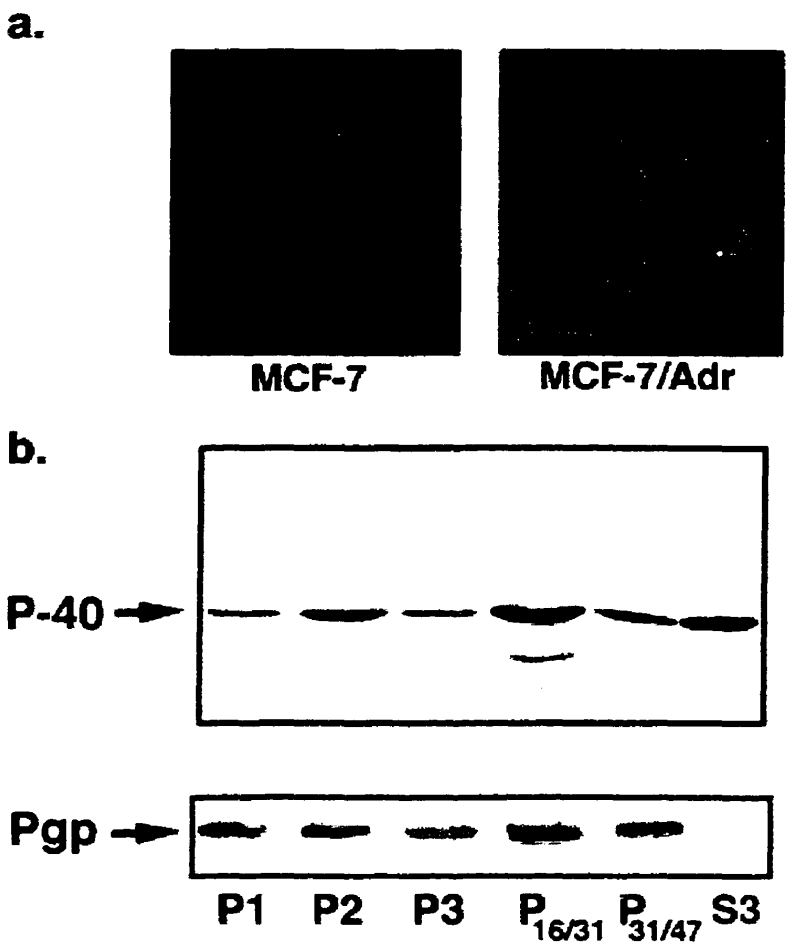


Figure 3. Subcellular localization of P-40 in *MDR* cells. Indirect immuno-fluorescence staining of MCF-7 and MCF-7/Adr with IPM96 mAb shows bright fluorescent staining at the plasma membrane and diffused staining in the cytoplasm in MCF-7/Adr cell **(a)**. No staining was observed in MCF-7 cells **(a)**. Figure 3b shows a Western blot analysis of enriched membrane (P1, P2, P3, P16/31 and P31/45) and soluble (S3) fractions from MCF-7/Adr cells (see Material and Methods). The nitrocellulose membrane with equal protein samples was probed for P-40 with (IPM96) and P-gp with (C219) mAbs **(b)**.

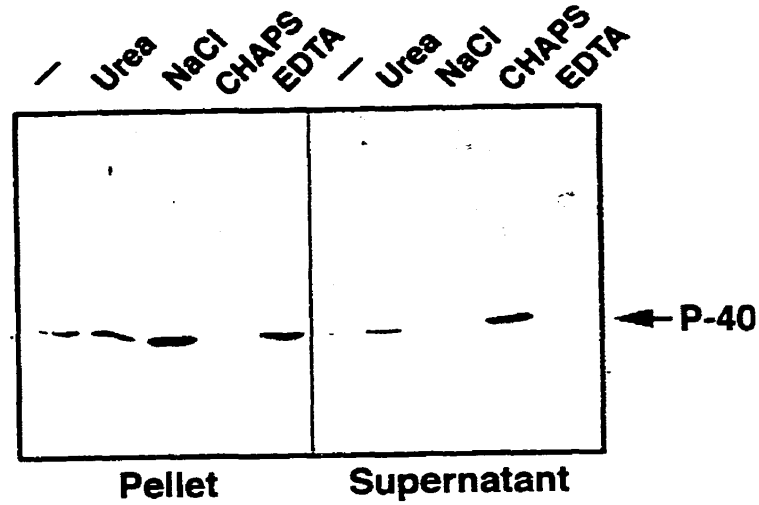


Figure 4. Effects of denaturing agents and detergents on P-40 interactions with the plasma membrane. Plasma membrane fractions were extracted with 6 M urea, 1.5 M NaCl, 5 mM EDTA, or 10 mM CHAPS. Figure 4 show a Western blot of the extracted (soluble) and the remaining membrane bound P-40 (pellet) probed with IPM96 mAb.

DISCUSSION

In this study, we describe the isolation of a monoclonal antibody (IPM96) that binds specifically to a 40 kDa protein (P-40) in *MDR* cells. We also show that P-40 is highly expressed in several *MDR* cell lines that overexpress P-gp or MRP. The co-expression of P-40 with P-gp or MRP is unlikely to be the result of co-amplification, since the *mdr1* (P-gp 1) and *mrp1* (MRP) genes map to chromosomes 7(q21 and q31) and 16 (p13.1), respectively (9, 37). Furthermore, the observed decrease of P-40 levels in a revertant cell line (H69/PR) derived from H69/AR, together with the above results, suggest a correlation between the P-40 expression and drug resistance in H69/AR cells. However, as the H69/PR cells were derived from H69/AR over a long period of cell culturing in the absence of drug selection, further studies are required to demonstrate direct correlation between P-40 and drug resistance.

The overexpression of P-40 in paclitaxel or cis-platinum selected cell lines (SKTax6a and $A2780^{CP}$), in the absence of a detectable levels of P-gp or MRP, supports the notion that P-40 alone may confer resistance to cytotoxic drugs. Although it remains to be determined if other *MDR*-associated proteins are overexpressed in these cell lines, earlier characterization of SKTax6a cells has demonstrated the presence of several isoforms of tubulins not expressed in the parental SKOV-3 cells (34). Similarly, the cis-platinum selected cells ($A2780^{CP}$) were also shown to have an enhanced DNA repair compared to the parental A2780 cells (25).

Earlier reports have described the co-expression of an 85 kDa (CD36) and 135-220 kDa (CD56) surface proteins with P-gp in several *MDR* tumour cell lines (38, 39). More recently (40), a 7 kDa membrane protein was also shown to be co-expressed with P-gp in $SKOV/VLB^{1.0}$ cells. Furthermore, Mirski et al. (41) have described the isolation of several monoclonal antibodies that recognized changes in proteins between sensitive and *MDR* human cells. The antigen (a 35 kDa soluble protein) recognized by one of the monoclonal antibodies was shown to be lipocortin II (42). However, except for the 85 kDa (CD36) and 25 kDa calcium binding protein (Sorcin), the effects of above proteins on the *MDR* phenotype have not been examined. Furthermore, it is not known if the above proteins are also co-expressed with MRP in *MDR* cells. The co-expression of P-40 with P-gp or MRP should allow us to address the question of whether P-40 is responsible for the observed differences in *MDR* levels

seen in P-gp or MRP transfectant versus *in vitro* selected cells that express equal levels of P-gp or MRP. Studies are ongoing to determine if P-40 interacts directly with P-gp and MRP.

Alternatively, P-40 may mediate a drug resistance phenotype independently of P-gp and MRP *MDR* mechanisms. For example, P-40 expression may be a first line of defense and as such precedes the expression of P-gp and MRP. In this respect, the low level of P-40 in the absence of P-gp in SKOV-3 parental cells is not surprising since SKOV-3 cells were previously shown to be moderately resistant to several cytotoxic drugs (e.g., diphtheria toxin, cis-platinum and adriamycin) (43). Moreover, selection of SKOV-3 cells for resistance to vinblastine or paclitaxel (SKOV/VLB$^{1.0}$ or SKTax6a) leads to much higher levels of P-40. In line with the above speculation, several studies have now demonstrated the co-expression of P-gp and MRP in *MDR* selected cell lines (44-47). Furthermore, it has been shown that MRP expression in those cell lines precedes that of P-gp at lower levels of drug resistance(45-47). Thus, in future studies, it would be of interest to determine if P-40 expression precedes that of P-gp or MRP at lower levels of drug resistance. These findings are important with respect to *MDR* reversal, since the drugs that reverse P-gp-mediated *MDR* are not good reversing agents of MRP-mediate *MDR* (48, 49).

Our subcellular fractionation and immunofluorescence data show P-40 to be associated with the plasma membrane and soluble fractions. However, the membrane associated P-40 was resistant to extraction with high salt or chelating agents and phosphatidylinositol (PI)-specific phospholipase C. The presence of 6 M urea caused some P-40 to dissociate from the membrane, while 10 mM CHAPS resulted in the complete release of P-40. The release of P-40 in the presence of 6 M urea but not with high salt or EDTA is not clear but it is consistent with hydrophobic interactions (50). Alternatively, the release of P-40 in the presence of a denaturing agent may be caused by proteolytic cleavage . At any rate, comparison of P-40 from soluble and plasma membrane pools by two-dimensional gel electrophoresis shows differences in pI (results not shown). Further biochemical analyses of these two pools of P-40 and the nature of interactions between P-40 and the cell membrane are the subject of further studies.

In conclusion, we show the overexpression of a 40 kDa protein in MDR cells in the presence and absence of P-gp or MRP. Although further studies are required to demonstrate a direct role for P-40,

if any, in drug metabolism and *MDR;* P-40 could modulate an *MDR* phenotype indirectly. For example, P-40 may be a component of the apoptosis signaling pathway. There is now growing evidence that changes in the levels or the functions of proteins involved in the signalling of apoptosis can confer an *MDR* phenotype on tumour cells (51, 52).

ACKNOWLEDGMENTS

The authors would like to thank their colleagues at the Institute of Parasitology for their careful reading of this manuscript. This research is supported by funds from the National Cancer Institute of Canada with funds from the Canadian Cancer Society to E.G. Research at the Institute of Parasitology is supported by Fonds FACA pour L'aidea La Recherche.

REFERENCES

1. Rock, D. B., Murray, K. J., Schultz, C. J., Lauer, S. J. & Wilson, J. F. (1996) *American J. Clinical Oncol.* **19,** 174-178.

2. DeVita, V. T. J. & Hubbard, S. M. (1993) *New Engl. J. Med.* **328,** 560-565.

3. Ferguson, P. J. & Cheng, Y.-C. (1989) *Cancer Bulletin* **41,** 7-13.

4. Bradley, G., Juranka, P. F. & Ling, V. (1988) *Biochem Biophys Acta* **948,** 87-128.

5. Pastan, I. & Gottesman, M. (1987) *New Engl. J. Med.* **316,** 1388-1393.

6. Tew, D. T. (1994) *Cancer Res.* **54,** 4313-4320.

7. Frelich, S. J. & Osheroff, N. (1995) *J. Biol. Chem.* **270,** 21429-21432.

8. Riordan, J. R. & Ling, V. (1979) *J. Biol. Chem.* **254,** 12701-12705.

9. Cole, S. P. C., Bhardwaj, G., Gerlach, J. H., Almquist, K. C. & Deeley, R. G. (1993) *Proc. Amer. Assoc. Cancer Res.* **34,** 579.

10. Scheffer, G. L., Wijngaard, P. L., Flens, M. J., Izquierdo, M. A., Slovak, M. L., Pinedo, H. M., Meijer, C. J., Clevers, H. C. & Scheper, R. J. (1995) *Nature Medicine* **1,** 578-578.

11. Dano, K. (1973) *Biochem. Biophys. Acta.* **323,** 466-483.

12. Almquist, K. C., Loe, D. W., Hipfner, D. R., Mackie, J. E., Cole, S. P. & Deeley, R. G. (1995) *Cancer Res.* **55,** 102-110.

13. Endicott, J. A. & Ling, V. (1989) *Ann. Rev. Biochem.* **58,** 137-171.

14. Gottesman, M. M. & Pastan, I. (1993) *Ann. Rev. Biochem.* **62,** 385-427.

15. Cole, S. P., Sparks, K. E., Fraser, K., Loe, D. W., Grant, C. E., Wilson, G. M. & Deeley, R. G. (1994) *Cancer Res.* **54,** 5902-5910.

16. Ueda, K., Cardarelli, C., Gottesman, M. M. & Pastan, I. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 3004-3008.

17. Gros, P., Neriah, Y. B., Croop, J. M. & Housman, D. E. (1986) *Nature* **323,** 728-731.

18. Zaman, G. J. R., Flens, M. J., Vanleusden, M. R., Dehaas, M., Mulder, H. S., Lankelma, J., Pinedo, H. M., Scheper, R. J., Baas, F., Broxterman, H. J. & Borst, P. (1994) *Proc. Nat. Acad. Sci. USA* **91,** 8822-8826.

19. Conter, V. & Beck, W. T. (1984) *Cancer Treat. Reports.* **68,** 831-839.

20.Batist, G., Tulpule, A., Sinha, B. K., Katki, A. G. & Myers, C. E. (1986) *J. Biol. Chem.* **261,** 15544-15549.

21.Bradley, G., Naik, M. & Ling, V. (1989) *Cancer Res.* **49,** 2790-2796.

22.Cole, S. P. C., Chanda, E. R., Dicke, F. P., Gerlach, J. H. & Mirski, S. E. L. (1991) *Cancer Res.* **51,** 3345-3352.

23.Dalton, W. S., Durie, B. G., Alberts, D. S., Gerlach, J. H. & Cress, A. E. (1986) *Cancer Res.* **46,** 5125-5130.

24.Marsh, W., Sicheri, D. & Center, M. S. (1986) *Cancer Res.* **46,** 4053-4057.

25.Jekunen, A. P., Christen, R. D., Shalinsky, D. R. & Howell, S. B. (1994) *Brit. J. Cancer* **69,** 299-306.

26. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193.**

27.Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350-4354.

28.Galfre, G. & Milstein, C. (1981) *Meth. Enzymology.* **73,** 3-46.

29.Georges, E., Zhang, J.-T. & V., L. (1991) *J. Cell. Physiol.* **148,** 479-484.

30.Laemmli, U. K. (1970) *Nature* **227,** 680-685.

31.Cole, S. P. C., Bharswaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M. V. & Deeley, R. G. (1992) *Science* **258,** 1650-1654.

32.Nakagawa, M., Schneider, E., Dixon, K. H., Horton, J., Kelley, K. & Morrow, C. (1992) *Cancer Res.* **52,** 6175-6181.

33.Futscher, B. W., Abbaszadegan, M. R., Domann, F. & Dalton, W. S. (1994) *Biochem. Pharmacol.* **47,** 1601-1606.

34.Rischin, D., Lee, G., Gurusinghe, A. & Woodcock, D. (1996) *Proc Annu Meet Am Assoc Cancer Res.* **37,** A2563.

35.Kawai, K., Kamatani, N., Georges, E. & Ling, V. (1990) *J. Biol. Chem.* **265,** 13137-13142.

36.Slapak, C. A., Mizunuma, N. & Kufe, D. W. (1994) *Blood* **84,** 3113-3121.

37.Trent, J. M. & Witkowski, C. M. (1987) *Cancer Genetics & Cytogenetics.* **26,** 187-190.

38.Scheper, R. J., Dalton, W. S., Grogan, T. M., Schlosser, A., Bellamy, W. T., Taylor, C. W., Scuden, P. & Spier, C. (1991) *Inter. J. Cancer* **48,** 562-567.

39.Sugimoto, Y., Hamada, H., Tsukahara, S., Noguchi, K., Yamaguchi, K., Sato, M. & Tsuruo, T. (1993) *Cancer Res.* **53,** 2538-2543.

40.Yang, X. & Page, M. (1995) *Cancer Letters* **88,** 171-178.

41.Mirski, S. E. L. & Cole, S. P. C. (1991) *Brit. J. Cancer* **64,** 15-22.

42.Cole, S. P., Pinkoski, M. J. & Bhardwaj, G. (1992) *Brit. J. Cancer.* **65,** 498-502.

43.Fogh, J. & Trempe, G. (1975) *Plenum Press, New York*, 155-159.

44.Feller, N., Kuiper, C. M., Lankelma, J., Ruhdal, J. K., Scheper, R. J., Pinedo, H. M. & Broxterman, H. J. (1995) *Brit. J. Cancer.* **72,** 543-549.

45.Van der Bliek, A. M., Meyers, M. B., Biedler, J. L., Hes, E. & Borst, P. (1986) *EMBO J.* **5,** 3201-3208.

46.Van der Bliek, A. M., Baas, F., Van der Velde-Koerts, T., Biedler, J. L., Meyers, M. B., Ozols, R. F., Hamilton, T. C., Joenje, H. & Borst, P. (1988) *Cancer Res.* **48,** 5927-5932.

47.Fairchild, C. R., Ivy, S. P., Kao-Shan, C. S., Whang-Peng, J., Rosen, N., Israel, M. A., Melera, P. W., Cowan, K. H. & Goldsmith, M. E. (1987) *Cancer Res.* **47,** 5141-5148.

48.Cole, S. P. C., Downes, H. F. & Slovak, M. L. (1989) *Brit. J. Cancer* **59,** 42-46.

49.Gollapupi, S. & Gupta, S. (1992) *Anticancer Res.* **12,** 2127-2132.

50.Nozaki, Y. & Tanford, C. (1963) *J. Biol. Chem.* **238,** 4074-4081.

51.Lowe, S. W., Ruley, H. E., Jacks, T. & Housman, D. E. (1993) *Cell* **74,** 957-967.

52.Lowe, S. W., Bodis, S., McClatchey, A., Remington, L., Ruley, H. E., Fisher, D. E., Housman, D. E. & Jacks, T. (1994) *Science* **266,** 807-810.

CHAPTER IV.

MANUSCRIPT III.

CONNECTING STATEMENT 2

Our previous study (Chapter III) has shown the overexpression of P-40 in MDR cells in the absence or presence of P-gp or MRP. Biochemical characterization of P-40 has revealed that P-40 is an intracellular protein associated with the plasma membrane and soluble fraction. More importantly, the expression of P-40 can be correlated with the level of drug resistance (Wang et al , 1997). It is very likely that, as a alternative mechanism, overexpression of P-40 may confer MDR. Although the expression pattern of P-40 led to its implication in MDR phenotype, further investigations, especially the molecular characterization of P-40 was required to demonstrate the role of P-40 in MDR. The establishment of P-40 cDNA transfectants in drug sensitive cells will be critical to evaluate the functional role of P-40 in the development of MDR phenotype. In this study, we described the molecular characterization of P-40 and elucidated the role of P-40 in MDR by a single gene transfer study.

Manuscript in preparation.

OVEREXPRESSION OF ANNEXIN I IN MAMMALIAN TUMOUR CELLS CONFERS DRUG RESISTANCE TO ANTICANCER DRUGS

YING WANG AND ELIAS GEORGES*

Institute of Parasitology, McGill University, Macdonald Campus, Ste-Anne de Bellevue, Quebec, Canada

* Corresponding author. Institute of Parasitology, McGill University, 21,111 Lakeshore road, Ste-Anne de Bellevue, Quebec, Canada H9X 3V9. Fax: 514-398-7857.
E-mail:Elias_Georges@maclan.McGill.CA

ABSTRACT

The development of multidrug resistant tumour cells during the chemotherapeutic treatment of cancer patients is a major obstacle in cancer treatment. Studies from *in vitro* selected cell lines have led to the identification of several cellular proteins that mediate resistance to anticancer drugs (eg. P-gp and MRP). We have previously identified a 40 kDa protein (named P-40) that is overexpressed in several MDR cell lines (Wang et al, 1997). In this study, we have further characterized this 40 kDa protein at molecular level. The cloning of the cDNA encoding P-40 revealed that P-40 is identical to annexin I, a substrate for the epidermal growth factor receptor tyrosine kinase. The identity of isolated P-40 cDNA and annexin I is also established based on; (a) the cross-reactivity of IPM96 monoclonal antibody with 40 kDa protein following the *in vitro* expression of P-40 cDNA; (b) the similarity of the molecular mass of P-40 and annexin I and (c) the distribution pattern of P-40 and annexing I in mammalian cells. Northern blot analysis of total RNA from drug sensitive and resistant cells revealed an increase in P-40 (or annexin I) in drug resistant cells. The northern blot results are also consistent with the Western blot data, especially those relating to the protein and mRNA levels of P-40 (or annexin I) in SKOV3 compared with SKOV/VLB1.0 cell line. Thus, the observed increase in P-40 (or annexin I) protein level in drug resistant cells is transcriptionally regulated. To determine the role of P-40 (or annexin I) in MDR, the effects of several anticancer drugs were compared between MCF-7 transfected with P-40 (annexin I) cDNA (P-40-MCF-7) versus vector only. Our results show that P-40-MCF-7 transfectant cells are 2 to 3- fold more resistant to adriamycin, actinomycin D, Taxol and cisplatin relative to the MCF-7 cells transfected with vector alone. Interestingly, P-40-MCF-7 cells did not show cross -resistance to vincristine and colchichine. Taken together, these results provide the first direct evidence for the role of annexin I in multidrug resistance.

INTRODUCTION

The ability of malignant cells to develop multidrug resistance (MDR) to cytotoxic drugs has become a major impediment in the chemotherapeutic treatment of cancer patients. Studies from *in vitro* selected MDR tumour cell lines have led to the identification of several cellular proteins which confer resistance to a variety of chemotherapeutic agents (Pastan and Gottesman, 1987; Bradley et al, 1988). The overexpression of P-glycoprotein (P-gp) and the Multidrug Resistance Protein (MRP) in cells selected with hydrophobic cytotoxic drugs (eg. Vinca alkaloid, anthracyclines and epipodophyllotoxins) have been shown to confer an MDR phenotype (Gottesman and Pastan, 1993; Endicott et al, 1989; Cole et al,1996). Both P-gp and MRP belong to a large family of ATP trafficking proteins that mediate the transport of numerous substrates ranging from ions to large peptides (Higgins 1992). In tumour cell lines, P-gp and MRP reduce the intracellular drug accumulation via an energy-dependent drug efflux mechanism (Shapiro and Ling, 1995; Doige and Sharom, 1992; Zaman et al, 1994).

P-gp and MRP are expressed to variable levels in normal tissues and are thought to mediate the transport of the normal cell metabolites, hormones and xenobiotics (Cordon-Cardo et al, 1990; Bradely et al, 1990; Thorgeirsson et al, 1987; Thiebaut et al, 1987). In agreement with the *in vitro* studies, disruption of both alleles of P-gp (mdr1) from the mouse genome has resulted in the accumulation or redistribution of drugs in many organs and tissues where P-gp is expressed (Schinkel et al 1997; Schinkel et al, 1994). High levels of P-gp expression has also been detected in more than 50 % of tumours including those derived from tissues in which P-gp gene is consistently activated, or those in which cell transforming events appear to be responsible for the activation of P-gp gene (Tishler et al, 1992; Abe et al, 1994; Baker et al, 1989; Belloni et al, 1989; Charpin et al, 1994; Fojo et al, 1987; Charpin et al, 1994; Henson et al, 1992; Hijazi et al, 1994; Mattern and Volm 1994). The correlation between the overexpression of P-gp and failure of chemotherapy and poor survival rates has been established for some hematopoietic tumours and childhood malignancies (Chan et al 1990). However, the lack of P-gp expression in other MDR tumours indicates that additional cellular changes can confer resistance to anticancer drugs (Lee et al, 1997; Baggetto 1997; Linn et al 1994; Lonn et al, 1994; Sognier et al 1994). Some of the cellular changes identified in drug resistant cells

include the overexpression of MRP (Loe et al 1996), alterations in glutathione-S-transferase activity or GSH levels (Tew, 1994), reduction in Topoisomerase II levels or activity (Frelich et al, 1995), overexpression of LRP (the Lung Resistance Protein, the component of human vaults (Scheffer et al, 1995), and changes in proteins mediating apoptosis or programmed cell death (Lowe et al, 1993; Lowe et al, 1994).

We have previously demonstrated the overexpression of a 40 kDa protein (P-40) with or without the expression of P-gp or MRP (Wang and Georges 1997). Our earlier biochemical characterization demonstrated the presence of P-40 in the membrane and cytosolic fractions in several MDR cell lines. In this report, we show that P-40 is identical to annexin I (also known as lipocortin). Annexin I is a member of a large family of Ca^{2+}-dependent phospholipid binding proteins with several predicted functions including intracellular membrane vesicular trafficking and exocytosis (Raynal and Pollard, 1994). However, annexin I has not been previously implicated in drug resistance. Moreover, we show that transfection of P-40 (or annexin I) cDNA into drug sensitive cells (MCF-7) confers resistance to structurally and functionally dissimilar drugs. Taken together, the results of this study demonstrate, for the first time, the role of annexin I in the MDR phenotype of tumour cells.

MATERIALS AND METHODS

Cell culture. Cell culture was performed under standard conditions in RPMI or α-MEM medium, containing 10% to 15 % fetal calf serum (Hyclon Inc.). Drug-sensitive cells (MCF-7, H69, SKOV3 and HL60) and their resistant mutants (MCF-7/Adr, H69/Adr, SKOV/VLB$^{1.0}$ HL60/AR) were grown in the absence of antibiotics. Briefly, cells were grown at 37^{0}C in humid atmosphere of 5% CO_2 and 95% air and passaged when cultures were 70-80% confluent for adherent cells or 1X10^6 cells/ml for cells in suspension. Drug-resistant cells were grown continuously with appropriate concentrations of cytotoxic drugs. All cells were routinely examined for Mycoplasma contamination by PCR (Mycoplasma PCR kit from Stratgene).

Screening an expression cDNA library with monoclonal antibody. The screening of an expression cDNA library constructed in λ-gt 11 using mRNA from HeLa cells was performed using a P-40 specific monoclonal antibody essentially as outlined in the manufacturer's protocol (Clonetech Laboartories Inc, U.S.A). About 5X10^5 plaques were plated using *Escherichia coli* Y1090 as host and screened with IPM96 monoclonal antibody. Briefly, plates containing phage plaques were incubated at 42^{o}C for 4 hours and then overlaid with a dried nitrocellulose filter saturated with 10 mM IPTG (isopropyl-B-D-thiogalactopyranoside). The agar plates were incubated for another three hours at 37 ^{o}C . To process the filters for antibody screening, double lifts from the agar plates were immediately immersed in a large volume of TNT buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20) and washed twice for 30 minutes at room temperature. Following one hour blocking with 5% skim milk in the presence of 1.5 mM of sodium azide, the nitrocellulose filters were incubated with TBST buffer containing 2 μg/ml of Protein G column purified IPM96 monoclonal antibody overnight at 4^{o}C. Filters were washed with TNT buffer and incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (Gibco BRL). Positive plaques were visualized by chemiluminescence using Amersham ECL kit (Amersham, Oakville, Ont.). The positive immunoractive plaques were verified by duplicate lifts and purified by subsequent rounds of screening using lower plaque density. The phage particles were eluted from the positive plaques in water and used as templates for PCR with the 3' and 5' insert screening

amplimer sequence of λ-gt-11 (Clonetech Laboratories Inc., U.S.A.). The DNA fragments from the PCR reactions were cloned into PCR II vector (Invitrogen) following standard procedures (Sambrook et al, 1989).

Nucleotide sequencing and computer sequence analysis. The above cDNA clones were sequenced by the dideoxy termination method using M13 universal primers and sequence specific primers via the automated DNA sequencing service at Sheldon Biotech Centre at McGill University and Core Facility of Queen's University at Kingston Ontario. Both strands of two different clones were completely sequenced. Computer analysis of the DNA and protein sequence was done with MacVector software and Blast search program.

Northern blot. For Northern blot analysis, total RNA was prepared from drug sensitive and their resistant sublines using the Trizol Reagent Kit (GibcoBRL, Gaithersburg, U.S.A) according to the manufacturer's directions. Approximately 10 μg of RNA samples from each cell line was separated by electrophoresis through a 1% agarose gel containing 10 mM NaH_2PO_4 and transferred to a Hybonds^{TM}-N probe membrane (Amersham, Oakville, Ont.) by pressure blotting with 20XSSC (1XSSC contains 140 mM NaCl, 32 mM Sodium Citrate, pH 7.4). The membrane was incubated in buffer A (50% formamide, 2.5XDenhardt's solution, 25 mg/ml denatured salmon sperm DNA, 1% SDS and 1.25X SSPE) containing $1X10^6$ cpm/ml of α-[^{32}P]dATP nick translated probe of P-40 cDNA at 42°C overnight. The low stringency sequential washes with 0.5 SSC were performed for 30 minutes at room temperature. A higher stringency wash was applied only if it was necessary. Quantification of the radioactive signals was carried out by scanning the resultant autoradiograph using NIH imaging software (NIH Image 1.59).

***In vitro* transcription and translation.** *In vitro* expression of cDNA clones was carried out using a rabbit reticulocyte lysate transcription and translation system (Promega Corporation. Madison, WI). Briefly, cDNA encoding for the full length P-40 was cloned into the *Not* I site of a pCDNA3 eukaryotic expression vector that contained the T7 and SP6 promoters (Invitrogen, Inc.). The

pCDNA3 with and without P-40 cDNA insert were then added into a coupled reticulocyte lysate transcription and translation system in the presence of [^{35}S]-methionine (Dupont/NEW, Mississauga, Ont). Following a two hour incubation at 30°C, *in vitro* synthesized proteins were analysed by Western blotting and immunoprecipitation with IPM-96 monoclonal antibody as previously described (Wang and Georges, 1997).

Gene transfection. MCF-7 cells were transfected with the vector (pCIN4) alone or vector containing P-40 cDNA using lipofectAMINE (Gibco, Burlington, Ontario, Canada) as outlined by manufacture's protocol. Briefly, 4 X 10^5 cells was plated in a 60 mm plate with complete medium and incubated overnight at 37°C. The plates were washed with serum-free α-MEM medium three times and overlaid with 1 ml of serum-free α-MEM containing 5 μg of supercoiled DNA in 10 μl of lipofectAMINE. After a 5 hour incubation, the medium was replaced with 5 ml of α-MEM medium supplemented with 10% fetal calf serum (Hyclone Laboratories) and cells were further cultured at 37°C for 24 hours. For stable transfectants, G418 (Gibco) was added to the cells at 1 mg/ml and cells were cultured continuously with G418 for another three weeks. Individual clones of MCF-7 cells stably transfected with pCIN4-P40 and pCIN4 only were obtained and subsequently expanded in the presence of G418 (1mg/ml). A population of cells exhibiting high expression of P-40 and a population of the cells transfected with pCIN4 vector alone were characterized by Western blotting and immunofluroresence.

Cytotoxicity assays. The chemosensitivity patterns of stably transfected MCF-7 cells with pCIN4 vector alone or full length P-40 cDNA were determined by a tetrazolium salt-based microplate assay as described previously (Pouliot et al 1997). Briefly, 100 μl aliquots of cells were plated into 96-well plates at 5000 cells per well. The cells were then incubated at 37°C for 24 hours before the addition of increasing concentration of different cytotoxic agents. Following a 72 hour incubation with different anticancer agents, 3-(4,5-Demethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma) was added to each well of the plates at a final concentration of 2.5 mg/ml. After 4 hour incubation, cells were solubilized by the addition of 50 μl of 10% Triton X-100 in 0.01 N HCl. The

96-well plates were heated in the microwave oven for 1 minute at the minimal power setting, and 10 µl of 100 % ethanol was added to disperse the bubbles formed during pipetting. Plates were read at 570 nm using an ELISA microplate reader. The effects of drugs on the viability of cells were expressed as the mean ± SD of two to three independent experiments in which triplicates were assayed.

Indirect immunofluorescence. Both *in vitro* selected P-40-expressing MDR cell lines and P-40 cDNA transfectants were used for indirect immunofluorescence analysis as described previously (Wang and Georges 1997). Briefly, cells were washed three times with PBS and prepared onto slides by cytospin centrifugation. Cytospins were air dried and fixed for 10 minutes in ice cold acetone. All subsequent steps were performed at room temperature. Slides were rinsed briefly in PBS and then incubated in a blocking solution containing 1% BSA in PBS for 20 minutes. A 100 µl of IPM96 monoclonal antibody (2µg/ml) was added to each slide and allowed to incubate for 30 minutes at room temperature. The washed cytospins were incubated with fluorescein FITC-conjugated goat antimouse IgG (Sigma) for 30 minutes. After several washes, slides were mounted in PBS containing 50 % glycerol and examined with a Nikon UFX-DX fluorescence microscopy fitted with a 60X immersion objective. Photographs were taken with Ektachrome Tungsten film (160 ASA) at 400X to 600X magnification.

RESULTS

Isolation and identification of P-40 cDNA clones. In a previous report (Wang et al, 1997), we had demonstrated the overexpression of a 40 kDa protein (P-40) in several MDR cell lines alone or together with Pgp or MRP. To further characterize the P-40 protein, it was of interest to isolate the cDNA clone that encodes its sequence. In this study, we have used a P-40 specific monoclonal antibody (IPM96) to screen a cDNA expression library prepared from HeLa cells. A total of 500,000 plaques from λ-gt 11 phage library were screened. Several positive plaques were obtained following the initial screening. Of the latter positive plaques, two positive clones were obtained after a sequential plaque purification and both inserts were isolated by PCR (see Materials Methods) The two positive clones encoded for the same 1.4 kb fragment that was subcloned into T/A PCRII vector. Sequence analysis of both cDNA clones showed an open reading frame of 344 amino acids which is consistent with the expected molecular mass of the protein (38.2 kDa versus 40 kDa). Comparison of P-40 nucleotide and amino acid sequences to other sequences in the DNA data bank, using a DNA search programs (Blast Search) revealed a complete identity of P-40 to annexin I* (Wallner et al, 1986, accession No emb/x059081/HSLIPCR).

To confirm the identity of the isolated P-40 cDNA as that of annexin I, a pCDNA3 construct containing a 1.417 kb fragment encoding the full length of P-40 (Annexin I) was expressed *in vitro* using T7 promoter directed transcription and translation reticulocyte lysate with [^{35}S]-Methionine. Figure 1a shows the immunoprecipitation of proteins with IPM96 monoclonal antibody from an *in vitro* expression reaction containing vector only (lane 1) or vector plus 1.4 kb insert (lane 2). As control for IPM96 monoclonal antibody, an irrelevant IgG_{2b} was used to immunoprecipitate proteins from a reaction mixture containing vector plus the 1.4 kb insert (Figure 2a). The results of figure 2a show a [^{35}S]-methionine labelled 40 kDa protein immunoprecipitated with IPM96 monoclonal antibody but not with irrelevant IgG_{2b}. Figure 2b shows Western blot analysis of the protein lysates identical to those in figure 2a, but probed with IPM96 monoclonal antibody or irrelevant IgG_{2b}. Taken together, these results confirm the identity of the 1.4 kb fragment as the annexin I gene.

To determine if the amino acid sequence of P-40 (or annexin I) cloned from HeLa cells is different

*accession number:emb/Xo5908/HSLIPCR

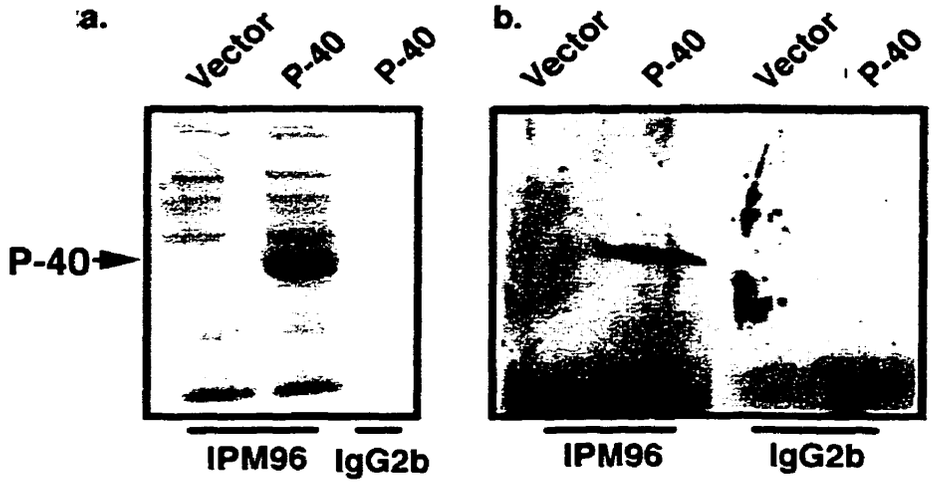


Figure 1. ***In vitro*** **expression of P-40 (or annexin I).** The *in vitro* expression of P-40 (or annexin I) cDNA was performed using the T7 promoter directed transcription in pCDNA3 cloning vector. Figure 2a shows *in vitro* transcribed and translated products containing vector only or vector plus 1.4 kb insert following immunoprecipitation with IPM96 monoclonal antibody. Figure 2b shows the same samples as in figure 2a but transferred to nitrocellulose membrane and probed with IPM96 monoclonal antibody or an irrelevant IgG_{2b}, respectively.

from that found in MCF-7/Adr cells, P-40 (or annexin I) was further cloned from MCF-7/Adr cells by RT-PCR using 5' and 3' primers encoding P-40 (or annexin I). Analysis of annexin I sequence from MCF-7/Adr cells revealed no differences from that isolated from HeLa cells (data not shown). In our previous study (Wang and Georges, 1997), the level of P-40 (annexin I) was compared between drug sensitive and resistant cells lines. Figure 2a shows a Western blot analysis of total cell lysates from drug sensitive (MCF-7, SKOV3 and H69) and their resistant (MCF-7/Adr, SKOV/VLB$^{1.0}$ and H69/AR) mutants probed with IPM96 monoclonal antibody. The results of the latter Western blot shows an increase in P-40 expression in resistant cells relative to the parental drug sensitive cells. The SKOV3 cells show lower levels of P-40 (or annexin I) than the resistant SKOV/VLB $^{1.0}$ cells. Interestingly, the SKOV3 cell line was originally derived from a patient with ovarian tumour that was considered clinical resistant to cisplatin and adriamycin (Fog and Trempe, 1975). To determine if the overexpression of P-40 is due to transcriptional modification, northern blot analysis was performed with RNA extracted from drug sensitive (MCF-7, SKOV3 and H69) and resistant (MCF-7/Adr, SKOV/VLB$^{1.0}$ and H69/AR) cells. The membrane was probed with ^{32}P labelled 1.4 kb fragment. The result in figure 2b shows a 1.6 kb mRNA band in MCF-7/Adr, H69/AR and SKOV/VLB$^{1.0}$ drug resistant cell lines but not in their drug sensitive parental cells with the exception of SKOV3 cells. In comparison with SKOV/VLB$^{1.0}$, a 4-fold decrease in P-40 transcript levels was observed in SKOV3 drug sensitive cells (Figure 3, lane 3 and lane 4). Taken together, the northern blot results clearly demonstrate that the observed increase in P-40 (or annexin I) in drug resistant cell lines is due to an increase in its mRNA levels. Furthermore, the northern blot results are consistent with the Western blot data, especially, those relating levels of P-40 (or annexin I) in SKOV3 versus SKOV3/VLB$^{1.0}$ cells. To determine if the above increase in mRNA levels in MDR cells relative to drug sensitive cells is due to gene amplification, genomic DNA from drug sensitive and resistant cells were isolated and analysed quantitatively by Slot blotting. Interestingly, no gene amplification of P-40 (or annexin I) was observed in resistant cells (data not shown).

Characterization of P-40 (or annexin I) MCF-7 stable transfectant cells. To determine if P-40 (or annexin I) alone confers resistance to anticancer drugs, a full length cDNA clone of P-40 (or

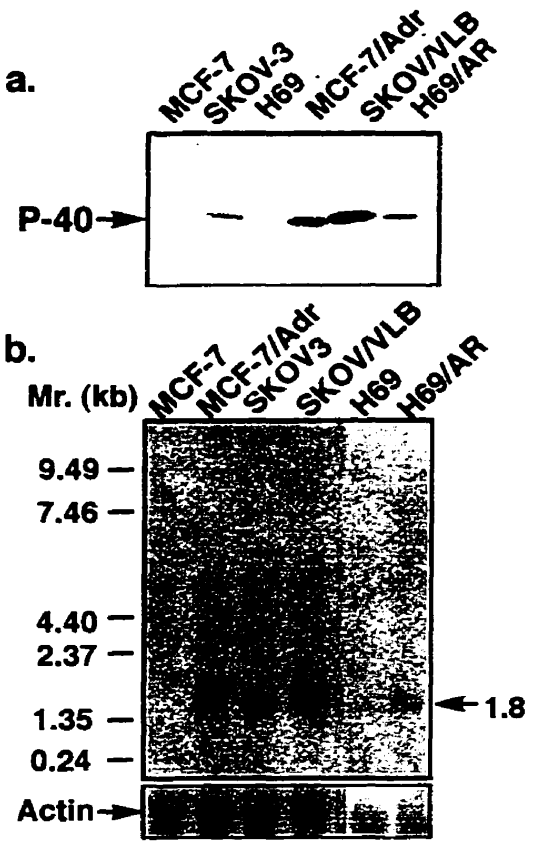


Figure 2. Analysis of protein and mRNA levels of P-40 (or annexin I) in drug sensitive and drug resistant cells. Total cell extract from drug sensitive (MCF-7, SKOV3 and H69) and drug resistant human MDR cell lines (MCF-/Adr, SKOV/VLB$^{1.0}$ and H69/AR) were fractionated on SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with IPM96 monoclonal antibody. P-40 (or annexin I) is seen in extracts from drug resistant cells. Low level of P-40 is detected in SKOV3 drug sensitive cells but not in MCF-7 or H69 cells. For mRNA levels in the same cell lines, total RNAs were resolved on agarose gel and transferred to nylon membrane and probed with ^{32}P-dATP labelled 1.4 kb fragment encoding for P-40 (or annexin I) and actin. Figure 3b shows that the expression of a 1.6 kb mRNA in drug sensitive and resistant MDR cells correlates with levels of P-40 (or annexin I) in the same cell lines.

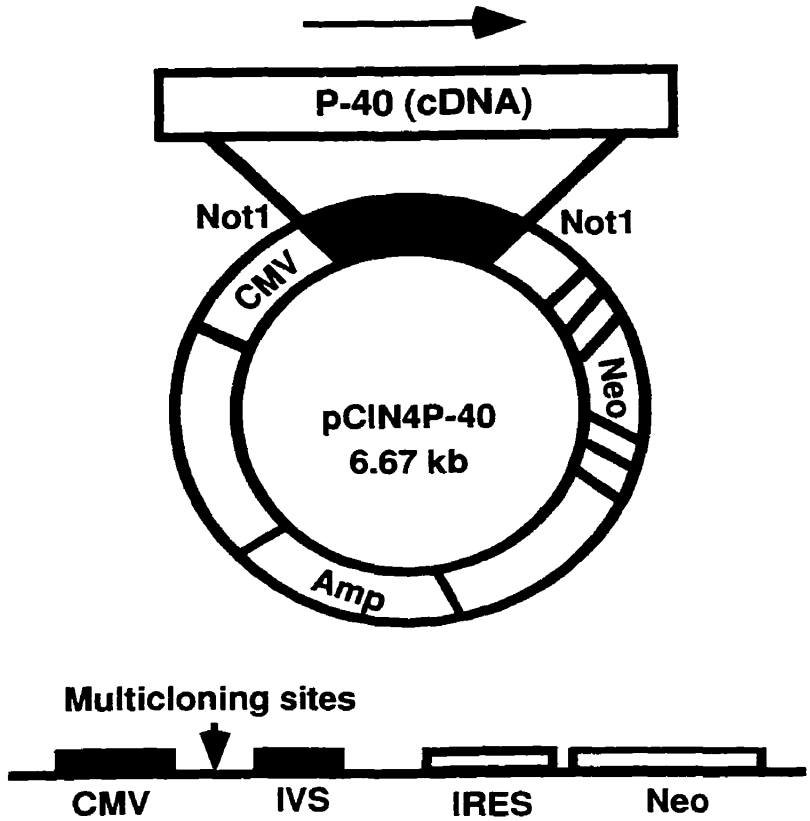


Figure 3. P-40 (or annexin I) construct pCIN4-P40. The full length P-40 (or annexin I) cDNA was cloned into the Not-1 site of eukaryotic expression vector pCIN4 in both sense and antisense orientations. This places the P-40 (or annexin I) adjacent to the viral CMV promoter, which directs its expression. The neomycin phosphotransferase gene is also present on the construct with its expression being driven by the same promoter element located ahead of multi-cloning site. (indicated in the map), thus providing G418 resistance to cells containing a construct. Please notice that, to facilitate translation of the second open reading frame (neomycin phosphotransferase), the encyphalomyocarditis virus internal ribosome entry sites has been inserted into the expression cassette immediately before the start codon of neomycin phosphotransferase nucleotide sequence. Intervening sequence (IVS) accompanies the transcription complex (P-40 cDNA and neomycin phosphotransferse gene) for maximal expression of mature transcripts.

annexin I) was cloned into pCIN4 eukaryotic expression vector (Figure 3) and transfected into MCF-7 drug sensitive cells. Stable transfectants of P-40 (or annexin I) MCF-7 cells (P-40-MCF-7) were selected in the presence of lethal concentration of G418. Figure 4a shows a Western blot analysis of lysates from P-40-MCF-7 cells probed with the IPM96 monoclonal antibody. The results of the latter Western blot shows P-40-MCF-7 cells to express 1/3 less P-40 than the MDR *in vitro* selected MCF-7/Adr cells. However, the distribution pattern of P-40 (or annexin I) in P-40-MCF-7 transfectants is similar to that in MCF-7/Adr cells (Figure 4b). As expected , no detectable levels of P-40 was observed in cells transfected with vector alone.

Cross resistance patterns of P-40 MCF-7 transfectants. Having established the expression of P-40 (or annexin I) in P-40-MCF-7 cells, it was of interested to known the effect of P-40 (or annexin I) on the sensitivity of MCF-7 cells to anticancer drugs. Figure 5 shows the results of the chemosensitivity assays using P-40-MCF-7 cells in the presence of increasing concentration of anticancer drugs to that of MCF-7 cells transfected with vector alone. Surprisingly, P-40-MCF-7 cells displayed low level of resistance to adriamycin, actinomycin D, Taxol and cisplatin (Figure 6) relative to the cells transfected with vector alone. However, P-40-MCF-7 cells did not show cross-resistance to colchicine and vincristine (data not shown) These results suggest that the overexpression of P-40 (or annexin I) does confer low level drug resistance to a variety of anticancer drugs.

Post-translational modification of P-40 (or annexin I) in resistant cells. Annexin I is a phosphoprotein phosphorylated at serine and tyrosine amino acids (Wang and Cretuz, 1994; Varticovski et al, 1988). Furthermore, it has been suggested that phosphorylation of annexin I at its N-terminal domain decreases its affinity for negatively charged phospholipids and its ability to cause the aggregation of synthetic lipid vesicles (Wang and Cretuz, 1994). Given the above results, concerning the role of P-40 in drug resistance, it was of interest to examine the post-translational modification of P-40 (or annexin 1) in MDR cells. The results in figure 6 show the immunoprecipitation of P-40 (or annexin I) with IPM96 monoclonal antibody or an irrelevant IgG_{2b}

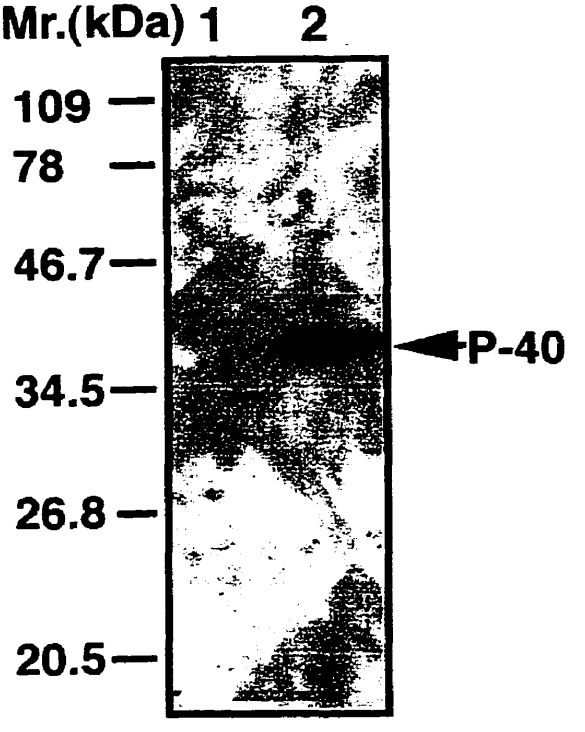
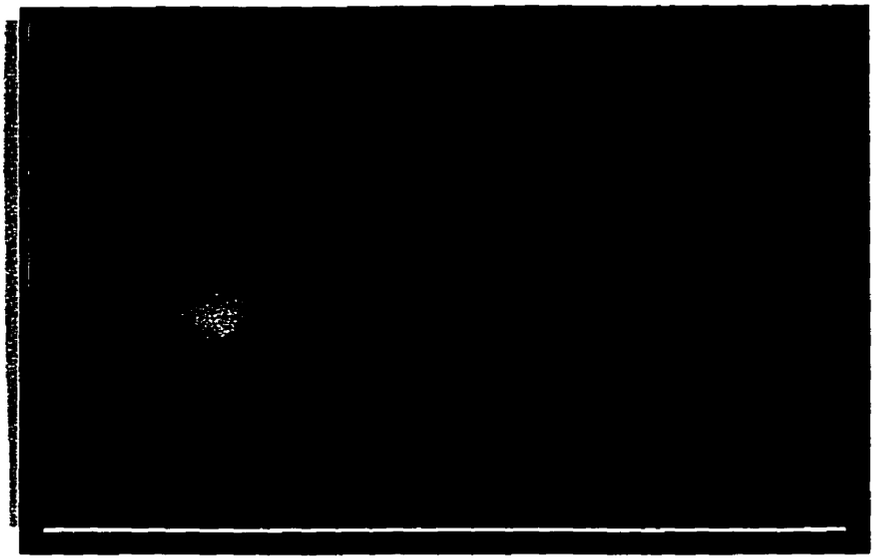
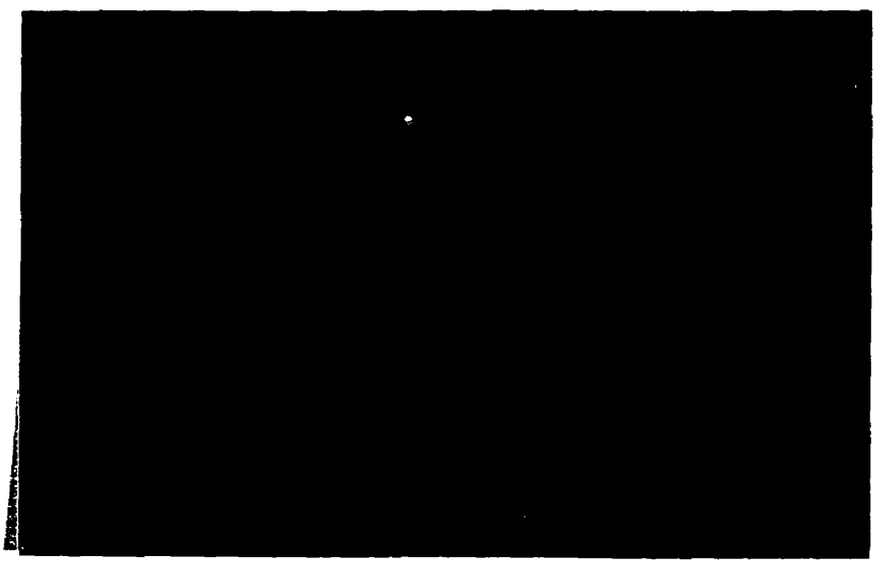


Figure 4a. Expression of P-40 (or annexin I) in MCF-7 stable transfectants. The expression level of P-40 (annexin I) in MCF-7 stable transfectants was determined by Western blot and indirect immunfluroresence. Figure 5a shows equal number of cells from MCF-7 transfected with vector alone (lane 1) or vector plus P-40 cDNA (P-40-MCF-7)(lane 2) were lysed and the total lysates were separated by 10% SDS PAGE and blotted onto PVDF membrane. The blot was probed with IPM96 monoclonal antibody. Figure 5b shows immunohistochemical staining of MCF-7 cells transfected with vector alone or vector plus P-40 cDNA. The expression of P-40 (or annexin I) in stable transfectants (P-40-MCF-7) was determined using the cytospins probed with IPM96 mAb and FITC conjugated goat anti-mouse secondary antibody as described in Materials and Methods.



MCF-7/Adr
(800X)



Vector-MCF-7
(600X)

P-40-MCF-7
(400X)

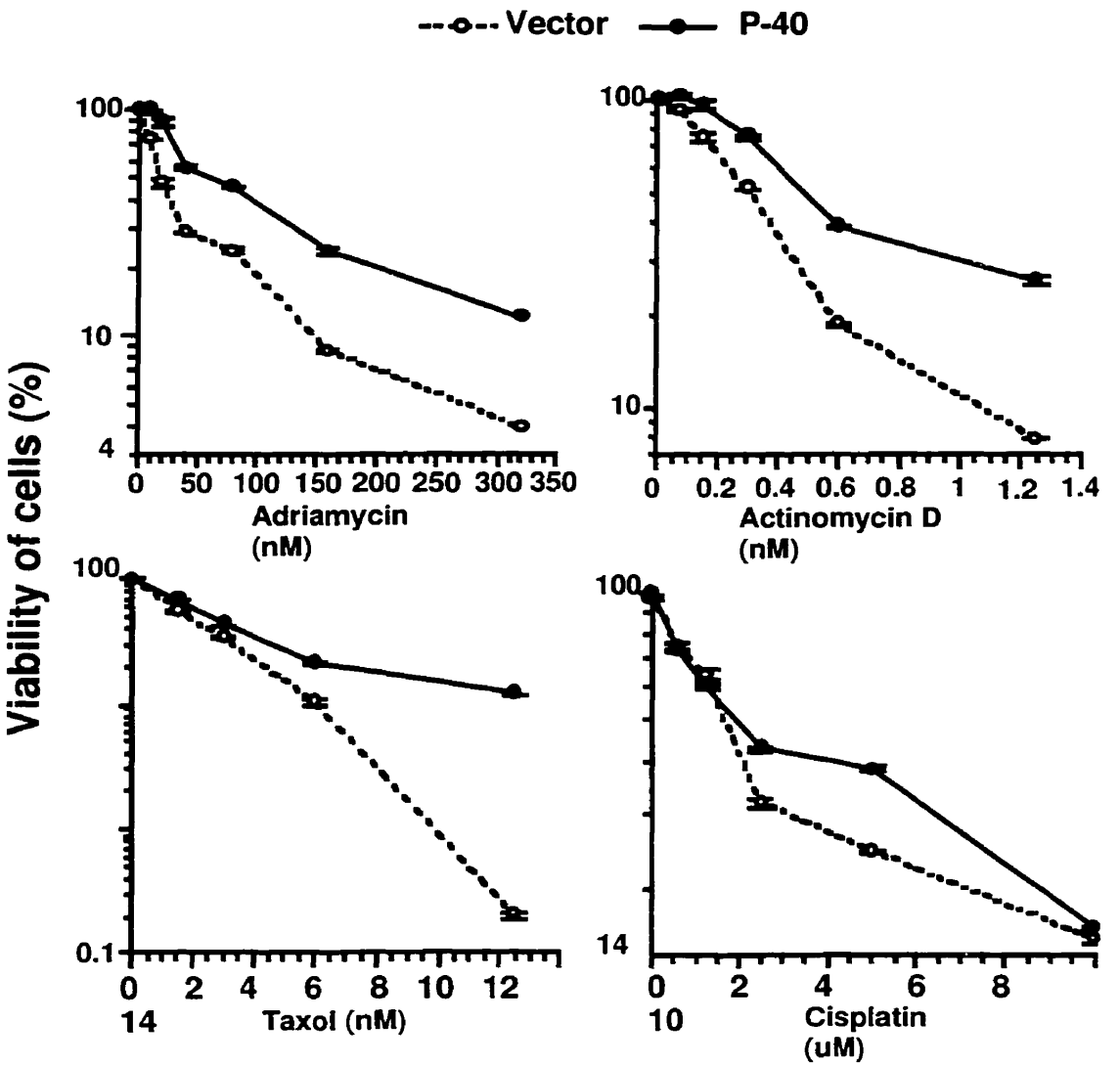


Figure 5. Effects of anticancer drugs on MCF-7 cells expressing P-40 (or annexin I). MCF-cells transfected with P-40cDNA or vector only were incubated in the absence and presence of increasing concentration of adriamycin, actinomycin D, Taxol and cisplatin. The sensitivity of cells to drugs was measured by the tetrazolium salt based assay as described in Materials and Methods. Experiments were performed in triplicates.

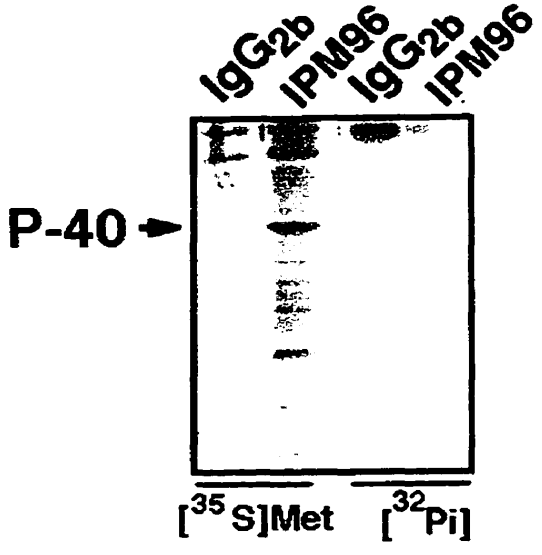


Figure 6. Post translational modification of P-40 (or annexin I) in MCF-7/Adr cells. Cells were metabolically labelled with [^{35}S]-methionine or ^{32}P-inorganic phosphate and the radiolabelled proteins were immunoprecipitated with an irrelevant IgG $_{2b}$ or IPM96 monoclonal antibody.

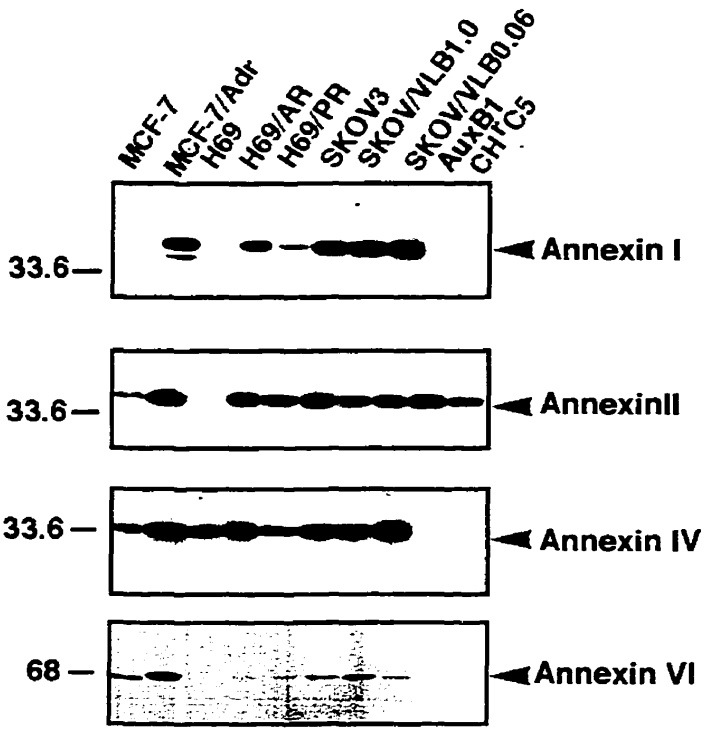


Figure 7. Expression of Annexin I, II, IV, and VI in drug sensitive and drug resistant cells. Total cell lyzates from drug sensitive (MCF-7, H69, SKOV3 and AuxB1) and resistant (MCF-7/Adr, H69/AR, SKOV/VLB$^{1.0}$ and ChrC5) or revertant (H69/PR) are resolved on SDS-PAGE and transferred to nitrocellulose membrane. The nitrocellulose membranes were probed with anti–annexin I, II, IV and VI monoclonal antibodies.

from MCF-7/Adr cells that have been metabolically labelled with [^{35}S]-methionine (lane 1 and lane 2) or [^{32}P] inorganic phosphate (lane 3 and lane 4). Interestingly, P-40 (or annexin I) is not phosphorylated in MCF-7/adr cells. Similarly, basal level of P-40 phosphorylation was not detected in other MDR cell lines (data not shown).

Overexpression of annexins in MDR cell lines. To determine if other members of the annexin family are similarly overexpressed in MDR cells relative to the parental drug sensitive cells, total cell lysates from drug sensitive (MCF-7, H69 and SKOV3) and resistant (MCF-7/Adr, H69/AR and SKOV3/VLB$^{1.0}$) cells were analysed by Western blotting with anti-annexin I, II, IV and VI monoclonal antibodies. The results (Figure 7) show a significant increase in the expression of annexin II and IV in the above MDR cell lines relative the parental drug sensitive cell lines. However, unlike annexin I, both annexin II and IV are expressed, at lower levels, in drug sensitive cells (figure 8). Of considerable interest is the levels of annexin I , II, and IV in a revertant cell line (H69/PR) derived from H69/AR cells that are less resistant to adriamycin (Figure 7).

DISCUSSION

In this study, we have used the monoclonal antibody IPM96, previously shown to bind to a 40 kDa protein in MDR cells, to screen a λ-gt 11 expression library. Having cloned the gene that encodes for P-40, we have determined that P-40 is identical to annexin I, a known substrate of epidermal growth factor receptor (Wallner et al, 1986). In addition, northern blot analysis using total RNA from drug sensitive and resistant cells confirmed the overexpression of P-40 (or annexin I) in MDR cells relative to their parental drug sensitive cells. The identity of P-40 as annexin I is consistent with the molecular masses of P-40 and annexin I. Furthermore, the cross-reactivity of IPM96 monoclonal antibody with P-40 *in vitro* expressed protein confirmed that P-40 is annexin I. Our earlier observations that P-40 was associated with the cell membrane and in the soluble fraction (Wang et al, 1997) is again consistent with the cellular distribution patterns of annexin I (Ranyl and Pollard, 1994). The fact that the extraction of membrane associated P-40 (or annexin I) was resistant to high salt and EDTA indicated that P-40 may be associated more tightly with membranes (Wang et al, 1997). Although we have demonstrated that hydrophobic forces may be responsible for the association of P-40 with the plasma membrane , the exact factors that govern this association remain unclear. Interestingly, a similar conclusion regarding annexin I resistance to extraction from the cell membrane was previously reported by others (Pollard and Rojas 1988; Rojas et al 1990). The latter possibility is likely given that annexins I, V, VI and VII are thought to possess transmembrane calcium channel activities. Also consistent with our assignment of P-40 as annexin I, is the 35 kDa proteolytic product which has been previously demonstrated to represent cleavage at the tail domain of annexin I (N-terminus) (Wang and Creutz 1994).

We have previously shown that P-40 (or annexin I) is highly expressed in several MDR cell lines relative to their parental drug sensitive cells (Wang et al, 1997). The MDR cell lines used in our study were previously shown to contain amplified copies of Pgp MDR-1 or MRP genes. The genes encoding Pgp, MRP and P-40 (or annexin I) are localized on chromosome 7, 16 and 9 respectively (Cole et al, 1993; Trent and Witkowski, 1987; Wallner et al, 1986). Therefore, the observed increase in P-40 protein is not due to a co-amplification of P-gp or MRP. In addition , our Slot blot results did not reveal the amplification of P-40 (or annexin I) gene in any of the MDR cell lines where P-gp

or MRP are amplified. Furthermore, P-40 (or annexin I) was detected in MDR cell lines that lacked detectable P-gp or MRP. The northern blot analysis of total RNA from drug sensitive and resistant cells revealed an increase in P-40 (or annexin I) mRNA levels in drug resistant cells. Thus, the observed increase in P-40 (or annexin I) protein level in resistant cells is transcriptionally regulated . Alternatively, the increased transcription or mRNA stabilization may govern the overexpression of P-40 in MDR cells. Of interest was the detectable increase in P-40 (or annexin I) mRNA in SKOV3 drug sensitive cells versus that in other drug sensitive cells. However, P-40 (or annexin I) mRNA in SKOV3 cells was four fold less than that in its drug resistant counterpart ($SKOV/VLB^{1.0}$). In particular, the northern blot results are consistent with the Western blot data, especially those relating to the protein and mRNA levels of P-40 or annexin I in SKOV3 compared with $SKOV/VLB^{1.0}$.

Annexin I is a member of a family of Ca^{2+}-dependent membrane binding proteins (Ranyl and Pollard, 1994). Annexins share a similar core domain with four to eight conserved repeats of 70 amino acids. The variability in the N-terminal domains of annexins is thought to be important in dictating the specific functions of annexins. Although the physiological functions of annexins are not clear, they have been implicated in Ca^{2+}-regulated exocytosis (Drust and Creutz, 1988; Creutz et al 1987). Annexin I has been shown to mediated the Ca^{2+}-dependent fusion of liposomes with isolated neutrophil plasma membrane (Meers et al, 192). In intact cells, annexins are generally phosphorylated in response to varieties of stimuli. Annexin I is phosphorylated by EGF receptor kinase at tyrosine residues found in N-terminus (Pepinsky et al, 1986) and by protein kinases C and A (Varticovski et al, 1988). Interestingly, phosphorylation of annexin I at the N-terminal domain by protein kinase C inhibits its ability to aggregate chromaffin granules and synthetic liposomes (Wang and Creutz, 1992). Our finding that annexin I is not phosphorylated in MDR cells is consistent with its increased capacity to cause aggregation of membrane vesicles.

The observed decrease in P-40 (or annexin I) expression in a revertant cell line(H69/PR) derived from H69/AR (Cole et al 1992) together with the increase in P-40 in $SKOV/VLB^{1.0}$ following *in vitro* selection from SKOV3 show a strong correlation between the overexpression of P-40 (or annexin I) and MDR . In this study, we show that the level of drug resistance of P-40 (or annexin

I) transfectants is 2 to 3 fold higher than that transfected with vector alone. It is likely that P-40 (or annexin I) confers lower level of drug resistance than P-gp or MRP (3 to 8 fold). However, unlike the high levels of drug resistance seen in *in vitro* drug selected MDR tumour cell lines, low level of drug resistance conferred by different cellular changes (such as P-40) are likely to be clinical relevant. Given the fact that P-40 is expressed at lower level in SKOV3 cells, which are clinically resistant to adriamycin and cisplatin, than that of SKOV/VLB$^{1.0}$ (1000 fold resistance to vinblastine), we speculate that the observed overexpression of P-40 (or annexin I) is probably an early event in the development of clinical drug resistance. Work is in progress in the investigation of P-40 in clinical tumour samples.

In this study, we show, for the first time, that P-40 (or annexin I) confers resistance to anticancer drugs. The results from the cytotoxicity assays indicate that the drug resistance profile of P-40 (or annexin I) transfectants is similar but not identical to P-gp or MRP transfectant cells. This apparent difference in the resistant spectrum may be important to the mechanism of P-40 (or annexin I) mediated drug resistance. The mechanism by which P-40 (annexin I) confers drug resistance to anticancer drugs is presently unknown. However, the fact that P-40 can not be labelled by photoactive analogues of cytotoxic agents (data not showed) and its amino acid sequence does not encode for an ATP binding domain suggested that P-40 (or annexin I) itself does not transport drugs directly to the extracellular environment. The intracellular distribution of P-40 in both *in vitro* selected P-40-expressing cells and in the transfectant cells suggests that the membrane localization of P-40 (annexin I) is essential for its function in drug resistance. Translocation of P-40 from the plasma membrane to cytoplasm after treatment with verapamil and EDTA has been observed in both P-40 expressing cell lines and P-40 cDNA transfectants (data not shown). This finding led us to speculate that P-40 may participate in sequestering drugs from their targets. Alternatively, the intracellular cation concentration (eg. Ca^{2+}) may be important for the functions of P-40 (or annexin I) in drug resistance. Work is in progress to determine if P-40 induced drug resistance can be reversed by MDR modulators. Given the role of annexins in promoting aggregation of membrane vesicles through Ca^{2+} dependent phospholipid binding, we speculate that P-40 (annexin I) confers a drug resistant phenotype by aggregation of drug filled membrane vesicles or exocytosis of such drug

filed vesicles. In support with this speculation is the observed increase in membrane voculisation in many MDR cell lines (Abbaszadegan et al, 1997; Slapak et al, 1992; Klohs et al, 1988). Furthermore, P-glycoprotein and MRP have been detected in the endosomal membranes in *in vitro* selected cell lines. Thus, P-40 (or annexin I) or other members of the annexin family could function together with P-glycoprotein or MRP to cause the aggregation and possibly exocytosis of drug filled vesicles.

In conclusion, Our studies have provided convincing evidence that annexin I is important in the development of MDR phenotype in cancer cells. The findings of this study are also important to our understanding of the functions of annexin family. Analysis of clinical tumour samples for P-40 (or annexin I) expression (primary or relapsed after chemotherapy) will provide further evidence for the diagnostic role of P-40 in clinical drug resistance.

REFERENCES

Abe, Y., Ohnishi, Y., Yoshimura, M., Ota, E., Ozeki, Y., Oshika, Y., Tokunaga, T., Yamazaki H., Ueyema, Y., Ogata, T., Tamaoki, N.,and Nakamura, M. P-glycoprotein-mediated acquired multidrug resistance of human lung cancer cells in vivo.British Journal of Cancer. 74(12):1929-34, 1996

Baggetto, L.G. Non P-glycoprotein novel proteins involved in human cancer multidrug resistance. [Review] Bulletin du Cancer. 84(4):385-90, 1997

Beck, W.T. The cell biology of multidrug resistance. 36 (18):2879-2887

Belloni, P.N., and Tressler, R.J. Microvascular endothelial cell heterogeneity: interactions with leukocytes and tumor cells. Cancer & Metastasis Reviews. 8(4):353-89, 1990

Bradley, G., Juranka, P.F., and Ling, V. Mechanism of multidrug resistance. Biochemica et Biophysica Acta. 948:87-128, 1988

Bradley, G. and Georges, E., Ling, V. Sex-dependent and independent expression of the P-glycoprotein isoforms in Chinese hamster. Journal of Cellular Physiology. 145(3):398-408, 1990

Chan, H.S., Haddad, G., Thorner, P.S., DeBoer, G., Lin, Y.P., Ondrusek, N., Yeger, H., and Ling V. P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma .New England Journal of Medicine. 325(23):1608-14, 1991

Chan, H.S., DeBoer G., Haddad ,G.,and Ling V. Multidrug drug resistance in pediatric sarcomas. Hematology - Oncology Clinics of North America. 9(4):889-908, 1995

Charpin, C., Vielh, P., Duffaud, F., Devictor, B., Andrac, L., Lavaut, M.N., Allasia, C., Horschowski, N., and Piana, L. Quantitative immunocytochemical assays of P-glycoprotein in breast carcinomas: correlation to messenger RNA expression and to immunohistochemical prognostic indicators. Journal of the National Cancer Institute. 86(20):1539-45, 1994

Cole, S.P., and Deeley, R.G. Multidrug resistance associated with overexpression of MRP. Cancer Treatment and Research. 87:39-62, 1996

Cordon-Cardo, C., O'Brien, J.P., Boccia, J., Casals, D., Bertino ,J.R., and Melamed, M.R Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. Journal of Histochemistry & Cytochemistry. 38(9):1277-87, 1990

Doige and Sharom ,F.J. Transport properties of P-glycoprotein in plasma membrane vesicles from multidrug resistant chinese hamster ovary cells. Biochimica et Biophysica Acta. 1109:161-171, 1992

Drust, D.S., and Creutz, C.E.,Aggregation of chromaffin granules by calpactin at micromolar levels of calcium. Nature. 331(6151):88-91, 1988

Fogh, J.and Trempe, G. Human Tomour Cells In Vitro. Plenum Press, New, York, 155-159, 1975

Fojo, A.T., Ueda, K., Slamon, D.J., Poplack, D.G., Gottesman, M,M., and Pastan, I. Expression of a multidrug-resistance gene in human tumors and tissues. Proceedings of the National Academy of Sciences of the United States of America. 84(1):265-9, 1987

Fredericks ,W.J., Chen ,Y.F., and Baker, R.M. Immunoblot detection of P-glycoprotein in human tumors and cell lines. Cancer Treatment & Research. 57:121-49, 1991.

Frelich, F.J , and Osheroff, N. Topoisomerase poisons: Harnessing th dark side of enxyme mechanism. Journal of biological chemistry. 270:21429-21432, 1995

Gottesman, M.M, and Pastan, I. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annual Review of Biochemistry. 62:385-427, 1993

Henson, J.W., Cordon-Cardo, C., and Posner, J.B. P-glycoprotein expression in brain tumors. Journal of Neuro-Oncology. 14(1):37-43, 1992

Higgins, C.F. ABC transporters: From microorganisms to man. Annual Review of Cell Biology. 8:67-113, 1992

Hijazi, Y.M., Axiotis, C.A., Navarro, S., Steinberg, S.M., Horowitz, M.E., Tsokos M.Immunohistochemical detection of P-glycoprotein in Ewing's sarcoma and peripheral primitive neuroectodermal tumors before and after chemotherapy American Journal of Clinical Pathology. 102(1):61-7, 1994

Lee, J.S. Scala, S., Matsumoto, Y., Dickstein, B., Robey, R., Zhan, Z., Altenberg, G., and Bates SE. Reduced drug accumulation and multidrug resistance in human breast cancer cells without associated P-glycoprotein or MRP overexpression. Journal of Cellular Biochemistry. 65(4):513-26, 1997

Linn, S.C., van Kalken, C.K., van Tellingen, O., van der Valk, P.. van Groeningen, C.J., Kuiper C.M., and Pinedo HM. Giaccone G.Clinical and pharmacologic study of multidrug resistance reversal with vinblastine and bepridil.Journal of Clinical Oncology. 12(4):812-9, 1994

Lonn, U., Lonn, S., Nilsson, B., and Stenkvist, B.Intratumoral heterogeneity for amplified genes in human breast carcinoma. International Journal of Cancer. 58(1):40-5, 1994

Loe, DW. , Deeley, R.G., and Cole SP. Biology of the multidrug resistance-associated protein,

MRP. [Review] European Journal of Cancer. 32A(6):945-57, 1996

Lowe, S.W., Bodis, S., McClatchey, A., Remington, L., Ruley ,H.E., Fisher, D.E., Housman, D.E,. and Jacks, T. p53 status and the efficacy of cancer therapy in vivo. Science. 266(5186):807-10, 1994

Lowe, S.W., Ruley, H.E., Jacks, T., and Housman, D.E. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents.Cell. 74(6):957-67, 1993

Mattern, J. and Volm ,M. Detection of resistance proteins in matched primary lung tumors and lymph |node metastases. Anticancer Research. 14(2A):417-9, 1994

Meers ,P., Mealy, T., Pavlotsky, N., and Tauber, A,I. Annexin I-mediated vesicular aggregation: mechanism and role in human neutrophils [published erratum appears in Biochemistry 1993 Biochemistry. 31(28):6372-82, 1992

Nooter, K.. and Sonneveld P.Clinical relevance of P-glycoprotein expression in haematological malignancies. [Review] Leukemia Research. 18(4):233-43, 1994

Pastan, I.and Gottesman, M.M. Multiple-drug resistance in human cancer. New England Journal of Medicine. 316(22):1388-1392, 1987

Pepinsky, R.B., and Sinclair, L.K. Epidermal growth factor-dependent phosphorylation of lipocortin. Nature. 321(6065):81-4, 1986

Pouliot, J.F., L'Heureux, F., Liu, Z.,Prichard, R.K., and Georges E. Reversal of P-glycoprotein-associated multidrug resistance by ivermectin. Biochemical Pharmacology. 53(1):17-25, 1997

Rees, S., Stables, J.C., Harris, S.H.,and Lee. M.G., Restronic Vector for teh creation of stable mamalian cell lines that predisposes all antibiotic resistant cells to express recomninant protein. BioTechniques 20:102-110,1996

Raynal, P., and Pollard, H.B.Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. Biochimica et Biophysica Acta. 1197(1):63-93, 1994

Sambrook ,J., Fritsh, E.H., and Maniatis, I.Molecular cloning: A laboratory Manual. Cold Spring Habarber Laboratory Press, Cold Spring Harbor, N.Y. 1989

Scheffer, G.L., Wijngaard, P.L., Flens, M.J., Izquierdo, M.A. Slovak, M.L., Pinedo ,H.M., and Meijer CJ. Clevers HC. Scheper RJ. The drug resistance-related protein LRP is the human major

vault protein Nature Medicine. 1(6):578-82, 1995

Schinkel, A.H., Mayer ,U., Wagenaar, E., Mol, C.A., van Deemter, L., Smit, J.J. van der Valk MA. Voordouw, A.C., Spits H., van Tellingen, O., Zijlmans, J.M., Fibbe, W,E., and Borst, P. Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. Proceedings of the National Academy of Sciences of the United States of America. 94(8):4028-33, 1997

Schinkel, A.H., Smit, J.J., van Tellingen. O., Beijnen, J.H., Wagenaar E. van Deemter L. Mol CA. van der Valk MA. Robanus-Maandag EC. and te Riele, H.P. et al. Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. Cell. 77(4):491-502, 1994

Sehested, M., Skovsgaard, T,. van Deurs, S.B., and Winther-Nielsen, H. Increased plasma membrane traffic in daunorubicin resistant P388 leukaemic cells. Effect of daunorubicin and verapamil. British Journal of Cancer. 56:747-751, 1987

Shapiro, A.B., and Ling, V. ATPase activity of purified and reconstituted P-glycoprotein from Chinese hamster ovary cells. Journal of Biological Chemistry. 269:3745-3754, 1994

Sognier, M.A., Zhang, Y., Eberle, R.L., Sweet, K.M., Altenberg, G,A.. and Belli JA.Sequestration of doxorubicin in vesicles in a multidrug-resistant cell line (LZ-100). Biochemical Pharmacology. 48(2):391-401, 1994

Tew, D.T. Glutathione-associated enzymes in anticancerdrug resistance. Cancer Research. 54:4313-4320, 1994

Thorgeirsson, S.S., Huber, B.E., Sorrell, S.. Fojo, A., Pastan, I., and Gottesman, M.M. Expression of the multidrug-resistant gene in hepatocarcinogenesis and regenerating rat liver. Science. 236(4805):1120-2, 1987

Tishler, D.M., Weinberg, K.I,. Sender, L.S., Nolta, J.A., and Raffel, C. Multidrug resistance gene expression in pediatric primitive neuroectodermal tumors of the central nervous system. Journal of Neurosurgery. 76(3):507-12, 1992

Varticovski, L., Chahwala, S.B., Whitman, M., Cantley, L., Schindler, D., Chow, E.P., Sinclair L.K.,.and Pepinsky, R.B. Location of sites in human lipocortin I that are phosphorylated by protein tyrosine kinases and protein kinases A and C. Biochemistry. 27(10):3682-90, 1988

Wallner, B.P., Mattaliano, R.J., Hession, C., Cate, R.L., Tizard, R., Sinclair, L., Foeller, C., Chow, E.P., Browing, J.L., and Ramachandran, K.L. et al. Cloning and expression of human lipocortin, a phospholipase A2 inhibitor with potential anti-inflammatory activity.Nature.

320(6057):77-81, 1986

Wang ,Y., Pan, X.Q. , Lheureux, F., and Georges, E. Overexpression of a 40-kDa protein in human multidrug resistant cells. Biochemical & Biophysical Research Communications. 236(2):483-8, 1997

Wang, W., and Creutz, C.E. Role of the amino-terminal domain in regulating interactions of annexin I with membranes: effects of amino-terminal truncation and mutagenesis of the phosphorylation sites. Biochemistry. 33(1):275-82, 1994

Wang, W., and Creutz, C.E.Regulation of the chromaffin granule aggregating activity of annexin I by phosphorylation. Biochemistry. 31(41):9934-9, 1992

CHAPTER V.

GENERAL DISCUSSION

The use of appropriate combination of anticancer drugs in chemotherapy has led to major improvements in the treatment of malignant tumours such as Hodgkin's disease and childhood leukaemia (Rack, et al 1996; DeVita and Hubbard, 1993). However, resistance to cytotoxic drugs in solid tumours , which are responsible for the 90% cancer related death, occurs frequently and remains a principal obstacle in the chemotherapeutic treatment of cancer patients. The search for mechanisms of resistance of cancers to chemotherapy has led to the elucidation of many cell-based genetic alterations including the reduced accumulation of drugs (Gerlach et al, 1986; Ling et al, 1983), altered drug metabolism (Batist et al, 1986; Vickers et al, 1989; Tew 1994), inhibition of apoptosis in response to cytotoxic drugs (Loew et al, 1993; Nunez et al, 1990; Conner et al, 1997) , changes to drug targets (Takano et al, 1991; Kavallaris, et al 1997) and enhanced repair of drug induced damage (Estaman 1991). By using tumour cell lines selected *in vitro* with anticancer drugs, two major mechanisms have been identified from multidrug resistant (MDR) cells. They are the P-glycoprotein (P-gp) and multidrug resistant protein (MRP). P-gp and MRP are multi-functional membrane transporters with broad substrate specificity ranging from ions to large peptides (Higgins, 1992). The function of P-gp as energy dependent efflux pump to reduce drug accumulation in their expressing cell lines has been well characterized at the biochemical, pharmacological and molecular levels. Direct evidence for the involvement of P-gp and MRP in mediating drug resistance to a number of anticancer drugs have been conclusively demonstrated with the single gene transfer studies (Gros et al, 1986; Ueda et al, 1987; Grant et al, 1994).

The abundant expression of P-gp and its transcripts have been observed in more than 50% of malignant tumours including intrinsically resistant cancers and tumours that have relapsed during or after chemotherapeutic treatment (Bergman et al, 1996; Goldstein et al, 1989; Weinstein et al, 1991). The overexpression of P-gp has been potentially linked with the failure of clinical chemotherapy and poor survival. Although P-gp functions as a drug efflux pump to reduce drug accumulation in MDR cells, little is known about the precise drug binding domain and how its ATPase activity is coupled to drug binding and transport. Studies from different research groups have attempted to correlate changes in the P-gp ATPase activity and its drug binding and transport

but the results are controversial. In particular, some of well-known P-gp substrates (eg, colchicine) do not stimulate P-gp ATPase activity (Shapiro and Ling 1994; Doige et al; 1992; Ambudkar et al, 1992). To further understand the correlation between P-gp ATPase activity and drug binding , we pursued the study using chemical modification of P-gp with N-ethylmaleimide (NEM) (chapter one).

NEM is a V-type ATPase inhibitor that exerts its inhibition of ATPase activity by modifying Cysteine residues in the ATP binding domain (Feng and Forgac 1994). Furthermore, NEM was shown to modify a single Cysteine residue in a recombinant N-terminal ATP binding domain of P-gp and to inhibit its ATPase activity (Dayan et al, 1996). To understand the effects of NEM on P-gp drug binding and transport in MDR cells, we first examined the changes in P-gp-drug interactions using a photoactive and radioactive drug (iodoarylaziodiprasozin, IAAP). In that study, we showed that treatment of MDR cells with 1 to 50 μM of NEM led to a concentration dependent increase in P-gp photoaffinity labelling with IAAP and increased [^{3}H]-vinblastine accumulation in drug resistant cells but not in drug sensitive cells. These results suggests that NEM treatment of MDR cells increased P-gp binding to IAAP likely by inhibiting its ATPase activity. By inactivating P-gp ATPase activity, IAAP accumulates in the plasma membrane and saturates P-gp drug binding sites. Alternatively, the increase in P-gp photoaffinity labelling may be due to changes in IAAP binding sites. To verify this possibility, IAAP photolabelled P-gp from MDR cells with or without NEM treatment were digested with *Staphylococcus aurous* V8 and the resultant photoaffinity labelled peptides were resolved by SDS-PAGE. Comparison of the pattern of the peptides from this study did not reveal differences between P-gp isolated from cells that were incubated with or without NEM. Thus, the observed increase in the drug binding in the presence of NEM is not due to the photolabelling of other sites in P-gp, but rather due to increased labelling of the same domains.

P-gp is shown to undergo phosphorylation at several Serine residues in the linker domain. Protein kinase C (PKC) catalysed P-gp phosphorylation is thought to affect the function of P-gp MDR function. Given that NEM causes a large increase in P-gp phosphorylation in MDR cells (Marsh and Center, 1985), it was of interests to determine if the increase in IAAP binding following NEM treatment of MDR cells is due to changes in P-gp phosphorylation states. Very interestingly, our

finding showed that NEM increased P-gp phosphorylation by inhibiting the turnover of phosphate. To determine if a higher phosphorylation state of P-gp is responsible for the increase in IAAP binding to P-gp in MDR cells, we investigated the drug binding capacity of P-gp in the presence of calyculin A (a phosphatase inhibitor). Our results showed that inhibition of P-gp phosphorylation with calyculin A did not show any changes in P-gp photoaffinity labelling in MDR cells. Taking together, our study indicates that inhibition of ATPase activity can contribute to the observed increased in drug binding in P-gp. Furthermore, inhibition of P-gp ATPase activity does not inhibit the capacity of P-gp to bind drugs.

The discovery of P-gp has revealed a fundamental mechanism by which cancer cells evade chemotherapy. Having demonstrated the role of P-gp in MDR *in vitro*, the validity of applying these findings to the clinical situations has been intensively investigated. A correlation between the overexpression of P-gp and failure of chemotherapy and poor survival has been established in hematopoietic tumours and childhood malignancies (Chan et al 1990). Unfortunately, a similar correlation between P-gp expression and clinical MDR has not been observed in other cancers. The recent finding that some tumour cell lines overexpress another membrane transporter (the multidrug resistance protein or MRP), suggested that other proteins or cellular alterations, alone or together with P-gp, could explain the MDR phenotype in some clinical tumours. This possibility is consistent with the finding that the levels of drug resistance in Pgp (or MRP) transfectants are much lower than those observed in *in vitro* selected cell lines that express similar levels of these protein (Cole et al, 1994; Ueda et al, 1987; Gros et al, 1986; Zaman et al, 1994). In an effort to identify these cellular changes, we have used an immuno-dot blot method to isolate a monoclonal antibody (IPM96) which recognized a 40 kDa protein (P-40) overexpressed in several MDR cells. In the second chapter of this thesis, we have shown that the overexpression of a 40 kDa protein correlates with MDR in several cell lines. The co-expression of P-40 with P-gp or MRP is unlikely to be the result of co-amplification, since the *mdr1* (P-gp) and *mrp1* (MRP) genes are mapped to chromosome 7 and 16, respectively. Furthermore, the overexpression of P-40 in a Taxol and a cisplatin selected MDR cell lines, in the absence of a detectable level of P-gp or MRP, supports the notion that the mode of action of P-40 in MDR is independent of P-gp or MRP. More importantly, overexpression of P-40

correlates with lower level of drug resistance, which is very similar to the clinical situation. Low levels of P-40 was detected in SKOV-3, a cell line that was previously shown to be moderately resistant to several cytotoxic drugs (eg, cisplatin and adriamycin; Fogh and Trempe, 1975). Moreover, selection of SKOV-3 cells for higher levels of drug resistance with Taxol or vinblastine led to increased levels of P-40. In agreement with the above speculation, several studies have now demonstrated the coexpression of P-gp and MRP in MDR cell lines and MRP expression in these cell lines precede that of P-gp (Brock et al 1995). Having a correlation between the overexpresion of P-40 and drug resistance, we predicted that P-40 may mediate a drug resistance phenotype independently of P-gp and MRP MDR mechanisms. However, given the fact that P-40 is co-expressed with P-gp or MRP in some MDR cell lines, it is likely that P-40 is a first line of defense that precedes P-gp or MRP expression. P-gp or MRP have been shown to mediate higher levels of drug resistance. Biochemical characterization of P-40 suggested that P-40 is a membrane associated protein . The fact that the extraction of membrane associated P-40 was resistant to high salt and EDTA indicates that the membrane portion of P-40 may be associated more tightly with the plasma membrane. Although we have demonstrated that hydrophobic forces may be responsible for the association of P-40 with plasma membrane, the nature of P-40 interaction with the plasma membrane in MDR cells is unknown. We speculate that the distribution pattern of P-40 in MDR cells may be important for its function. Furthermore, we have demonstrated that P-40 does not undergo major post-translational modifications (e.g., glycosylation or phosphorylation, unpublished data) in these MDR cells.

Although the expression pattern of P-40 in MDR cells indicated that it may be important in MDR, further molecular studies were required to demonstrate the role of P-40 in MDR. The molecular cloning of P-40 cDNA has unveiled that P-40 is annexin I, a substrate for epidermal growth factor receptor tyrosine kinase. The identity of the isolated cDNA of P-40 and annexin I was established based on (a) the similarity of the molecular mass of P-40 and annexin I; (b) the cross-reactivity of IPM96 monoclonal antibody with a 40 kDa protein following the *in vitro* expression annexin I cDNA and (c) the distribution pattern of P-40 and annexin I in mammalian cells.

Briefly, P-40 (or annexin I) is a member of the annexin family of structurally related Ca^{2+}-

dependent phospholipid binding proteins. The biochemical features of annexin I have been extensively studied. However, the exact biological role of this family of proteins in cellular metabolism is not known. *In vitro* studies have implicated annexin I in several functions that include intracellular vesicular trafficking (Creutz 1992), membrane fusion during exocytosis (Creutz et al, 1987; Drust and Creutz, 1988), mitogenic signal transduction (Hollenberg, et al 1988), inhibition of phospholipase A2 activity (Huang et al, 1986) and the transport of ions across the cell membrane (Rojas et al 1990). Annexin I has also been associated with cell growth, differentiation and tumourgenesis (Reinhard et al, 1993; Masaki et al, 1996). In our study, we show for the first time, a direct role of P-40 (or annexin I) in the expression of drug resistance in tumour cells. Having established a stable P-40 (or annexin I) transfectant cell line, we show that overexpression of P-40 (or annexin I) in drug sensitive cells (MCF-7) does confer low levels of drug resistance to several cytotoxic drugs. This observation supports our previous speculation based on the expression pattern of P-40 in SKOV3 (a clinical resistant cell line).

As mentioned in the third chapter of this thesis, the mode of action by which P-40 (or annexin I) leads to low level of drug resistance is not known. Furthermore, as it was not possible to photolabel P-40 with photoactive analogues of cytotoxic drugs, we predict that P-40- mediated MDR does not involve direct binding to cytotoxic drugs. However, given the role of P-40 (annexin I) in promoting the aggregation of membrane vesicles, it is likely that P-40 (or annexin I) confers drug resistance by promoting the aggregation of drug filed membrane vesicles or exocytosis of such drug filed vesicles. In support of this notion, increased membrane voculisation has been observed in many MDR cell lines (Beck,1987; Sehested et al, 1987). According to amino acid sequence analysis, annexin I can be phosphorylated by PKC. Our study suggests that basal level of phosphorylation of P-40 can not be detected in MDR cells. Several possibilities may explain the lack of P-40 phosphorylation in MDR cells. It has been previously reported that phosphorylation of annexin I by PKC inhibits its ability to aggregate membrane vesicles (Wang and Creutz, 1992). Thus, an unphosphorylated P-40 (or annexin I) in MDR cells may increase its capacity to aggregate membrane vesicles or increased exocytosis.

The mechanisms that are responsible for MDR have been studied for decades. The observation

from *in vitro* and clinical trials have provided strong evidence that clinical chemodrug resistance is complex. Cisplatin resistance is a very serious problem in the treatment of gynecologic malignancies, especially in the ovarian carcinoma. In addition to decreased drug accumulation(Gately et al, 1993; Zheng et al, 1995), enhanced DNA repair (Estaman 1991, Estaman and Schulte 1988) and enhanced inactivation of drugs (Ishikawa 1994, Godwin et al 1992), a very notable aspect in these tumours is the mutation of p53 that results in the loss ability of p53 to transactivate its target gene such as Bax to prevent transition of the cell cycle (Perego et al 1996), as well as increased level of Bcl-2 protein (Eliopulos et al 1995,). Again, in human AML HL60 resistant subline, HL60/TAX1000, overexpression of P-gp and Bcl-2/Bcl-xl were determined and co-overexpression of these proteins was thought to be responsible for the impaired paclitaxel accumulation and paclitaxel induced apoptosis (Huang et al 1997). Taxol is another powerful cytotoxic agent used in the treatment of ovarian cancer cells. Resistance to taxol and its synthetic analogs has been correlated with different mechanisms. For example, overexpression of different classes of β-tubulins and alteration of ratio of different classes of β-tubulin have been observed in experimentally selected Taxol resistant cell lines and in the ovarian cancer cells (Kavallaris et al, 1997). It has been proposed that altered expression of distinct isotypy β-tubulin could modify tubulin/microtubule dynamics or stability of microtubule in such a way that the action of taxol is diminished. In addition to the alteration of expression of β-tubulin isotypes, overexpression P-gp and Bcl-2 has also been associated with Taxol resistance in cancer cells (Horwitz sb et al 1993). Very recently, a transfectant that contains wide type p53 made by transferring a trans-dominate negative mutated p53 gene that had a specific mutations abrogating both wild type p53 trans-activation and repression functions was characterized. It was also observed that the latter p53 transfectant had a remarkable increase in P-gp expression. Functional assay showed a typical multidrug resistant fashion of P-gp whereby the transfectant was cross-resistant to vinblastine, vincristine, actinomycin D and VM-26. Furthermore, the drug sensitivity in this cell line was restored by addition of calcium channel blocker, reserpine (Thottassery et al, 1997). Given the observation from our study, it is logical to believe that MDR is often a multifactorial process and any single mechanism will not be enough to elucidate the MDR phenotype of tumour cells, in the clinical situations. Although the mechanisms described above are

fundamentally different, there is no doubt that they can act together to cause drug resistance in clinical cancer chemotherapy. Consideration of these mechanisms in the design of drug and treatment should allow for circumvention of MDR in clinical cancer chemotherapy.

REFERENCES

Ambudkar ,S.V., Lelong, I.H., Zhang, J.. Cardarelli, C.O., Gottsman ,M.M., and Pastan I. Partial purification and reconstitution of human multidrug resistant pump: Characterization of drug-stimulate ATP hydrolysis Proceedings of the National Academy of Sciences of the United States of America. 89:8472-8476, 1992

Batist, G., Behrens, B.C., Makuch, R,. Hamilton, T.C., Katki, A.G., Louie, K.G., Myers ,C.E., and Ozols RF. Serial determinations of glutathione levels and glutathione-related enzyme activities in human tumor cells *in vitro* . Biochemical Pharmacology. 35(13):2257-9, 1986
Beck WT. The cell biology of Multidrug Resistance. Biochemical Pharmacology. 36:2879-2887, 1987

Brock, I., Hipfner, D.R., Nielsen, B.S., Jensen, P.B., Deeley ,R.G., and Cole, S,P Sehested M. Sequential coexpression of the multidrug resistance genes MRP and mde 1 and their products in VP-16-selected H69 Small Cell Lung Cancer Cells. Cancer Research. 55:459-462, 1995

Chan, H.S., Thorner, P.S., Haddad ,G., and Ling V. Immunohistochemical detection of P-glycoprotein: prognostic correlation in soft tissue sarcoma of childhood. Journal of Clinical Oncology. 8(4):689-704, 1990

Connor, T.M., Knudson, C,M., Korsmeyer, S.J., and Lowe ,S.W. bax-deficiency promotes drug resistance and oncogenic transformation by attenuating p53-dependent apoptosis. Proceedings of the National Academy of Sciences of the United States of America. 94(6):2345-9, 1997

Creutz,C.E., Pazoles, C.J., Pollard, H.B., Identification and purification of an adrenal medullary protein (synexin) that causes calcium-dependent aggregation of isolated chromaffin granules. Journal of Biological Chemistry. 253(8):2858-66, 1978

Creutz, C.E. The annexins and exocytosis.Science. 258(5084):924-31, 1992

Doige, C.A., Yu. X., .and Sharom, F.J. ATPase activities of partially purified P-glycoprotein from multidrug resistant Chinese Hamster ovary cells. Biochemical and Bipphysical Acta. 1109:149-160, 1992

Dayan, G., Baubichon-Cortay, H., Jault, J.M., Cortay, J.C., Deleage, G.. Di , Pietro,and A. Recombinant N-terminal nucleotide-binding domain from mouse P-glycoprotein. Overexpression, purification, and role of cysteine 430.Journal of Biological Chemistry. 271(20):11652-8, 1996

Eastman, A., and Schulte, N. Enhanced DNA repair as a mechanism of resistance to cis-diamminedichloroplatinum(II). Biochemistry. 27(13):4730-4, 1988

Eastman ,A. Mechanisms of resistance to cisplatin.Cancer Treatment & Research. 57:233-49, 1991.

Eliopoulos, A.G., Kerr, D.J., Herod ,J., Hodgkins, L.. Krajewski ,S., Reed ,J.C., and Young, LS. The control of apoptosis and drug resistance in ovarian cancer: influence of p53 and Bcl-2. Oncogene. 11(7):1217-28, 1995

Drust, D.S., and Creutz, C.E. Aggregation of chromaffin granules by calpactin at micromolar levels of calcium. Nature. 331(6151):88-91, 1988

Feng, Y.,and Forgac, M. Inhibition of vacular H(+)-ATP ase by disufide bond formation between cysteine 254 and cysteine 532 in subunite A. Journal of Biological Chemistry. 269:13224-13230, 1994

Fogh, J., Trempe, G., and Plenum . In: Human Tumour cells In vitro. Plenum Press, New York, 155-159
:155-159,1975

Georges, E. , Sharom, F.J., and Ling, V. Multidrug resistance and chemosensitization: therapeutic implications for cancer chemotherapy. Advances in Pharmacology. 21:185-220, 1990.

Gerlach, J.H., Kartner, N., Bell, D.R., and Ling .V Multidrug resistance. Cancer Surveys. 5(1):25-46, 1986.

Godwin, A.K., Meister, A., O'Dwyer, P.J., Huang, C.S., Hamilton, T.C., and Anderson, M.E. High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. Proceedings of the National Academy of Sciences of the United States of America. 89(7):3070-4, 1992

Goldstein, L.J., Galski, H., Fojo, A., Willingham ,M., Lai, S.L., Gazdar, A., Pirker, R., Green, A., Crist, W., and Brodeur, G.M., et al. Expression of a multidrug resistance gene in human cancers. Journal of the National Cancer Institute. 81(2):116-24, 1989

Grant, C.E., Valdimarsson, G., Hipfner, D.R., Almquist, K,C., Cole, S.P., and Deeley, R.G. Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. Cancer Research. 54(2):357-61, 1994

Gros ,P.. Croop, J., and Housman D. Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. Cell. 47(3):371-80, 1986

Higgins, C.F.The ABC transporters: from mircoorganisms to man. [Review].Annual review of Cell Biology. 8:67-113, 1992

Huang, K.S., Wallner, B.P., Mattaliano ,R.J,. Tizard, R.. Burne, C.. Frey, A.. Hession, C. McGray P. Sinclair, L.K., and Chow, E.P. . Two human 35 kd inhibitors of phospholipase A2 are

related to substrates of pp60v-src and of the epidermal growth factor receptor/kinase. Cell. 46(2):191-9, 1986

Huang, Y., Ibrado, A.M., Reed ,J.C,. Bullock, G., Ray, S., Tang, C.,and Bhalla, K. Co-expression of several molecular mechanisms of multidrug resistance and their significance for paclitaxel cytotoxicity in human AML HL-60 cells. Leukemia. 11(2):253-7, 1997

Kavallaris, M., Kuo ,D.Y.S., Burkhart, C.A., Regl, D.L., Norris, M.D., Haber, M.. and Horwitz SB.Taxol-resistant epithelial ovarian tumors are associated with altered expression of specific beta-tubulin isotypes. Journal of Clinical Investigation. 100(5):1282-93, 1997

Ling, V., Kartner, N., Sudo, T., Siminovitch, L,. and Riordan, J.R, Multidrug-resistance phenotype in Chinese hamster ovary cells. Cancer Treatment Reports. 67(10):869-74, 1983

Lowe, S.W., Ruley, H.E,. Jacks, T., and Housman, D.E. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell. 74(6):957-67, 1993

Marsh, W., and Center, M,S. Evidence for the involvement of two distinct membrane proteins in

adriamycin resistance in Chinese hamster lung cells. Cancer Research. 45(12 Pt 1):6088-92, 1985

Masaki, T., Tokuda, M. , Ohnishi, M., Watanabe, S., Fujimura, T., Miyamoto, K., Itano ,T., Matsui, H., Arima, K., Shirai, M., Maeba, T., Sogawa, K., Konishi, R., Taniguchi, K., Hatanaka Y. Hatase, O., and Nishioka, M. Enhanced expression of the protein kinase substrate annexin in human hepatocellular carcinoma. Hepatology. 24(1):72-81, 1996

Moss, S.E (ed) Annexins. 1992, Portland Press Ltd., London

Nunez, G., London, L., Hockenbery, D., Alexander, M., McKearn, J.P., and Korsmeyer, S.J. Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. Journal of Immunology. 144(9):3602-10, 1990

Perego, P., Giarola, M., Righetti, S.C., Supino, R., Caserini, C., Delia, D., Pierotti, M.A. Miyashita, T., Reed, J.C. and Zunino F. Association between cisplatin resistance and mutation of p53 gene and reduced bax expression in ovarian carcinoma cell systems. Cancer Research. 56(3):556-62, 1996

Rojas, E., Pollard, H.B., Haigler, H.T., Parra, C.,Burns, A.L. Calcium-activated endonexin II forms calcium channels across acidic phospholipid bilayer membranes. Journal of Biological Chemistry. 265(34):21207-15, 1990

Shapiro, A.B., and Ling, V. Using purified P-glycoprotein to understand multidrug resistance.

[Review] Journal of Bioenergetics & Biomembranes. 27(1):7-13, 1995

Sehested, M., Skovsgaad, T., Van Deurs ,B.,and Winther-Nielsen H. Increased plasma membrane traffic in daunorubicin resistant P338 leukaemic cells. Effect of daunorubicin and verapmil. Brtish Journal of Cancer. 56:747-751, 1987

Takano, H., Kohno, K., Ono, M., Uchida, Y., and Kuwano, M. Increased phosphorylation of DNA topoisomerase II in etoposide-resistant mutants of human cancer KB cells. Cancer Research. 51(15):3951-7, 1991.

Tew, K.D. Glutathione-associated enzymes in anticancer drug resistance. Cancer Research. 54(16):4313-20, 1994

Thottassery, J., Zambetti, G., Arimori, K., Schuetz, E., and Schuetz, J. p53 regulates expression of the endogenous mdr gene. Procedings of 88th annunal meeting of American Association for Cancer Research. Abstract 1686, 1997

Ueda, K. , Pastan, I., and Gottesman, M.M. Isolation and sequence of the promoter region of the human multidrug-resistance (P-glycoprotein) gene.Journal of Biological Chemistry. 262(36):17432-6, 1987

Vickers, P.J., Dufresne, M.J., and Cowan, K.H. Relation between cytochrome P450IA1 expression and estrogen receptor content of human breast cancer cells. Molecular Endocrinology. 3(1):157-64, 1989

Wang, W., and Creutz, C.E. Role of the amino-terminal domain in regulating interactions of annexin I with membranes: effects of amino-terminal truncation and mutagenesis of the phosphorylation sites.Biochemistry. 33(1):275-82, 1994