P-GLYCOPROTEIN-MEDIATED MULTIDRUG RESISTANCE EXPRESSION AND FUNCTIONAL EFFLUX IN ACUTE MYELOGENOUS LEUKEMIA

KITTIPONG DHANUTHAI

A THESIS SUBMITTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(CLINICAL PATHOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2000

ISBN 974-663-922-6

Thesis

entitled

P-GLYCOPROTEIN-MEDIATED MULTIDRUG RESISTANCE EXPRESSION AND FUNCTIONAL EFFLUX IN ACUTE MYELOGENOUS LEUKEMIA

Kittiporg Dhanuthai
Mr.Kittiporg Dhanuthai
Candidate

Suntaree Apibal
Assoc.Prof.Suntaree Apibal, M.Sc.
Major-Advisor

Surapon W. Pai
Lect.Surapon Worapongpaiboon, M.D.
Co-advisor

Budsaba Rerkamnuaychoke
Assoc.Prof.Budsaba Rerkamnuaychoke, D.M.Sc.
Co-advisor

Liangchai Limlomwongse
Prof.Liangchai Limlomwongse
Ph.D.
Dean
Faculty of Graduate Studies

Malai Vorachit
Assoc.Prof.Malai Vorachit, D.Sc.
Chairman
Master of Science Programme
in Clinical Pathology
Faculty of Medicine
Ramathibodi Hospital

Thesis

entitled

P-GLYCOPROTEIN-MEDIATED MULTIDRUG RESISTANCE EXPRESSION AND FUNCTIONAL EFFLUX IN ACUTE MYELOGENOUS LEUKEMIA

Was submitted to the Faculty of Graduate Studies, Mahidol University

for the degree of Master of Science (Clinical Pathology)

on

April 24, 2000

Kittipong Dhanuthai
.....
Mr.Kittipong Dhanuthai
Candidate

Suntaree Apibal
.....
Assoc.Prof.Suntaree Apibal, M.Sc.
Chairman

Surapon W. Bk
.....
Lect.Surapon Worapongpaiboon, M.D.
Member

Kovit Pattanapanyasat
.....
Prof.Kovit Pattanapanyasat, Ph.D.
Member

Budsaba Rerkamnuaychoke
.....
Assoc.Prof.Budsaba Rerkamnuaychoke, D.M.Sc.
Member

.....
Prof.Liangchai Limlomwongse, Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

Prakit Vathesatogkit
.....
Prof.Prakit Vathesatogkit
M.D., A.B.I.M.,
Dean
Faculty of Medicine
Ramathibodi Hospital
Mahidol University

ACKNOWLEDGEMENT

This thesis could not be possibly completed without the assistance of many persons. I would like to express my sincere gratitude and deep appreciation to Assoc. Prof. Suntaree Apibal, my principal advisor, for her guidance, advice, supervision and encouragement. She was incredibly kind and supportive. I am equally grateful to Dr.Surapon Worapongpaiboon for his invaluable advice, supervision and dedication. He stood side by side with me in the experiment.

I am greatly in debt to Dr.Kovit Pattanapanyasat for teaching me both the theory and practice of flow cytometry used in this experiment. He also gave helpful guidance as well as morale support.

I am also grateful to Dr.Budsaba Rerkamnuaychoke and all members of the Human Genetics Division, Ramathibodi Hospital for providing the drug sensitive cell line, K562, the development of the drug resistant cell line, and the access to their laboratory facilities.

My appreciation is also extended to all members of the Division of Hematology, Division of Blood Bank, Division of Immunology, Ramathibodi Hospital for the kind provision of the specimen, the access to their laboratory facilities, and tremendous assistance.

Kittipong Dhanuthai

4036032 RACP/M: MAJOR: CLINICAL PATHOLOGY; M.Sc. (CLINICAL PATHOLOGY)
KEY WORDS : MULTIDRUG RESISTANCE/ P-GLYCOPROTEIN/ FUNCTIONAL EFFLUX ACTIVITY/ ACUTE MYELOGENOUS LEUKEMIA
KITTIPONG DHANUTHAI: P-GLYCOPROTEIN-MEDIATED MULTIDRUG RESISTANCE EXPRESSION AND FUNCTIONAL EFFLUX IN ACUTE MYELOGENOUS LEUKEMIA. THESIS ADVISORS; SUNTAREE APIBAL, M.Sc. (CLINICAL PATHOLOGY), SURAPON WORAPONGPAIBOON, M.D., BUDSABA RERKAMNUAYCHOKE, D.M.Sc.
88 P. ISBN 974-663-922-6

Chemotherapy is the preferred treatment for patients with acute myelogenous leukemia. The major obstacle for the successful chemotherapeutic treatment is the development of multidrug resistance. P-glycoprotein (P-gp) is believed to mediate the multidrug resistance by acting as an energy-dependent drug efflux pump. This study was designed to use flow cytometer to detect P-gp expression using antibody MRK16 with biotin-streptavidin signal amplification as well as functional efflux activity using rhodamine 123 as P-gp substrate and cyclosporin A as MDR modulator. The results showed that P-gp and functional efflux activity were both detected in 45.83% of cases. P-gp was strongly correlated with functional efflux activity ($p<0.001$), but not with age ($p=0.155$), gender ($p=0.682$), CD34 expression ($p=0.423$), WBC count ($p=0.146$) nor FAB subtype ($p=0.331$). Functional efflux activity was not correlated with age ($p=0.155$), gender ($p=0.682$), CD34 expression ($p=0.105$), WBC count ($p=0.481$) nor FAB subtype ($p=0.596$). The identification of P-gp and functional efflux activity has several applications. They can be used as a prognostic indicator and a guide for clinician in selecting the regimens incorporating the MDR modulators or the use of alternative chemotherapeutic agents which are not the substrate of the P-gp.

4036032 RACP/M: สาขาวิชา: พยาธิวิทยาคลินิก; วท.ม. (พยาธิวิทยาคลินิก)

กิตติพงษ์ ดนุไทย : การแสดงออกของการดื้อต่อยาหลายชนิดและการขับสารออกจากเซลล์เนื่องมาจากพี-ไกลโคโปรตีนในมะเร็งเม็ดโลหิตขาวแบบเฉียบพลันชนิดมัยอีลอยด์ (P-GLYCOPROTEIN-MEDIATED MULTIDRUG RESISTANCE EXPRESSION AND FUNCTIONAL EFFLUX IN ACUTE MYELOGENOUS LEUKEMIA) คณะกรรมการควบคุมวิทยานิพนธ์: สุนทรี อภิบาล, M.Sc. (CLINICAL PATHOLOGY), สุรพล วรพงศ์ไพบูลย์, M.D., บุษบา ฤกษ์อำนวยโชค, D.M.Sc. 88 หน้า. ISBN 974-663-922-6

เคมีบำบัดเป็นการรักษาที่นิยมสำหรับผู้ป่วยมะเร็งเม็ดโลหิตขาวแบบเฉียบพลันชนิดมัยอีลอยด์แต่อุปสรรคสำคัญสำหรับการรักษาด้วยเคมีบำบัดคือการดื้อต่อยาที่ใช้ในการรักษาหลายๆ ชนิด พี-ไกลโคโปรตีนเป็นสาเหตุสำคัญที่ทำให้เกิดการดื้อต่อยาหลายชนิดโดยทำหน้าที่ขับยาออกจากเซลล์โดยขบวนการที่ต้องอาศัยพลังงาน การศึกษานี้ใช้โฟลซัยโตมิเตอร์เพื่อตรวจการแสดงออกของพี-ไกลโคโปรตีน โดยใช้แอนติบอดี MRK 16 ร่วมกับการขยายสัญญาณโดยไบโอ-ตินและ สเตรปอวิดิน รวมทั้งการตรวจความสามารถในการขับยาออกจากเซลล์โดยใช้โรดามีน 123 เป็นซับสเตรทของพี-ไกลโคโปรตีนและซัยโคลสปอริน เอ เป็นตัวปรับเปลี่ยนการดื้อต่อยาหลายชนิด ผลการศึกษาพบว่าทั้งพี-ไกลโคโปรตีนและความสามารถในการขับยาออกจากเซลล์จะตรวจพบได้ 45.83% ของจำนวนคนไข้ทั้งหมด พี-ไกลโคโปรตีนมีความสัมพันธ์อย่างมากกับการขับยาออกจากเซลล์ ($P < 0.001$) แต่ไม่มีความสัมพันธ์กับ อายุ ($p=0.155$), เพศ ($p=0.682$), การแสดงออกของ CD34 ($p=0.423$), จำนวนของเม็ดเลือดขาว ($p=0.146$), หรือชนิดย่อยตาม FAB ($p=0.331$) การขับยาออกจากเซลล์ไม่มีความสัมพันธ์กับอายุ ($p=0.155$), เพศ ($p=0.682$), การแสดงออกของ CD34 ($p=0.105$), จำนวนของเม็ดเลือดขาว (0.481) หรือ ชนิดย่อยตาม FAB ($p=0.596$) การตรวจหาพี-ไกลโคโปรตีนและการขับยาออกจากเซลล์จะสามารถนำไปใช้ประโยชน์ได้หลายประการโดยอาจใช้เป็นดัชนีเพื่อพยากรณ์การดำเนินไปของโรค, เพื่อเป็นแนวทางให้แพทย์เลือกแผนการรักษาที่มีตัวปรับเปลี่ยนการดื้อต่อยาหลายชนิดหรือการเลือกใช้ยาที่ไม่ได้เป็นซับสเตรทของพี-ไกลโคโปรตีน

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	: Alanine
ABC	: ATP-binding cassette
ADM	: Adriamycin
AML	: Acute myeloid leukemia
ALL	: Acute lymphocytic leukemia
ALLN	: N-acetyl-leucyl-norleucinal
AMPPNP	: Adenyl-5'-yl imidodiphosphate
ANAE	: α-Naphthyl acetate esterase
ANLL	: Acute non-lymphocytic leukemia
Arg	: Arginine
Asn	: Asparagine
Asp	: Aspartic acid
ATP	: Adenosine triphosphate
BCEC	: Brain capillary endothelial cell
CD	: Cluster of differentiation
cDNA	: complementary deoxyribonucleic acid
CFTR	: Cystic fibrosis transmenbrane conductance regulator
CHAP	: 3-[(3-Cholamidopropyl)dimethylaminonio]-1-propanesulfonate

CLL : Chronic lymphocytic leukemia

CML : Chronic myelogenous leukemia

CsA : Cyclosporin A

D : Aspartic acid

Dau : Daunorubicin

DNA : Deoxyribonucleic acid

E : Glutamic acid

EDTA : Ethylene diamine tetraacetic acid

F : Phenylalanine

FAB : French-American-British

FITC : Fluorescein isothiocyanate

G : Glycine

Glu : Glutamine

GTP : Guanosine triphosphate

H : Hydrogen

^{3}H : Tritium

HA : hemagglutinin

I : Isoleucine

^{125}I : Radioactive iodine

IgG : Immunoglobulin G

K : Lysine

L : Leucine

μm : micrometer

M : Methionine

MDR : Multidrug resistance

ml : millilitre

N : Nitrogen

N : Asparagine

NBD : Nucleotide-binding domain

NHL : Non-Hodgkin lymphoma

NIH : National Institute of Health

nm : nanometer

P : Proline

PE : Phycoerythrin

P-gp : P-glycoprotein

PKC : Phosphokinase C

Q : Glutamine

R : Arginine

RNA : Ribonucleic acid

RT-PCR : Reverse transcriptase polymerase chain reaction

S : Serine

SD : Standard deviation

SDS : Sodium dodecyl sulphate

Ser : Serine

T : Threonine

Thr : Threonine

Tyr : Tyrosine

TM : Transmembrane

V : Valine

Val : Valine

vs. : Versus

WBC : White blood cell

Y : Tyrosine

CHAPTER I

INTRODUCTION

Background

Leukemia constitutes the major disease of the hematopietic system. It is a malignant tumor characterized by replacement of normal bone marrow elements with abnormal or neoplastic cells. These leukemic cells are frequently encountered in the peripheral blood and sometimes invade the reticuloendothelial tissue. Chemotherapy remains to be the main treatment of choice for leukemia (1,2,3,4). Most regimens for the treatment of leukemia trend to use as many active non-crossresistant agents as possible and to use them as early as possible, and as intensively as possible (5). Despite recent advances in cancer chemotherapy, not all patients achieve complete remission. In AML, 60-70% of patients with de novo AML initially achieve complete remission. Remission is sustained in less than 25% of patients and the remainders relapse and eventually die of the disease (6). In ALL, the complete remission rate is much higher than in AML such as 88% by Linker et al (7) or 91% by Kantarjian et al (8), so this study will focus on AML patients since these patients are more problematic in term of complete remission and the development of resistance to chemotherapeutic drugs. Resistance to cytotoxic drugs used in the treatment regimens is one of the major obstacles to successful treatment in hematologic malignancies. The drug resistance phenomenon can be divided into 2 categories; primary or inherent drug resistance and acquired drug resistance. In the primary drug resistance, the patient does not respond to the initial implementation of

the chemotherapy. In the acquired resistance, the patient shows initial response to chemotheropeutic agents, but the tumor subsequently recurs and the chemotherapy is no longer effective. Different mechanisms of drug resistance such as defective drug metabolism, increased drug inactivation, altered DNA repair and multidrug resistance have been identified (9,10), but the best characterized and well-known one is the so-called multidrug resistance (MDR) (11). This process is not specific for a single drug, but the tumor cells showing the MDR phenotype are resistant to a variety of structurally and functionally unrelated drugs (12). These drugs include naturally occurring antineoplastic drugs such as anthracycline, vinka alkaloids (vincristine, vinblastine), and epipodophylotoxins, which are commonly used to treat these hematologic malignancies (13,14).

In de novo AML, P-glycoprotein (P-gp) positivity were variously reported as 30% by Guerci et al by flow cytometry using monoclonal antibody MRK16 (15), 46% by Gruber et al by RNAse protection assay (16), 47% by Campos et al (17) and 55% by Leith et al by flow cytometry using monoclonal antibody MRK16, anti-CD33 and anti-CD34 (6), 56% by Wood et al by immunocytochemistry using antibody JSB-1 (18), 43% and 73% by Del Poeta et al using flow cytometry and monoclonal antibody C219 and monoclonal antibody JSB1 respectively (19).

In secondary AML, the P-gp was reported as 80% by Holmes et al using slot blot analysis (20). In relapsed AML, P-gp was reported as 46.67% by Gruber et al using quantitative RNA hybridization (16), 51.0% by Nussler et al using flow cytometry and monoclonal antibody C219 (21), 57% by Hart et al using immunocytochemistry and monoclonal antibody JSB-1 (22), 70% by Guerci et al using flow cytometry with monoclonal antibody MRK-16 (15) or from 61 to 100%

by Musto et al by immunocytochemistry (23).

P-gp expression was associated with an adverse clinical outcome in term of complete remission rate, remission duration and duration of overall survival (6,19,24,25,26). Complete remission rate was significantly lower in P-gp-positive than in P-gp-negative cases (32% Vs 81%) (17). Complete remission rate decreased as expression of MDR1 increased as evidenced by the study of Marie et al which showed that the complete remission rate among patients with low MDR1 expression was more than twofold higher than in those with increased MDR1 expression (27). The study of Del Poeta et al demonstrated that a higher rate of relapse among MDR-positive compared to MDR-negative patients could be observed when using both antibody C219 and antibody JSB1 (80% vs. 44% and 52% vs. 27% respectively) Furthermore, the survival rate and the disease-free survival were significantly shorter in patients with C219-positive and in JSB1-positive (19).

In Thailand, there are a large number of acute leukemia patients in both the children and in adult population. After receiving chemotherapeutic agents, some of the patients still do not respond to the chemotherapeutic agents. Clinical resistance has been attributed, to a large extent, to the emergence of drug resistance mediated by P-gp. Numerous compounds such as cyclosporin A and verapamil have been identified to be able to block the efflux activity mediated by MDR1 gene product and reverse cellular resistance to chemotherapeutic agents (28,29). This suggests that clinical drug resistance in human tumors overexpressing P-gp may be potentially overcome through the concomitant administration of MDR reversing agent along with chemotherapeutic drugs.

In Thailand, the studies of multidrug resistance in hematologic malignancies especially in leukemia are still in infancy stage. To the best of my knowledge, this study is the first of its kind in acute myelogenous leukemia. There has been little, if any, information about the incidence and other clinical significance such as the relationship between the P-gp expression and the effect on the complete remission, the relapse rate, the duration of disease-free period or the survival of the patients. This study may produce some of this information in the Thai population which may be used to improve the quality of the patient care, to compare to other studies conducted in the past or to be used as data base for future researches in this field.

OBJECTIVES

1. To determine the incidence of P-gp expression in AML patients.
2. To determine the functional efflux activity in AML patients.
3. To correlate P-gp expression and functional efflux activity in AML patients.

CHAPTER II

LITERATURE REVIEW

1. Structure

The P-gp encoded by MDR1 gene contains 1280 amino acids and consists of two approximately equal parts of about 605 amino acids which share considerable amino acid sequence homology with each other. Based on the hydropathy plots, each half of the protein can be subdivided into a short hydrophilic region at the amino terminus, a long hydrophobic region and a long relatively hydrophilic region near the carboxy terminus. These regions are referred to as the amino terminus (residues 1-48), hydrophobic region1 (residues 49-350), and hydrophilic region 1 (residues 351-637) in the amino terminal half, and as the linker (residues 638-708), hydrophobic region 2 (residues 709-993), and hydrophilic region 2 (residues 994-1280) in the carboxy terminal half. Each hydrophobic domain contains six transmembrane segments, whereas the hydrophilic domains include potential nucleotide-binding sites (30). Each transmembrane segment is predicted to be 21 amino acids long (31) and it is linked to each other by either the extracellular or intracellular loops. The transmembrane segments are designated TM1 to TM6 in the amino terminal half and TM7 to TM12 in the carboxy terminal half (30). The two homologous halves of the P-gp are joined to each other by a highly charged linker domain (32). On the amino terminus are the N-linked glycosylations (33). The region of close identity between the two halves are large cytoplasmic loops containing putative nucleotide-binding

sites (30).

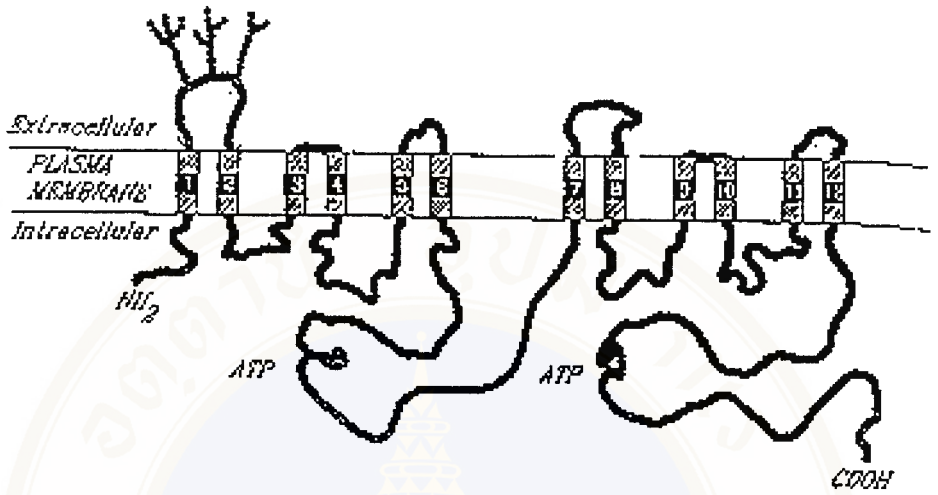

Figure 1 The proposed structure of P-glcoprotein (34).

From the experiment performed by Yoshimura and their colleagues in which they raised antibodies against the amino acid sequences of the putative ATP binding site in the amino terminus half called anti-P and to the amino acid sequences of the putative ATP binding sites in both the amino terminus and the carboxy terminus half called anti-C, respectively. Digestion with trypsin after the resistance cells were permeabilized cleaved the P-gp molecule into 2 fragments designated P1 in the amino terminus half and P2 in the carboxy terminus half. The two antibodies and the antibody C219 did not stain the intact cells while they stained the plasma membrane of cells permeabilized by methanol indicating that ATP binding sites were located intracellularly. Anti-C and C219 precipitated both P1 and P2 fragments while anti-P precipitated only the P1 fragment indicating that P1 and P2 fragments corresponded to the amino-terminal half and carboxy-terminal half of the P-gp respectively. P-gp and its tryptic fragment P1 labeled with (^{3}H)glucosamine were immunoprecipitated

with all three antibodies, while P2 fragment were not indicating that P1 fragment was glycosylated, but P2 fragment was not. From these experiments, it was concluded that the ATP binding sites were located intracellularly (35). The two nucleotide-binding domains (NBD) of P-gp share amino acid sequence identity with each other and the equivalent domains of other ABC transporters. These domains bind and hydrolyse ATP and couple the hydrolysis of ATP to solute translocation across the membrane (36). Although the mechanism of energy transduction is unknown, it is likely to involve ATP hydrolysis-induced conformational changes. The binding of substrate to P-gp is known to stimulate ATP hydrolysis (37,38) and the conformational changes occur following the drug binding and ATP hydrolysis (39,40).

From the experiment of Yoshimura et al mentioned above, it was concluded that P-gp possessed the glycosylation which was located extracellularly in the amino-terminal half of the molecule (35). The protein sequence of MDR1 contains ten potential N-glycosylation sites, Asn-X-Ser/Thr (41). Seven of these sites are located on the cytoplasmic side of the membrane, and therefore are not expected to be glycosylated because glycosylation only occurs on extracellular site (33). The remaining three sites, corresponding to residues 91-91, 94-96, and 99-102, are all clustered in the first extracytoplasmic domain so they are potential N-glycosylation sites (30).

From the results of the study by Schinkel et al, conflicting data on the significance of N-glycosylation for P-gp function were obtained. On one hand, the results indicated that N-glycosylation was not strictly essential for P-gp function. They found that the clones having a certain amount of unglycosylated P-gp in the

plasma membrane could display their drug resistance pattern which was indistinguishable from that of clones having glycosylated wild-type P-gp in the plasma membrane. Thus, once a certain amount of glycosylation-deficient P-gp was present in the plasma membrane, its drug transport function appeared to be normal. On the other hand, transfection experiment of the mutant cDNA having codons coding for asparagine residues substituted by all 3 codons coding for glutamine residues yielded drug resistance clones with a much lower frequency than transfection of wild type cDNA. This indicated that N-glycosylation did have considerable effect on the overall activity of P-gp. They could reconcile these differences by assuming that N-glycosylation improved the efficacy of routing or sorting of P-gp or that it stabilized P-gp, but it did not have direct effect on drug transport capacity. For instance, N-glycosylation may prevent P-gp from ending up or getting stuck in the wrong subcellular compartments. It might help stabilize correct folding or protect P-gp en route to the plasma membrane from degradation by luminal proteases. The transfection of the cDNA which resulted in the deletion of 20 amino acids in the first extracellular loop severely affected the drug transport activity. Since the loss of N-glycosylation could not account for the decrease in activity, the deletion was thought to have direct effect on the drug transport efficacy of the mutated protein. Due to the deletion , the P-gp may assume a stressed conformation, which no longer allowed the protein to easily undergo the conformational changes associated with drug transport. In this view, this part of the first extracellular loop just functioned as a spacer element, which was preferentially glycosylated for other reasons (42).

In human MDR1 P-glycoprotein, Ser661 was identified by Chambers et al (32) and Ser667 was inferred from analysis of a relevant synthetic peptide substrate

(43) as phosphorylation sites on the linker region of P-gp by phosphokinase C. Goodfellow et al (44) mutated the phosphokinase C phosphorylation sites of P-glycoprotein and found that the ability of the mutated P-glycoprotein to confer drug resistance, or to mediate (^{3}H)vincristine accumulation in secretory vesicle, was indistinguishable from that of the wild type P-gp. The only difference was that the mutated P-gp could not be phosphorylated by proteinkinase C anymore. Mutation of the phosphorylated sites did not alter P-gp expression or its subcellular locatization. The transport properties of the mutant and the wild type proteins were indistinguishable. Thus, phosphorylation of the linker region of P-gp by proteinkinase C did not affect the rate of drug transport (44,45). The ability of the cystic fibrosis gene product CFTR which fell into the same ABC transporter protein family as P-gp to regulate heterologous channels also depended on phosphorylation of a domain which separated the two halves of the molecule (46) so phosphorylation of the linker region of P-gp appeared to modulate the activity of P-gp as a channel regulator, rather than as an active transporter (44,45).

Kast et al inserted a small antigenic peptide epitope(YPYDVPDYAIEGR) containing part of the hemagglutinin (HA) of influenza virus at different positions of the MDR3 protein. Intracellular and extracellular localization of this tag in the full-length protein was determined in intact or permeabilized cells by immunofluorescence by the use of monoclonal antibody specific for the HA epitope. The results indicated that the intervening segments separating TM1-TM2 and TM5-TM6 were located extracellularly while the segments linking TM2-TM3 and the one located downstream of TM6 were located intracellularly (47). Similarly, the insertion of epitope tag in the predicted intracellular or extracellular loops of the protein

indicated that intervening segment separating TM4-TM5, TM10-TM11 and downstream of TM12 were located intracellularly, but the segments linking TM7-TM8, TM9-TM10 and TM11-TM12 were located extracellularly (48).

Loo et al constructed cDNA of P-glycoprotein which was devoid of cysteine residues and infected it into drug sensitive cells. This process conferred drug resistance to drug sensitive cells indicating that cysteine did not play a direct role in the mechanism of drug transport. However, cysteines appeared to contribute to folding and stability of P-gp since maturation of the cysteine-less P-gp was slower than the wild type as evidenced by the pulse-chase assay. In addition, the relative half life of the mutant P-gp was shorter than that of wild type P-gp. To study the topology of P-gp in the membrane, site-directed mutagenesis was used to reintroduced cysteine residues into loops between transmembrane segments of the cysteine-less P-glycoprotein which was subsequently probed by membrane-permeant or impermeant thiol-specific reagents. If the cysteine in the loops between transmembrane segments were located on the extracellular side, it would rapidly be biotinylated in the presence of low concentration of biotin maleimide which specifically reacted with a thiol-specific compound and biotinylation was blocked when cells were preincubated with membrane-impermeant silbenedisulfonate maleimide because stilbenedisulfonate maleimide already reacted with the cysteine residues so there was no cysteine residue left to react with biotin maleimide. On the contrary, biotinylation of cysteine in the loops located on the intracytoplasmic side required a relatively higher concentration of biotin maleimide and biotinylation was not blocked by the membrane-impermeant maleimide because it could not go across the membrane to react with the cysteine residues located on the intracellular side.

From these experiments, they concluded that the segments between TM5-TM6, TM7-TM8 and TM11-TM12 were located extracellularly while the segments between TM2-TM3,TM4-TM5,TM8-TM9 as well as the carboxy terminus were located intracellularly. The loops between transmembrane segments TM3-TM4 as well as TM9-TM10 were predicted to be located extracellularly, but reintroduction of cysteines into these loops resulted in no detectable biotinylation. The possible explanation was that these loops were believed to be too short and may not protrude significantly above the lipid bilayer, making them inaccessible to labeling with biotin maleimide (49).

Zhang et al conducted an experiment in which the region from TM 10 to the carboxy terminus of MDR1 was replaced by the corresponding sequences from MDR3. The resultant chimeric protein was expressed, but non-functional. By using progressively smaller replacements, they showed that replacements limited to TM12 markedly impaired resistance to actinomycin D, vincristine and doxorubicin. This phenotype was associated with an impaired ability to photoaffinity label the chimeric P-gp with (^{125}I)iodoaryl azidoprazosin. In contrast, replacement of the loop between TM11-TM12 appeared to demenstrate a more efficient drug pump for actinomycin D, doxorubicin, but not vincristine. These results suggested that amino acids within and immediately N-terminal to the last transmembrane domain of P-gp were part of the drug-binding pocket and were in close proximity to photoaffinity drug-labeling domains (50).

In overall shape, P-gp approximates a cylinder of about 10 nm in diameter with a maximum height of about 8 nm compared to a depth of the lipid bilayer of about 4 nm. This suggests that about one-half of the molecule is within the

membrane. When viewed from the extracellular surface of the membrane, P-gp possesses a large central pore of about 5 nm in diameter. The ring of protein surrounding the central pore is roughly hexagonal. The aqueous pore open at the extracellular face of the membrane is much larger in diameter (~5nm) than is required for the passage of known P-gp substrates. Such a large pore, if open across the membrane, would destroy the permeability barrier. However, the pore is closed at the cytoplasmic face of the membrane. This closure is presumably achieved by the two intracellular lobes (putative nucleotide-binding domains) and the hydrophilic cytoplasmic loops that separate the 12 membrane-spanning segments. Thus, P-gp forms a large aqueous chamber within the membrane and opens to the extracellular milieu (33). In addition, an opening to the lipid phase, within the plane of the membrane, is also apparent. This opening is consistent with the flippase model for P-gp and data show that substrates can gain access to the pore-translocation pathway from the lipid phase (51,52).

2. Function

P-gp encoded by MDR1 gene is believed to function as an energy-dependent efflux pump that can pump a variety of structurally and functionally unrelated lipophilic compounds out of the cells in a manner analogous to that defined for active transport proteins, thus reducing their intracellular concentrations (29,30,53,54). This model proposes that substrates bind to specific domains of the P-gp, which subsequently undergoes an energy-dependent conformational change, can later be released on the exterior side of the membrane (29) via a pore or channel formed by the molecule's multiple transmembrane domains using energy derived from P-gp-mediated ATP hydrolysis (55). Efforts to map the drug-binding domains

of P-glycoprotein by photoaffinity drug analogs and site-directed mutagenesis suggested that P-gp contained multiple nonoverlapping or partially overlapping drug-binding sites, each having different affinities for different drugs or classes of drugs (56,57). Unlike other carrier molecules, P-gp is far less selective with regard to substrate (29). There are a number of reasons to support this proposition.

1. P-gp is a transmembrane protein which possesses 2 potential nucleotide binding sites (30).

2. The biochemical analysis of photoaffinity-labeled tryptic P-gp peptides identified by epitope mapping revealed that two short segments near TM6 and TM12 were major drug binding sites (35,58,59).

3. Both ATP and GTP can provide energy for the transport reaction (60).

4. P-gp has been shown in biochemical studies to hydrolyze ATP (61).

5. Using plasma membrane vesicles prepared from multidrug-resistant cells, it was possible to demonstrate ^{3}H-vinblastine transport into into inside-out vesicles. This transport system was dependent on the constant supply of an energy source as it occurred against concentration gradient. Non-hydrolyzable ATP analogs such as AMPPNP did not support this type of transport (61,62).

6. In the study that monitored the uptake of CsA by primary cultured bovine brain capillary endothelial cell (BCEC) expressing P-gp showed that the steady-stage uptake of CsA was increased significantly to approximately 4-fold when cellular ATP was depleted by treating with 2,4-dinitrophenol, suggesting that the efflux process was ATP dependent(63).

7. The amino acid sequence of human P-gp shares significant homology with the gram-negative bacterial periplasmic transport system, especially the

hemolysin B of *Escherichia coli*, whose function is to transport alpha-hemolysin B out of the cell (30,64). Twelve putative membrane spanning domains, six in each half, are believed to anchor the P-gp in the plasma membrane and are thought to form a channel through which substrates are extruded from the cell (65).

8. P-gp function can be blocked or reversed by a large group of structurally unrelated so-called modulator such as the calcium channel blocker (66), the immunosuppressive cyclosporin A (67) and its analogs (68) and the natural hormone progesterone (69). These all seem to compete for drug binding sites on P-gp (70).

Based on the known widespread distribution of p-gp in a host of human and rodent tissues, normal physiologic functions of this multidrug transporter have been proposed (71). These widespread distribution suggest that P-gp may be utilized for different purposes by different cells (10). The proposed functions of P-gp include

1. Excretion of toxic or potentially toxic substances. The polarized expression of P-gp on the proximal tubules of kidney, the apical biliary surface of hepatocytes and luminal surface of the epithelail cells of colon and jejunum suggests either a physiologic role in extruding potentially toxic xenobiotics or mutagenic chemicals (72,73). In addition, certain endogenous metabolites that are substrates for the multidrug transporter would be handled in a similar manner (71).

2. Protection of sensitive organs as in the blood-brain, blood-testis, and blood-placental barriers which may explain the failure of drugs to penetrate into these tissues, thus allowing them to remain as pharmacologic sanctuaries for malignant cells (10,74). Mouse mdr1a P-gp is abundant in the blood-brain barrier and its absence leads to highly increased levels of drugs ivermectin, vinblastine, digoxin and cyclosporin A in the brain(75) suggesting that P-gp may serve to extrude

various toxic compounds from the central nervous system and other pharmacologic sanctuaries (29).

3. Maintenance of homeostatic levels of steroid hormones. High levels of P-gp expression in the adrenal gland suggests a role in steroid secretion (71,76) For example, deletion of one copy of the mdr1b gene in adrenal Y-1 cells affects steroid secretion (71).

4. Cell volume regulation. A volume-regulated chloride channel activity has been observed in P-gp expressing cells (77). This activity is clearly related to expression of P-gp since the addition of antisense oligonucleotide that reduced P-gp expression eliminated the chloride channel activity (71) or the demonstration of inhibitory action against chloride channel activity by addition of antibody to P-gp (78). The question is P-gp acts as the chloride channel protein or as the regulator of the chloride channel protein. Since the Hela cells which do not possess the P-gp could demonstrate large volume-activated chloride currents which were indistinguishable from those seen in NIH-3T3MDR1 cells so P-gp should not act as a chloride channel protein (45). There are eight serine/threonine residues in the linker region which correspond to consensus PKC phosphorylation sites. PKC-dependent phosphorylation of the linker region modulates the activity of human P-gp as a chloride channel regulator (45). P-gp expression not only confers PKC-sensitivity on volume-regulated chloride channels, it also enhances chloride channel activity by reducing the osmotic gradient required to activate these channels(45,79). The chloride channel regulator and the drug transport activity of P-gp are distinct and separable. Drug transport requires ATP hydrolysis while only ATP binding is sufficient to activate the chloride channel since the nonhydrolyzable ATP analogs

sufficient to activate the chloride channel since the nonhydrolyzable ATP analogs can substitute authentic ATP for the normal chloride channel activity. Furthermore, mutations in the ATP-binding domain that permits ATP binding but prevents ATP hydrolysis allow chloride channel activation to proceed normally, but prevent drug transport. Thus , ATP binding is sufficient for channel activation whereas drug transport requires ATP hydrolysis (80).

3. Antibodies to P-glycoprotein encoded by MDR1 gene

Up until now, several antibodies against P-gp have been discovered. The use of monoclonal antibodies is integral to many aspects of patient care in acute leukemia. Immunophenotyping allows rapid and accurate diagnosis in the majority of cases. Immune marker analysis has been used as prognostic factors and to detect minimal residual disease. New treatment strategies using monoclonal antibodies to select stem cells prior to transplantation have been developed (81). Monoclonal antibodies provide the most exact method for determining the expression of different P-gp genes in normal tissues, tumor tissues and in MDR cell lines (55). Monoclonal antibody can be divided into those reacting with cytoplasmic or extracytoplasmic protein domains. Monoclonal antibody reacting to cytoplasmic epitopes usually recognizes a determinant conserved during evolution while the species-specificity seems to be characteristic of the monoclonal antibodies reacting to extracellular epitopes (82).

The monoclonal MM4.17 is an IgG 2a subclass of antibody (83). The MM4.17 epitope is constituted by the continuous-linear TRIDDPET amino acid sequence (residues 750-757 of the human MDR1- P-glycoprotein). This continuous-

linear epitope belongs to the fourth loop of the P-glycoprotein extracellular domain. The monoclonal antibody MM4.17 recognizes only the human MDR1- P-gp isoform (84).

The monoclonal antibody 4E3 is an IgG2a subclass of antibody which specifically recognizes the external epitope on the human mdr1 P-gp, but not the mdr3 gene product. Binding of the monoclonal antibody to multidrug-resistant cells does not specifically affect the intracellular accumulation or potentiate the cytotoxicity of daunorubicin in multidrug-resistant cells so this antibody can be used for analyzing P-gp expression and for isolating live cells expressing the P-gp without significantly affecting the efflux function of the transporter (85,86).

The murine monoclonal antibody F4 is the IgG1 subclass of antibody. It detects an extracellular epitope localized in or near the third and/or sixth extracellular transmembrane loops of P-gp. Monoclonal antibody F4 reacts strongly with drug resistant cells and insignificantly with their drug sensitive counterparts. Monoclonal antibody F4 detects an extracellular epitope of P-gp which is different from the ones recognized by other major monoclonal antibodies directed against P-gp such as C219, MRK16, JSB-1, C494 and HYB-241 (87).

The monoclonal antibody C219 is an IgG2 subclass of antibody (88). It binds strongly to amino acid sequence VQEALD in the intracellular loop of the carboxy terminal half of P-glycoprotein with Val-506 and Asp-511 representing two critical amino acids required for antigen recognition. An almost homologous amino acid sequence VQAALD recognized by monoclonal antibody C219 is also found in the amino terminal half of P-glycoprotein (89). The monoclonal antibody C219 was reported to crossreact with the MDR3 gene product (89,90) as well as proteins other

than P-gp such as the heavy chain of muscle myosin in the cardiac and skeletal muscle (91). Strict correlations were noted either between C219 negativity and FAB M3 subtype or between C219 positivity and FAB M5 subtype (19,92,93).

The monoclonal antibody JSB-1 belongs to an IgG1 subclass of antibody. It was established by immunizing mice with colchicine-resistant Chinese hamster ovary cell line,CHrC5. It does not bind to the surface of intact, multidrug resistant cells. After some degree of permeabilization, however, strong cytoplasmic staining, preferentially at the cytoplasmic aspect of the plasma membrane, could be detected. The monoclonal antibody JSB-1 reacts with an epitope closely linked to the one recognized by monoclonal antibody C219. Differences between monoclonal JSB-1 and C219 include their isotype (IgG1 and IgG2 respectively) (83) as well as the better sensitivity of monoclonal JSB-1(19). It somehow crossreacts with pyruvate carboxylase, an mitochondrial enzyme. This crossreactivity was partially abolished by preincubating monoclonal antibody JSB-1 with a 1000-fold molar excess of monoclonal antibody C494 epitope-specific peptide (PNTLEGN) indicating that the epitope of monoclonal antibody JSB-1 may either overlap with or be in close proximity to that of monoclonal antibody C494 (94).

The monoclonal antibody UIC2 is an IgG2a subclass of antibody (95). It is reactive against the extracellular moiety of P-gp and it inhibits P-gp-mediated efflux by trapping the P-gp in a transient conformation which prevents it from functioning (96). Potentiation of cytotoxicity by UIC2 was observed with drugs associated with MDR (vinblastine, vincristine, colchicine, taxol, doxorubicin, etoposide, actinomycin D, puromycin and gramicidin D) (95). UIC2 reactivity with P-gp was increased by the addition of several ATP-depleting agents and by mutational inactivation of both

nucleotide-binding domains of P-gp (96).

The monoclonal antibody MRK16 is an IgG2a subclass of antibody. It was developed by producing the hybridoma obtained from the cell fusion of the mouse spleen cells after immunization with intact adriamycin-resistant human myelogenous leukemia K562/ADM and the mouse myeloma cell line. The initial characterization of this antibody demonstrated its ability to differentially stain impermeabilized drug-sensitive and MDR human cell lines. Moreover, it immunoprecipitated a 170-kDa protein which was identified to be P-gp 170 (97). The monoclonal antibody MRK16 epitopes are located on the first and the fourth extracellular domains of P-glycoprotein. They are residues 99-119 (NDTGFFMNLEEDMTRYAYYYS) and residues 737-756 (GVFTRIDDPETKRQNSNLFS) respectively. These epitopes are discontinuous and are separated by about 625 residues. The results from epitope mapping of the MRK16 monoclonal antibody suggested that the two extracellular loops (first and fourth) which were separated in the linear sequence of P-gp must be in close spatial proximity in the native complex in order to form a single antibody epitope. The binding property of MRK16 is conformationally sensitive (34) since MRK16 immunoprecipitated P-gp from selected detergents such as deoxycholate and CHAPS, but not from Nonidet P-40 and Triton X-100 (61,97). MRK16 recognizes 3-D structure of intact P-gp and does not bind to P-gp in Western blot analysis nor cells pretreated with SDS or methanol. (98). The essential residues found in the first extracellular loop of the class I isoform of the P-gp are Glu^{108}, Asp^{110}, Thr^{112} and Tyr^{114}. Similarly, Arg^{741}, Asp^{743}, Glu^{108}, Thr^{747}, and Arg^{749} are the essential residues on the fourth extracellular loop of class I human P-gp. The monoclonal antibody MRK16 is speicfic for human MDR1 gene product since it binded to only human

MDR1 not MDR3 P-gp isoforms and it did not recognize P-gp from monkey, horse, pig and rabbit adrenal gland (34). Most hematological tumor cells express the Fc receptor at different levels, so the blocking process is often used to eliminate the non-specific binding of antibody to Fc receptor. However, it is difficult to completely block the binding of MRK16 to the Fc receptor. The problem of the non-specific binding of MRK16 to the Fc receptor is eliminated by using MRK16F(ab)$_2$ fragments. For example, the human promyelocytic leukemia cell line, HL-60 produced undetectable mRNA level by reverse-transcriptase polymerase chain reaction (RT-PCR) but intact MRK16 significantly reacted with HL-60. When MRK16F(ab)$_2$ fragments were used, it did not stain HL-60 cells. The reactivity of MRK16F(ab)$_2$ fragments with respective MDR-1 positive cells, K-562/ADM and KYO-1, was same as that of intact MRK16. These results indicated that the affinity of MRK16F(ab)$_2$ fragments to the P-glycoprotein was not markedly influenced by the trypsin-digestion of intact MRK16 (98).

The monoclonal antibody MH-162 is a chimeric recombinant antibody in which the antigen-recognizing variable regions of MRK-16 are joined to the constant regions of human antibody. The avidity of the chimeric antibody to the K562/ADM cell antigen is similar to that of the all-mouse antibody MRK-16 as determined by enzyme-linked immunosorbent assay and also by competition inhibition cell binding radioimmuneassay(99). MH-162 augments the killing activity of human effector cells against drug-resistant tumor cells more effectively than the all-mouse monoclonal antibody MRK-16(100).

The monoclonal antibody MRK17 is an IgG1 subclass of antibody which recognizes the same antigen as monoclonal MRK16 and it was reported to have no

effect on drug accumulation or the potentiation of drug cytotoxicity, but was found to inhibit the growth of MDR cells (97).

The monoclonal antibody C32-binding domain covers a stretch of 13 amino acids (GDNSRVVSQDEIER), with four critical amino acids associated with its binding-Asp-427 and Val-431 from the first domain and Glu-436 and Glu-438 from the second domain. The monoclonal antibody C32 recognizes a sequence conserved in hamster class I and II P-gp isoform, but not class III P-gp isoform or the human and mouse P-gp (89).

The monoclonal antibody C494 recognizes the amino acid sequence PNTLEG with Thr^{323} and Glu^{325} as the critical residues. This amino acid sequence is located intracellularly in the carboxy terminal half of P-gp. The amino terminal half of P-gp does not contain a homologous sequence for the monoclonal antibody C494 epitope. The monoclonal antibody C494 is gene specific. It binds to sequence present only in the class I P-gp isoform of hamster and human (89). The monoclonal antibody C494 strongly crossreacts with human pyruvate carboxylase (101).

The monoclonal antibody 57 is an IgG2a subclass of antibody. It recognizes a peptide determinant of the extracellular P-gp subunit and it is specific for only human P-gp. It did not bind to drug sensitive cells in the study of Cenciarelli et al while it stained 98-100% of highly multidrug resistant cell variants. It also demonstrated a unique ability to detect small variations in P-glycoprotein levels as determined by the study in KB and CEM cell variants using flow cytometric analysis. Since the epitope of monoclonal antibody 57 is located extracellularly, this monoclonal antibody can be used not only to detect P-gp on intact, living cells and tissues, it can also be used in conjunction with effector cells to kill MDR cells, thus

suggesting an alternative therapy to eradicate cancer bearing P-gp on their surfaces (102).

The monoclonal antibody HYB-241 is an IgG1 subclass of antibody (103). It recognizes the extracellular epitope of P-gp. (104) It was an effeective modulator of P-gp, causing the increase in actinomycinD levels in the transfectant line by 6-fold, vincristine levels by 2-fold, and vinblastine levels by 3-fold. It also lowered the 50% inhibitory concentration values of actinomycin D by 11-fold, vincristine by 6-fold, and vinblastine by 2-fold. These results indicated that monoclonal antibody HYB-241 may be an effective chemosensitizer of actinomycin D, vincristine, and vinblastine. The proposed mechanism for this was that the binding of monoclonal antibody to the extracellular epitope of P-gp may induce a conformational change in the molecule which had an impact on the ability of the molecule to transport some drugs (103).

4. P-glycoprotein from bacteria to human

P-gp encoded by MDR1 gene shares dominal organization and significant sequence similarities, particularly in the nucleotide binding domains, with several putative membrane transport proteins of bacteria such as hemolysin B of *Escherichia coli* or of unicellular eukaryotes such as STE6 of *Saccharomyces cerevisiae*, pfmdr of *Plasmodium falciparum* and ltpgp of *Leishmania tarentolae* (65,105). A remarkable degree of sequence homology is found between P-gp and the bacterial transport protein HlyB, which is responsible for the export of HlyB from strains of hemolytic bacteria *Escherichia coli* (106). Chloroquine-resistant and chloroquine-sensitive *Plasmodium falciparum* strains accumulate chloroquine at equivalent rates;

however, the chloroquine-resistant strains are able to efflux the drug 40-50 times more rapidly (107). The transport system found in *Plasmodium falciparum* has many striking similarities to the drug efflux mediated by P-gp (108). STE6 protein from *Saccharomyces cereviciae* functions to export the hydrophobic mating factor in a manner analogous to the efflux of hydrophobic cytotoxic drugs performed by the related mammalian P-gp (109).

P-gp are encoded by a small family of closely related mdr genes. These consist of 3 members in rodents and 2 members in human (110). In mouse, these genes are designated mdr1, mdr2 and mdr3. In hamster, these genes are designated pgp1, pgp2 and pgp3 (111). In human, the genes are designated MDR1 and MDR2 (110). Hybridization of primate and rodent DNAs with single-exon probes corresponding to the human MDR1 and MDR2 genes demonstrated that rodent genes contain 2 genes corresponding to the human MDR1 but only one gene corresponding to MDR2. Among the 2 rodent homologs of the human MDR1 gene, one gene hybridized with human MDR1 probe more strongly than did the other gene. Analysis of sequence similarities among the known MDR cDNA sequences indicated that the first gene most probably corresponded to the gene previously designated pgp1 in hamster and mdr3 in mice and the second gene corresponded to the gene designated pgp2 in hamster and mdr1 in mice (112). A human MDRcDNA sequence which shares homology with the hamster pgp3 gene, was designated MDR3 by Van der Blick et al (113). Sequence comparison showed that the MDR3cDNA sequence in fact corresponded to the previously described MDR2 gene(114). Chin et al proposed to call the first rodent gene mdr1a and the second rodent gene mdr1b (112). The

human MDR2 gene corresponds to the gene previously designated pgp3 in hamsters (115) and mdr2 in mice (64). The above information can be summarized in Table 1.

Table 1. The different mammalian P-glycoprotein genes(65).

P-gp class	Human	Mouse	Hamster	Involved in multidrug resistance
I	MDR1	mdr3 (mdr1a)	pgp1	Yes
II		mdr1 (mdr1b)	pgp2	Yes
III	MDR3	mdr2	pgp3	

The possible evolutionary mechanisms that may be responsible for the creation of present day mdr1 gene are.

1. The gene could have evolved from the duplication of an ancestor gene already containing approximately half the number of introns of the mouse gene. The observation that both halves of the protein are encoded by a similar number of exon (14 vs 13) and that at least one intron pair (no 13 and 26) is located at exactly the same position within the nucleotide-binding folds would support this proposal.

2. An ancestral mdr gene containing a very limited set of introns would have duplicated prior to fusion and subsequently acquired additional introns now present in

the mouse mdr1 gene. This model would account for the lack of precise conservation of exon/intron boundaries that should be expected from the duplicationof an ancestral gene containng a complete set of introns (116).

5. Expression of P-glycoprotein in normal tissues and in tumors

5.1 Expression of theP-glycoprotein 170 in normal tissues.

The expression of P-gp in normal human tissues and in a number of human tumor samples has been documented. High level of MDR1 gene expression is found in the adrenal cortex (76), the apical membrane of the brush border of the proximal convoluted tubules, apical domain of collecting ducts and thick limb of Henle's loop of the kidney (117), the biliary front of hepatocytes and the apical surface of epithelial lining of the small biliary ductules in liver, apical surface of the columnar epithelial cells of colon, jejunum and placenta (118,119). Intermediate levels of MDR1 gene expression is found in the adrenal medulla, trachea, lung and prostate (10). Low or undetectable levels of MDR1 expression are seen in many other organs such as skin, skeletal muscle, heart, spleen, esophagus, stomach, ovary, spinal cord and bone marrow. Heyden et al summarized the expression of MDR1 gene in Table 2.

Table 2 MDR1 expression in normal tissues (10).

High MDR1 expression		Moderate MDR1 expression		Low MDR1 expression
Adrenal cortex		Adrenal medulla		Skin
Kidney	Renal proximal tubules	Trachea	Apical epithelial cells	Skeletal muscle
Liver	Bile canalicular face of Hepatocytes	Lung (major bronchi)		Heart
		Prostate	Glandular	Spleen
Placenta	Trophoblasts			Esophagus
Colon	Luminal surface of the			Stomach
Small bowel	Mucosa			Ovary
Brain	Capillary endothelial cells			Spinal cord
Testis	Endothelial cells			Bone marrow
Pancreas	Luminal surface of epithelial Cells of small ductules			

5.2 Expression of the MDR1 gene in hematological malignancies.

Many patients with hematological disorders develop resistance to the drugs used in the treatment of their diseases. Most of the drugs used for the treatment of hematological malignancies have been implicated in MDR (10). In normal hematopoietic cells (bone marrow, spleen, peripheral blood lymphocytes), only low to very low MDR1 gene expression levels are found (120,121). However, in almost all types of leukemias, multiple myelomas and non-Hodgkin's lymphoma, either untreated or treated, elevated MDR1 levels have been reported. The MDR1 levels can range from low to high (10). Goldstein et al classified hematological malignancies into 3 defined groups according to different levels of MDR expression (122). First, MDR1 levels were usually elevated in untreated, intrinsically drug-resistant tumors. The tumors that belonged to this group was CML in blastic crisis. Second, the MDR1 levels were occasionally elevated in untreated cancers. The tumors that belonged to this group were adult acute lymphocytic leukemia(ALL), adult acute nonlymphocytic leukemia (ANLL), indolent Hodgkin's lymphoma. Third, MDR1 levels were also increased in some cancers at relapse after chemotherapy, including ALL and ANLL (10). Lum et al included chronic lymphocytic leukemia (CLL) in the first group and non-Hodgkin's lymphoma (NHL) in the third group of acquired resistance (123).

6. The clinical problem of multidrug resistance

Chemotherapy has been proven to be effective in several disseminated cancers, and has led to the cure of many childhood and adult cancers such as leukemias, lymphomas, sarcomas, choriocarcinoma, and testicular cancers (71). The emergence and outgrowth of populations of drug-resistant tumor cells constitute a

major limitation to the chemotherapeutic treatment of human cancers. Drug-resistant cell populations often display cross-resistance to compounds unrelated in structure and intracellular target to which they have not been previously exposed (64). These compounds include hydrophobic natural p roducts derived from plants, or micro-organism, semi-synthetic analogs of such products, and synthetic organic compounds. These compounds can be classified as

6.1 Anticancer drugs

Vinca alkaloid, e.g. vinblastine

Antracycline, e.g. doxorubicin

Epipodophylotoxins, e.g. etoposide

Antibiotics, e.g. actinomycin D

Others, e.g. mitomycin C, taxol, topotecan, mitramycin

6.2 Other cytotoxic agents

Antimicrotubule drugs, e.g. colchicine, podophyllotoxin

Protein synthesis inhibitors, e.g. puromycin,emetine

DNA intercalators, e.g. ethidium bromide

Toxic peptide, e.g. valinomycin,gramicidin D, N-acetyl-leucyl-norleucinal (ALLN)

6.3 Agents that reverse drug resistance

Calcium channel blockers, e.g. verapamil, nifedipine, dihydroperidines, azidopine

Antiarrhythmics, e.g. quinidine, amiodarone

Antihypertensives, e.g. reserpine

Antibiotics, e.g. hydrophobic cephalosporins

Antihistamines, e.g. terfenadine

Immunosuppressants, e.g. cyclosporin A, FK506, Rapamycin

Steroid hormone, e.g. progesterone

Modified steroid, e.g. tirilazad, tamoxifen

Lipophilic cations, e.g. tetraphenylphosphonium

Diterpenes, e.g. forskolin

Detergents, e.g. tween-80

Antidepressants, e.g. tioperidone

Antipsychotics, e.g. phenothiazines

Many other hydrophobic, amphipathic drugs and their analogs (71)

7. P-glycoprotein modulator

Since the discovery of the multidrug resistance phenotype by Biedler and Riehm in 1970 (124), a lot of efforts have been made to reverse or overcome this phenomenon. One of the efforts was to make use of the compounds that can reverse the multidrug resistance phenotype in the clinical settings. A host of several compounds that can reverse the multidrug resistance are referred to by a variety of names such as chemosensitizers, multidrug resistance modulator or multidrug resistance reversing agent(73). These compounds share nothing in common except that they are all lipophilic and many are heterocyclic, positively charged substances (29). These multidrug resistance modulators can be classified into 6 groups based upon their primary pharmacologic activity (3).

7.1 Calcium channel blockers

Verapamil

Nicardipine

Niguldipine

Bepridil

PAK-200

Ro 11-2933

7.2 Calmodulin antagonists

Trifluoperazine

Prochlorperazine

Fluphenazine

Trans-Flupenthixol

7.3 Immunosuppressive drugs

Cyclosporin A

SDZ PSC 833

SDZ 280-446

7.4 Steroids and hormonal analogs

Progesterone

Tamoxifen

Toremifene

Megestrol acetate

7.5 Dipyridamole

Dipyridamole

IBW 22

7.6 Miscellaneous compounds

Quinidine

Chloroquine

Terfenadine

Reserpine

Two of these multidrug resistance modulators are worth mentioning. One is verapamil. Verapamil was the first compound which was identified to have the ability to reverse the multidrug resistance (66). It still remains as an important standard agent for comparison of potency and mechanism for all subsequently developed multidrug resistance modulators (29). The other compounds are cyclosporin A and its analogs. Cyclosporin A was found to be effective in reversing the multidrug resistance at concentration lower than previously identified multidrug resistance modulators (0.5-3.0 μm) (125). The cyclosporin analog such as PSC 833 may offer the bright prospect for the reversal of multidrug resistance because of its increased potency and specificity for P-gp-mediated multidrug resistance and its non-immunosuppressive property (29).

8. Mechanism of multidrug resistance modulation

The primary mechanism by which most MDR modulators are believed to antagonize MDR is through direct inhibition of drug efflux mediated by P-gp, resulting in restoration of cytotoxic drug concentrations in MDR cells. MDR modulators may block cytotoxic drug efflux by acting as competitive or noncompetitive inhibitors, perhaps by binding to similar drug/substrate binding sites or to other chemosensitizer binding sites that cause allosteric changes resulting in

inhibition of cytotoxic drug binding or transport (29). In support of this model, many studies have now demonstrated that certain MDR modulators may directly bind to cellular membranes enriched for P-gp in a specific and saturable manner, and that this binding may be inhibited by other MDR modulators and by chemotherapeutic drugs(126,127). In addition, radiolabeled, photoactivatable MDR modulator analogs irreversibly bind to P-gp and this may be effectively inhibited by many other MDR modulators (128).

Recent studies utilizing P-gp molecules containing various mutations support the notion that more than one site of interaction for substrates and inhibitiors exists. For example, substitution of a serine residue within the 11th predicted transmembrane domain of murine P-gp by any of the six other amino acids resulted in both positive and negative effects on the modulatory abilities of different MDR modulators (129). Similarly, diverse effects were seen for the capacity of different MDR modulators to inhibit drug efflux mediated by P-gp isoforms differing at a codon within the third transmembrane domain(130). Also, photoactivatable binding of the MDR modulator azidopine to P-gp has identified two distinct sites within the P-gp molecule, one between residues 198 and 440 of the amino half and the other within the carboxy portion of the protein (58).

Despite the seemingly bright prospect for the reversal of multidrug resistance in vitro, the in vivo situation is overshadowed by the toxicity of these multidrug resistance modulators. The plasma level required to elicit the reversal effect for the multidrug resistance may be unattainable in patients because of the inherent toxicity (55) such as heart block, congestive heart failure, a fall in systolic blood pressure below 80 mm Hg(131,132), nausea, vomiting, or mucositis (133).

Another potential problem with the multidrug resistant modulator is the interaction with the P-gp in noncancerous tissue. Inhibition of P-gp function in liver or kidney may alter the excretion of anticancer drugs and finally increase their tissue concentration and toxicity (134). Another brilliant means of employing the multidrug resistance modulators apply the same strategy proposed for the combination of chemotherapy by using the cocktail of several multidrug resistance modulators with nonoverlapping toxicity to achieve an overall reversal of multidrug resistance effect greater than that with individual compounds at higher doses (29).

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Subjects

The patients eligible for this study were 24 cases of untreated patients with the clinical diagnosis of acute myelogenous leukemia and the relapsed acute myelogenous leukemia patients. Informed consent was obtained from each patient before entering this study.

Twenty four donors from Blood Bank were used as our normal group. Informed consent was obtained from each donor before entering this study.

1.2 Cell line

- K562 (Drug sensitive cell line)
- K562/Dau (Drug resistance cell line)

1.3 Reagents

- Trypan blue (Sigma Diagnostics, U.S.A.)
- Rhodamine123 (Sigma Diagnostics, U.S.A.)
- Cyclosporin A (Sandoz, Switzerland)
- Monoclonal antibody MRK16 (Kamiya Biomedical Company, Seattle, U.S.A.)
- Biotin-goat antimouse IgG (Immunotech, Marseille, France)
- PE-conjugated streptavidin (Immunotech, Marseille, France)
- Daunorubicin

- PBS (Ca^{++} and Mg^{++}free) pH 7.2
- Histopaque (Sigma Diagnostics, U.S.A.)
- RPMI 1640 (GibcoBRL, U.S.A.)
- DMSO (Amresco, U.S.A.)
- NaN_3
- Sheath fluid
- Sodium hypochlorite
- Distilled water
- Fetal calf serum (GibcoBRL, U.S.A.)

1.4 Equipment

- Deep freezer (Forma Scientific, U.S.A.)
- Automate cell counter (Cell-Dyn, Abbott, U.S.A.)
- Falcon tube
- Adjustable autopipette 1-10 µl
- Adjustable autopipette 5-40 µl
- Rack
- Pipette tip
- Pipette 1, 5, 10 ml
- Beaker
- Centrifuge
- Refrigerator
- Refrigerated centrifuge (Beckman GFR, U.S.A.)
- Shaker waterbath (GFL 1083, Germany)
- FACScan (Becton Dickinson, U.S.A.)

2. Sample collection

Five ml of bone marrow or peripheral blood was drawn into the syringe. The syringe precoated with heparin was used in case of bone marrow aspiration while EDTA tube was used in case of peripheral blood. Both the bone marrow and the peripheral blood were sent immediately to the laboratory. Upon arrival, small amount of sample was sent to the automate cell counter and for Wright stain of the bone marrow or peripheral blood smear (Figure 2 and 3) while the rest was sent for the centrifugation at 2500 rpm for 10 minutes, then collected the interface between serum and the red blood cells.

3. Criteria for specimen rejection

- Insufficient quantity of cells or cell of interest can preclude testing.
- Clotted, partially clotted, or hemolysed specimens.
- Specimen age exceeds 24 hours.
- Specimens with low viability (<85%) may be rejected based on pathologist review.
- Refrigerated peripheral blood or bone marrow specimens.

4. Specimen preparation

- Three ml of Histopaque was added to a 15 ml conical centrifuge tube.
- Three ml or more of blood or bone marrow was carefully overlayed upon the Histopaque.
- The centrifuge tube was centrifuged at 1000 rpm 30 minutes at room temperature.
- Plasma was aspirated and discarded to within 0.5 mm of mononuclear layer.

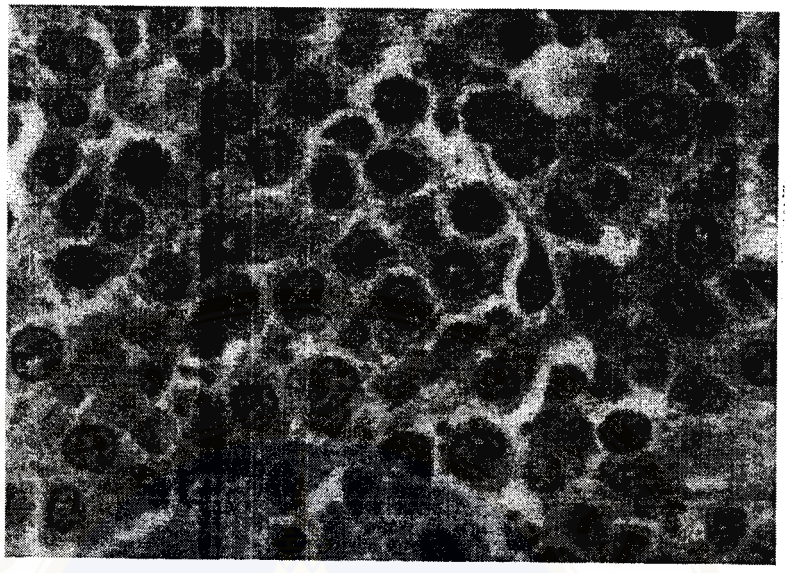

Figure 2 The bone marrow smear shows numerous monoblasts and promonocytes. The monoblasts are characterized by cells with large, round nuclei containing nucleoli and numerous azurophilic granules in the cytoplasm. The promonocytes are characterized by cells with cleaved nuclei and abundant cytoplasm.

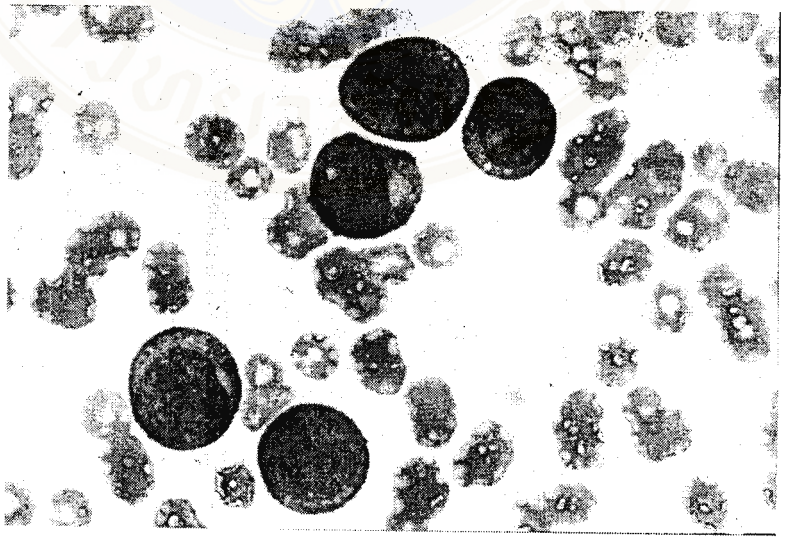

Figure 3 The peripheral blood smear shows numerous myeloblasts. Myeloblasts are characterized by cells with fine chromatin and prominent nucleoli in the nucleus and blue-grey cytoplasm.

- The mononuclear cell layer was aspirated and transferred to Falcon tube.
- The cells was washed by addition of PBS with 2% FCS and centrifuged at 2000 rpm 10 minute 2 times.plasm.

5. Specimen storage

In case the specimen could not be processed within 6 hours, it would be stored in the storage medium by the following procedure

- The storage medium(RPMI:DMSO:FCS=8:1:1)was added to the sample in a ratio of 1:1.
- The solution was mixed and aliquoted into several smaller tubes.
- After that, they were transferred to -4°c for 15 minutes, then -10 °c for 15 minutes and finally to -70 °c.

6. Specimen retrieval

- The sample was retrieved from the -70 °c freezer and placed in the prewarmed 37 °c waterbath until the sample was completely thawed.
- The sample was left in the waterbath for 1 hour.
- Three ml of PBS-2%FCS was added to the sample and the sample was centrifuged at 2000 rpm for 10 minutes, then removed supernatant for 2 times.

7. Determination of cell viability

Viability of the cells was tested employing Trypan blue (Sigma, U.S.A.). Ten µl of 1% Trypan blue was added to 100 µl of cell suspension and incubated at room temperature for 5 minutes. Place an aliquot of dye-cell suspension mixture on a slide and counted the number of dead cells in 100 total cells. The criteria for distinguishing viable cells from dead cells was that the viable cells appeared clear

under microscope compared to the blue nonviable cells. The percentage of viable cells was determined by utilizing a hemocytometer. The acceptable level was at least 85% viable cells. If the sample contained less than this desirable level, it would not be processed further.

8. Specimen processing

Both the samples that were processed within 6 hours and the freezed samples after thawing would be processed by the same following procedure.

8.1 The adjustment of cell concentration

8.1.1 WBC < 2,000 cells/ml

- One to three ml of sample was placed in a 15 ml conical centrifuge tube, then filled the tube with PBS and centrifuged at 2,000 rpm with cap on for 5 minutes.
- The supernatant was discarded, then resuspended WBC sediment in filtered PBS-50%FCS.
- Cells at 200 µl aliquot was counted and the cell concentration was adjusted to 5×10^6 cells/ml.

$$\text{Desired volume (ml)} = \frac{\text{Volume of cell suspension} \times \text{WBC of cell suspension}}{5\times10^6}$$

8.1.2 WBC > 20,000 cells/ml

- Sample was diluted with filtered PBS-50% FCS into the following dilution.

WBC	Dilution
20,000-40,000	1:2
40,000-60,000	1:3
> 60,000	Process as in 8.1.1

- Cells at 200 µl aliquot was counted and the cell concentration was adjusted to 5×10^6 cells/ml.

9. Procedure for CD34 staining

- Ten µl of FITC-conjugated anti-CD34 was added to100 µl of sample.
- The sample was incubate at 4°c in the dark for 15 minutes.
- Three ml of PBS-2%FCS was added to the sample and the sample was centrifuged at 2500 rpm for 10 minutes, then discarded the supernatant for 2 times.
- One ml of PBS with 2% FCS was added to the sample.

10. Procedure for MRK16 staining

- Fifty µl of MRK16 (25 µg/ml) was added to the sample (135).
- The sample was incubated on ice for 30 minutes.
- Three ml of PBS-2%FCS was added to the sample and the sample was centrifuged at 2500 rpm for 10 minutes, then discarded the supernatant for 2 times.
- Twenty five µl of biotin-goat antimouse IgG2a was added to the sample.
- The sample was incubated on ice for 20 minutes.
- Three ml of PBS-2%FCS was added to the sample and the sample was centrifuged at 2500 rpm for 10 minutes, then discarded the supernatant for 2 times.
- Fifty µl of goat serum was added to the sample.
- The sample was incubated on ice for 10 minutes.
- Three ml of PBS-2%FCS was added to the sample and the sample was centrifuged at 2500 rpm for 10 minutes, then discarded the supernatant

- Fifty µl of PE-conjugated streptavidin was added to the sample.
- The sample was incubated on ice for 20 minutes.
- Three ml of PBS-2%FCS was added to the sample and the sample was centrifuged at 2500 rpm for 10 minutes, then discarded the supernatant for 2 times.
- One ml of PBS with 2% FCS was added to the sample.

11. Procedure for functional efflux activity

- Cells (5×10^6) in RPMI was incubated with 10% FCS at 37°c for 1 hour.
- Rhodamine123 (150 ng/ml) was added and the sample was incubated at 37°c for 30 minutes (12).
- The sample was centrifuged at 2500 rpm 10 minutes and the supernatant was discarded.
- Five ml of RPMI with 10%FCS was added to the sample and the sample was centrifuged at 2500 rpm for 10 minutes then the supernatant was discarded for 2 times.
- Five ml of RPMI with 10%FCS was added to the sample.
- The specimen was divided into 2 aliquots.
- Aliquot I was resuspended in 5 ml of RPMI with 10% FCS.
- Aliquot II was resuspended in 5 ml of RPMI with 10% FCS and cyclosporin A (2500 ng/ml) (136).
- Aliquot I and II were incubated in 37°c shaker waterbath for 3 hours.
- Both aliquot I and II were centrifuged at 2500 rpm for 10 minutes and the supernatant was discarded.
- Five ml of RPMI with 10% FCS was added to the sample and the sample

was centrifuged at 2500 rpm for 10 minutes, then the supernatant was discarded

In this study, we used 24 blood samples from the donors of blood bank as our normal group. The donor blood samples were processed by the same procedures as in the patient samples.

12. Specimen analysis

Peripheral blood and bone marrow smears were classified according to FAB classification (137) which was based mainly on morphology and the cytochemistry. The myeloblasts of FAB M1-M3 show heavy granules by Sudan Black B, but more intense in FAB M3. They show weak diffuse cytoplasmic staining by β-glucuronidase. The myeloblasts of FAB M4&5 appears as in FAB M1-3. The monoblasts show scattered granules by Sudan Black B, brownish cytoplasmic staining by ANAE and intense diffuse cytoplasmic staining by β-glucuronidase. The megakaryoblasts of FAB M7 was determined by morphology and confirmed by CD41 staining. Stained cells were analysed on a FACScan analyzer (Becton Dickinson, U.S.A.). The excitation wavelength of 488 nm was emitted from the high power argon laser light source. Detectors were placed at the opposite end of the light source for the measurement of the cell size and at right angle to the light path for the measurement of the internal structure/granularity, respectively. After excitation, fluorescences attached to antibodies emitted characteristic spectra: FITC at 530 nm and PE at 575 nm. FITC emission was reflected toward the appropriate photomultiplier tube by 550 nm dichroic long band-pass filter, whereas PE fluorescence was collected as unreflected light behind the 600 nm dichroic short band-pass filter. Further selection of FITC and PE emission signals was achieved by

the addition of 525 and 575 nm band-pass filters, respectively. Spectral overlap between green (FITC) and red (PE) signals was electronically compensated. Forward and side scatter signals were collected using linear scales while fluorescence signals were collected on logarithmic scales. The instrument was calibrated making use of calibrite flow cytometer alignment beads (Becton Dickinson, U.S.A.).

Data was collected on 10,000 ungated events. The blast population was gated using standard two dimensional light scatter dot plot of forward angle light scatter versus orthogonal light scatter. Threshold was set to gate out red blood cells and cell debris. The gated cells were presumably to be the blast population based upon the fact that the sample contained at least 75% blast population, the size and granularity they possessed and the characteristic of CD45 staining. Controls were established by substituting MRK16 with IgG2a. Negative control was established using K562 cells stained with the same procedures as with the patient samples. Positive control was established using K562 with stepwise selection with daunorubicin stained with the same procedures as with the patient samples (138).

12.1 Criteria for positive antiCD34 and MRK16 staining

Specimens were considered positive when the percentage of cells expressing fluorescence intensity beyond that of control exceeded 20 percent (135,138).

12.2 Criteria for positive functional efflux activity

Any sample which showed the percentage difference of mean fluorescence intensity (MFI) beyond the mean of the percentage difference of the mean fluorescence intensity of the normal donor group plus 1 SD was considered positive.

The percentage difference of the mean fluorescence intensity =

$$\frac{\text{MFI with CsA-MFI without CsA} \times 100}{\text{MFI with CsA}}$$

13. Statistical analysis

The flow cytometric data were analyzed by LYSIS II software program. The correlations between P-glycoprotein expression, functional efflux activity, age, gender, CD34 expression, WBC count and FAB subtype were determined by Pearson Chi Square or Fisher Exact Test, whichever was more appropriate.

CHAPTER IV

RESULTS

Twenty four AML pateints were studied. These consisted of 9 males (37.5%) and 15 females (62.5%) with the mean age at study entry of 35.19 years, ranging from 1 to 73 years. Twenty-one patients (87.5%) were de novo AML while 3 patients (12.5%) were relapsed AML. Most of the cases were classified as FAB M4 (50.0%) followed by FAB M2 (22.7%) as shown in Table 3.

Data was not available for all parameters: P-gp expression, functional efflux activity, CD34 expression, age, gender, WBC count and FAB subtype in all cases either because the specimens received contained insufficient cells for all analyses.

The drug sensitive cell, K562, which was used as the negative control showed the negative MRK16 staining and functional efflux activity while the drug resistance cell, K562/dau, which was used as the positive control showed positive MRK16 staining and functional efflux activity as shown in Figure 4,5,6 and 7 respectively.

1. MDR1 gene expression

Eleven cases (45.8%) demonstrated positive MRK16 staining as shown in Table 4 and Figure 8. P-gp expression was not associated with age (p= 0.155), gender (p= 0.682), CD34 expression (p= 0.423), WBC count (p= 0.146), nor FAB subtype (p = 0.331) as shown in Table 5,6,7,8,9 respectively.

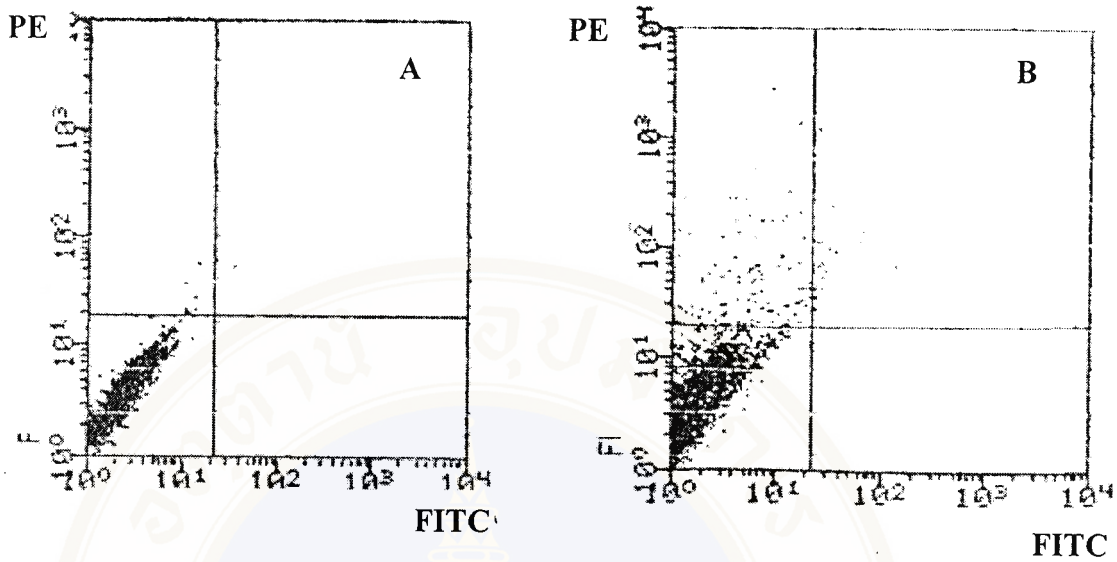

Figure 4 The detection of P-gp in K562 cell. The fluorescence intensity of FITC in the x axis vs. that of PE in the y axis of K562 cell with IgG2a (A) and MRK16 (B).

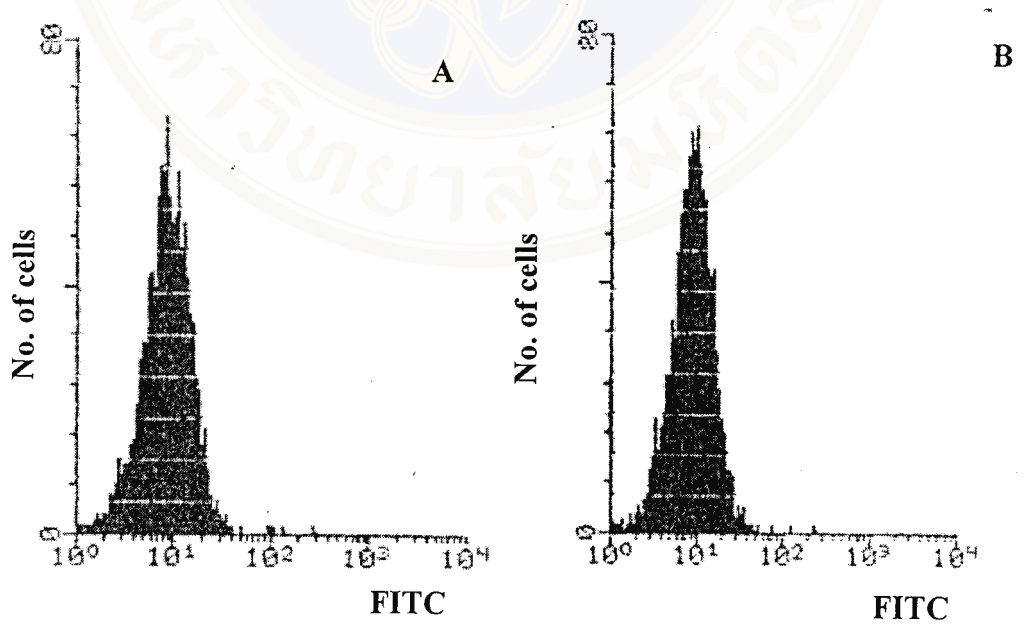

Figure 5 The detection of functional efflux activity in K562 cell. The fluorescence intensity of FITC in the x axis vs. the number of cell in the y axis of K562 cell without cyclosporin A (A) and with cyclosporin A (B).

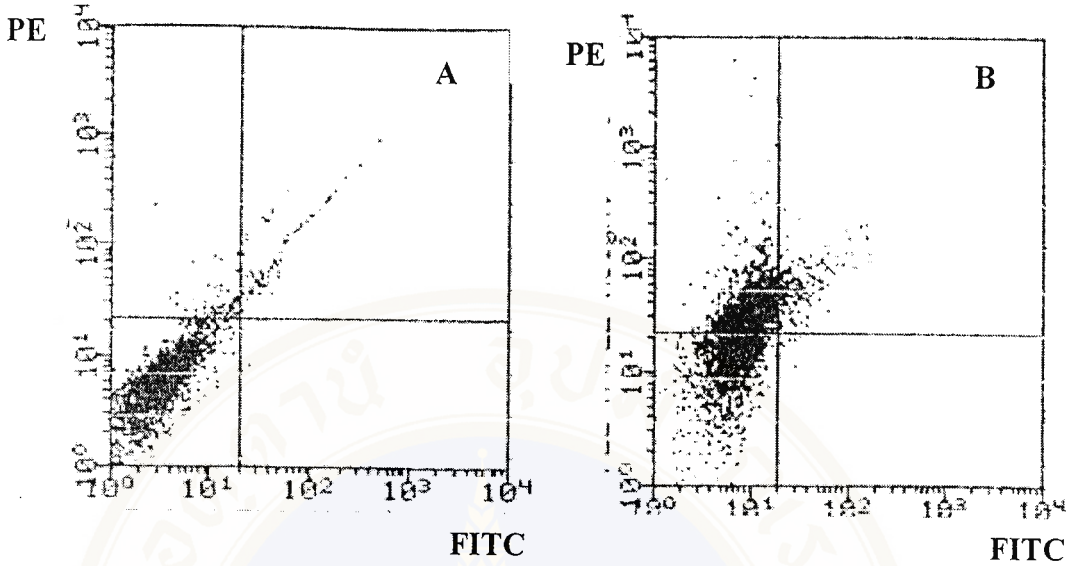

Figure 6 The detection of P-gp in K562/Dau. The fluorescence intensity of FITC in the x axis vs that of PE in the y axis of K562/Dox cell with IgG2a (A) and MRK16 (B).

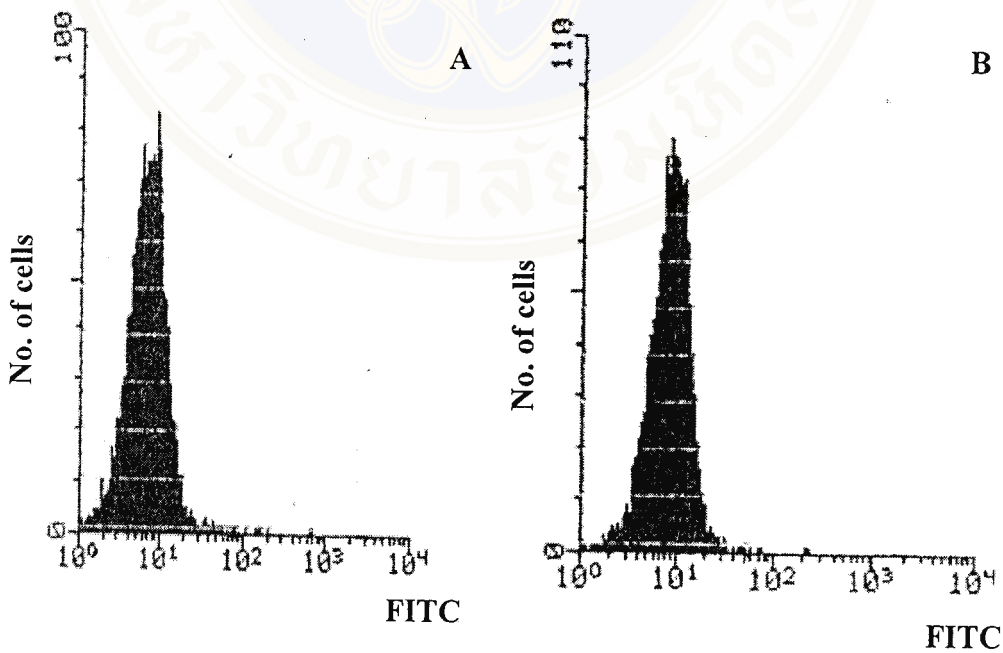

Figure 7 The detection of functional efflux activity in K562/Dau. The fluorescence intensity of FITC in the x axis vs. the number of cell in the y axis of K562/Dox cell without cyclosporin A (A) and with cyclosporin A (B).

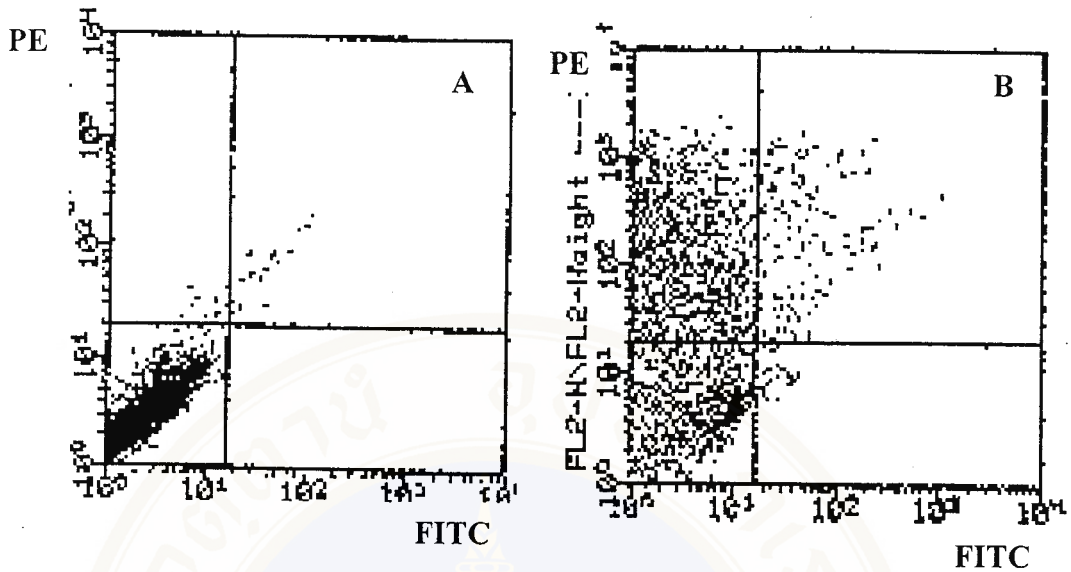

Figure 8 The detection of P-gp in the patient's sample. The fluorescence intensity of FITC in the x axis vs that of PE in the y axis of patient's sample with IgG2a (A) and MRK16 (B).

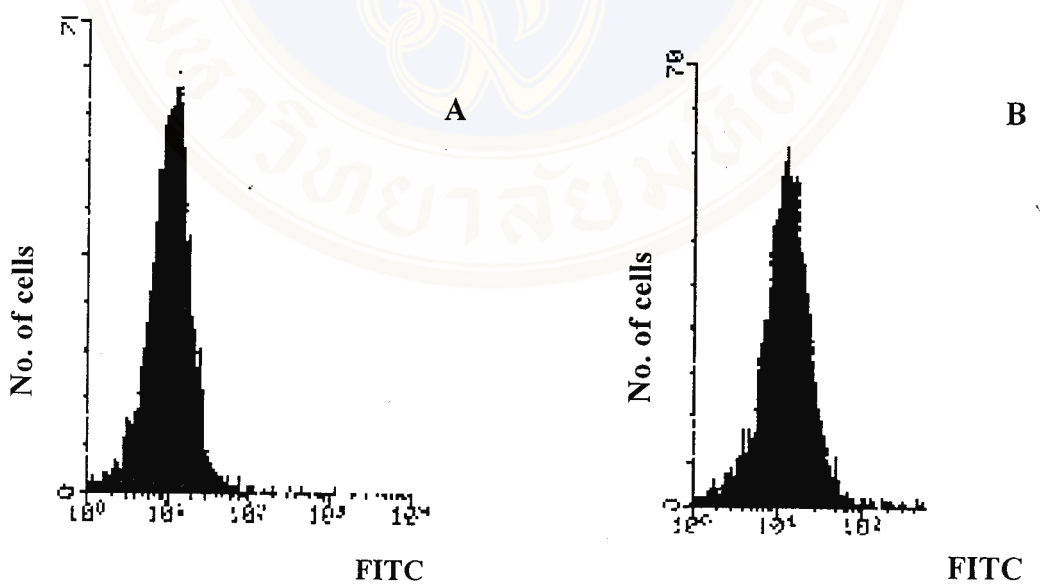

Figure 9 The detection of functional efflux activity in the patient's sample. The fluorescence intensity of FITC in the x axis vs the number of cell in the y axis of patient's sample without cyclosporin A (A) and with cyclosporin A (B).

2. Functional dye efflux

Functional dye efflux, which was inhibited by cyclosporin A, was detected in 11 cases (45.8%) as shown in Table 4 and Figure 9. The functional efflux activity was strongly correlated with MDR1 gene expression; 10 cases (90.91%) out of 11 MDR1-posiive cases were efflux positive, while 1 case (7.69%) out of 13 MDR-negative cases were efflux positive. ($p< 0.001$) as shown in Table 10. However, discrepant cases were identified. These consisted of 1 MDR-negative/efflux-positive case as well as 1 MDR-positive/efflux negative case.

The functional efflux activity was not associated with age ($p= 0.155$), gender ($p=0.682$), CD34 expression ($p= 0.105$), WBC count ($p=0.481$) nor FAB subtype ($p=0.596$) as shown in Table 11,12,13,14,15 respectively.

Table 3 Data of the patients' age, gender, WBC count, FAB subtype and AML type.

Patient	Age	Gender	WBC count ($\times 10^3$cells/ml)	FAB subtype	AML type
Patient 1	24	F	42.9	M4	De novo
Patient 2	15	M	55.7	M4	De novo
Patient 3	15	F	37.9	M4	De novo
Patient 4	-	F	6.23	M4	De novo
Patient 5	1	F	-	-	De novo
Patient 6	22	F	9.46	M3	De novo
Patient 7	45	F	52.5	M4	De novo
Patient 8	45	F	201.0	M1	De novo
Patient 9	27	M	72.3	M3	De novo
Patient 10	10	F	94.9	M2	De novo
Patient 11	12	F	25.5	M4	De novo
Patient 12	61	M	29.43	M2	De novo
Patient 13	68	M	223.0	M4	Relapsed
Patient 14	70	M	38.1	-	Relapsed
Patient 15	50	F	0.55	M4	De novo
Patient 16	13	M	6.99	M2	De novo
Patient 17	72	M	81.7	M1	De novo
Patient 18	37	F	7.10	M4	De novo
Patient 19	30	F	37.06	M5	De novo
Patient 20	73	F	5.63	M2	De novo

Table 3 (Continued) Data of the patients' age, gender, WBC count, FAB subtype and AML type.

Patient	Age	Gender	WBC count ($\times 10^3$cells/ml)	FAB subtype	AML type
Patient 21	-	M	21.5	M2	De novo
Patient 22	1	M	4.8	M7	De novo
Patient 23	28	M	7.8	M4	Relapsed
Patient 24	42	M	10.5	M4	De novo

Table 4 Data of the patients' P-gp expression, functional efflux activity and CD34 expression.

Patient	P-gp expression	Functional efflux activity	CD34 expression
Patient 1	–	–	+
Patient 2	+	+	+
Patient 3	–	–	+
Patient 4	–	–	–
Patient 5	+	+	+
Patient 6	–	–	–
Patient 7	–	–	+
Patient 8	+	–	–
Patient 9	–	–	–
Patient 10	+	+	+
Patient 11	+	+	–
Patient 12	+	+	+
Patient 13	+	+	+
Patient 14	+	+	+
Patient 15	+	+	–
Patient 16	–	–	+
Patient 17	+	+	+
Patient 18	–	+	+

+ = Positive - = Negative

Table 4 (Continued) Data of the patients' P-gp expression, functional efflux activity and CD34 expression.

Patient	P-gp expression	Functional efflux activity	CD34 expression
Patient 19	–	–	+
Patient 20	–	–	+
Patient 21	–	–	–
Patient 22	–	–	–
Patient 23	–	–	–
Patient 24	+	+	+

+ = Positive - = Negative

Table 5 The crosstabulation between P-glycoprotein expression and age.

Age	P-glycoprotein expression		Total
	Positive	Negative	
1-54	7	11	18
>= 55	4	1	5
Total	11	12	23

Fisher's Exact Test p = 0.155

Table 6 The crosstabulation between P-glycoprotein expression and gender.

Gender	P-glycoprotein expression		Total
	Positive	Negative	
Male	6	5	11
Female	5	8	13
Total	11	13	24

Fisher's Exact Test p = 0.682

Table 7 The crosstabulation between P-glycoprotein expression and CD34 expression.

CD34 expression	P-glycoprotein expression		Total
	Positive	Negative	
Positive	8	7	15
Negative	3	6	9
Total	11	13	24

Fisher's Exact Test p = 0.423

Table 8 The crosstabulation between P-glycoprotein expression and WBC count.

WBC count	P-glycoprotein expression		Total
	Positive	Negative	
0-10,000	1	7	8
10,001-20,000	1	0	1
20,001-30,000	2	1	3
30,001-40,000	1	2	3
40,001-50,000	0	1	1
>50,000	5	2	7
Total	10	13	23

Pearson Chi-square $p = 0.146$

Table 9 The crosstabulation between P-glycoprotein expression and FAB subtype.

FAB subtype	P-glycoprotein expression		Total
	Positive	Negative	
M1	2	0	2
M2	2	3	5
M3	0	2	2
M4	5	6	11
M5	0	1	1
M7	0	1	1
Total	9	13	22

Pearson Chi-square $p = 0.331$

Table 10 The crosstabulation between P-glycoprotein expression and functional efflux activity.

Functional efflux activity	P-glycoprotein expression		Total
	Positive	Negative	
Positive	10	1	11
Negative	1	12	13
Total	11	13	24

Pearson Chi-square $p < 0.001$

Table 11 The crosstabulation between functional efflux activity and age.

Age	Functional efflux activity		Total
	Positive	Negative	
1-54	7	11	18
>= 55	4	1	5
Total	11	12	23

Fisher's Exact Test p = 0.155

Table 12 The crosstabulation between functional efflux activity and gender.

Gender	Functional efflux activity		Total
	Positive	Negative	
Male	6	5	11
Female	5	8	13
Total	11	13	24

Fisher's Exact Test p = 0.682

Table 13 The crosstabulation between functional efflux activity and CD34 expression.

CD34 expression	Functional efflux activity		Total
	Positive	Negative	
Positive	9	6	15
Negative	2	7	9
Total	11	13	24

Fisher's Exact Test p = 0.105

Table 14 The crosstabulation between functional efflux activity and WBC count.

WBC count	Functional efflux activity		Total
	Positive	Negative	
0-10,000	2	6	8
10,001-20,000	1	0	1
20,001-30,000	2	1	3
30,001-40,000	1	2	3
40,001-50,000	0	1	1
>50,000	4	3	7
Total	10	13	23

Pearson Chi-square $p = 0.481$

Table 15 The crosstabulation between functional efflux activity and FAB subtype.

FAB subtype	Functional efflux activity		Total
	Positive	Negative	
M1	1	1	2
M2	2	3	5
M3	0	2	2
M4	6	5	11
M5	0	1	1
M7	0	1	1
Total	9	13	22

Pearson Chi-square $p = 0.596$

CHAPTER V

DISCUSSION

Although a great number of studies have been conducted to analyze the expression of P-glycoprotein in hematologic malignancies and to determine its clinical relevance, accurate measurement of P-gp in clinical samples still remains a controversial issue (12). Detection methods and diagnosis of the MDR phenotype in clinical samples have not been standardized and agree upon so far (73). Up until now, there have been a number of diagnostic methods to detect MDR phenotype in the RNA level: Northern blot, slot blot, in situ hybridization, RNAse protection assay, reverse transcriptase-polymerase chain reaction as well as in the protein level: Western blot, immunohistochemistry, immunocytochemistry, flow cytometry, to choose from. No single diagnostic method is flawless. Each method carries with it advantages as well as disadvantages.

This study using flow cytometry technique to measure the percentage of cells expressing fluorescence intensity beyond that of control more than 20 percent revealed that P-gp was detected in 11 cases (45.83%) of the combined de novo and relapsed AML cases. In fact, P-gp has been previously reported in virtually wide variety of ranges because of the differences in the method of detection: Northern blot, slot blot, in situ hybridization, RNAse protection assay, reverse transcriptase-polymerase chain reaction, Western blot, immunohistochemistry, immunocytochemistry, flow cytometry. Even within a single method such as flow cytometry, different centers use different protocols. A number of factors between

different protocols contribute to the differences in the percentage of P-gp reported such as the type of antibody, the concentration of antibody, the cut-off point, the use of direct or indirect technique, the incubation period or the incubation temperature.

Our result confirmed the previous studies that P-gp expression was related to functional efflux activity:$MRK16^{+}/efflux^{+}$ or $MRK16^{-}/efflux^{-}$. Only a few cases were atypical cases in which functional drug efflux and MDR1 gene expression were discordant. For example, there were cases which demonstrated $MRK16^{-}$/efflux+ as well as $MRK16^{+}$/efflux-. The plausible explanation for these are

1) The detection method used is not sensitive enough to detect the low level of P-gp present in the clinical samples (6).

2) Non-MDR1-mediated mechanisms of drug resistance are operating (6) since reduced intracellular drug accumulation in MDR^{-} cell lines is well described, indicating the existence of alternative drug efflux mechanisms (139,140,141,142). In addition, Ivy et al reported a case in which a P-gp-negative patient showed efflux which was not modulated by verapamil (143). Norgaard et al detected cases with undetectable expression of MDR1 gene, but showed drug resistance towards daunorubicin, doxorubicin, etoposide and mitoxantrone (144).

3) There may be an alteration in the epitope which monoclonal antibody MRK16 recognizes (143).

4) The MDR1 protein level is too low.

5) The protein is modified in some ways to an inactive or nonfunctional form (145,146).

In order to improve the sensitivity and the reliability of the detection method, some authors advocate the use of a panel of antibodies such as C219, JSB-1 and MRK16 (8,147)

The association between P-gp and CD34 expression is still a controversy. Some reports showed the closed relationship to CD34 expression (139,143,148), but not in others (149). In this study, we found that P-gp expression was not associated with CD34 expression. In a similar fashion, functional efflux activity was not associated with CD34 expression either.

Leith et al stated that AML in the elderly was frequently resistance to chemotherapy and that the overall outcomes remain extremely poor (136) so this study try to establish whether P-gp expression has the association with age or not. From the result of this study, it was shown that P-gp expression was not associated with age. Further more, P-gp expression was found to be unassociated with gender, WBC count and FAB subtype.

The striking feature concerning the FAB subtype was the unusually high proportion of FAB M4 in this study. This particular subtype accounted for 50.0% of all the cases. This figure was consider high compared to previous studies such as 6.67% by Macfarland et al (150), 9.95% by Leith et al (136), 22.0% by Martinez et al (151), 23.21 by Pokatikorn et al (152), 29.27% by Pallis et al (146), 30.0% by Chevillard et al (153). This finding either reflects the most common subtype of AML among the Thai population or it may just be a coincidence. Whatever is true awaits further and larger-scaled study. There have been reports describing the strong relationship between the P-gp-negativity and FAB M3 (19,92,93). The two cases of FAB M3 in this study did show P-gp-negativity as well as negative functional efflux activity. This data lend support for the reason why AML FAB M3 generally responds well to chemotherapy (154,155).

This study was in accordance with previous reports in that the percentage of P-gp positivity among 2 of the 3 relapsed cases (66.67%) was higher than that of the de novo cases (45.83%) (15,21,22,143,150). The majority of the relapsed cases showed functional efflux activity. This indicates that P-gp not only presents, but functions as well in the relapsed cases. This fact provides the reason for the generally observed poor outcome in relapsed cases compared to the de novo cases. At first, the number of leukemic cells bearing the MDR1 gene may be minute. Upon exposing to the chemotherapeutic agents, the leukemic clones bearing the MDR1 gene may survive the hostile environment imposed upon them by the chemotherapeutic agents administered, while other clones not possessing the MDR1 gene are less tolerant and pass away. Owing to the selective process, the MDR1-bearing clones ultimately outnumber the non-MDR1-bearing clones. Another point to be considered is that the MDR1 clones may evolve from the non-MDR1 clones upon exposing to the chemotherapeutic agents as there have been reports mentioning the development of the drug resistant cell lines from the drug sensitive cell lines by stepwise selection with chemotherapeutic agents. In this manner, the chemotherapeutic agents behave as a double-edged sword. They kill the leukemic cells on one hand, while promoting the development of the drug resistant cells on the other. The future rests on the researcher's hands to find ways and means to make the utmost use of the former while minimizing or preventing the latter from happening.

The identification of the MDR-positive tumor cells has several potential applications. It may be used as prognostic indicator to predict response to chemotherapy, to guide the clinician in recruiting patients who are strong candidates for treatment regimens incorporating the MDR modulators since the initial institution

of treatment, at which time the tumor burden is small and fewer mechanisms of drug resistance may be present to circumvent the multidrug resistance that may develop if the MDR modulators are not used since the beginning of the treatment or to identify the MDR-negative patients who should not be exposed to the MDR modulators because such drugs may not be of benefit to the patient (6,9). List and colleagues incorporated cyclosporin A into the treatment regimen and found an improved overall response rate of 69% (156). In addition, 4 of 5 MDR-positive leukemic patients in the study of Leith et al subsequently relapsed with MDR^- disease, suggesting that the therapy eliminated the MDR-positive blasts (6). A similar result was achieved by Marie and colleagues (27). These studies suggest that protocols which incorporate MDR modulators may benefit patients with MDR^+ AML. All of these would reduce the treatment failure, the expense especially for the cost in treating multidrug resistant patients. Furthermore, toxicity, morbidity and mortality would also be lessened.

In addition to MDR modulators, the use of chemotherapeutic drugs which are not substrates of MDR1 protein such as high dose cytosine arabinoside, alkylating agents or the anthracycline derivative aclarubicin which is chemically different from the classic anthracyclines (daunorubicin and doxorubicin) and could circumvent the drug resistance of MDR cell lines resistant to daunorubicin and doxorubicin, may offer another alternative for treating MDR-positive tumor cells (144,157).

CHAPTER VI

CONCLUSION

In this study, we tried to detect P-glycoprotein (P-gp) expression and functional efflux activity by the use of flow cytometer. We found that

1. P-gp was detected in 45.83% of the cases.
2. Functional efflux activity was detected in 45.83% of the cases.
3. P-gp expression was strongly correlated with functional efflux activity ($p< 0.001$).
4. P-gp expression was not correlated with age ($p=0.155$), gender ($p=0.682$), CD34 expression ($p=0.423$), WBC count ($p=0.146$) nor FAB subtype ($p=0.331$).
5. P-gp expression was detected more frequently in relapsed cases than in de novo AML cases.

The identification of biologic and clinical features associated with a poor prognosis is an essential step for developing a rational approach to improve the poor therapeutic outcome of AML patients. The identification of P-gp expression along with the functional efflux activity are particularly interesting since they suggest that therapies incorporating MDR1 modulators may be potentially beneficial to AML patients. The use of MDR1 modulators might have a tremendous impact on the AML patients who have MDR^+ leukemic cells since these MDR1 modulators can enhancing their cytotoxicities.

REFERENCES

1. Kantarjian HM. Adult acute lymphocytic leukemia: Critical review of current knowledge. Am J Med 1994;97:176-84.
2. Head DR, Behm FG. Acute lymphoblastic leukemia and the lymphoblastic lymphomas of childhood. Semin Diagn Pathol 1995;12:325-34.
3. Cripe LD. Adult acute leukemia. Curr Probl Cancer 1997;21:1-64.
4. Silverman LB, McLean TW, Gelber RD, Donnelly MJ, Gilliland DG, Tarbell NJ, et al. Intensified therapy for infants with acute lymphoblastic leukemia: results from the Dana-Farber Cancer Institute Consortium. Cancer 1997;80:2285-95.
5. Chabner BA, Bates SE, Fojo AT, Spolyar M, Wilson WH. Drug resistance in adult lymphomas. Semin Hematol 1994;31:70-87.
6. Leith CP, Chen IM, Kopecky KJ, Appelbaum FR, Head DR, Godwin JE, et al. Correlation of multidrug resistance(MDR1) protein expression with functional dye/drug efflux in acute myeloid leukemia by multiparameter flow cytometry: identification of discordant MDR-/efflux+ and MDR+/efflux- cases. Blood 1995;86:2329-42.
7. Linker CA, LevittLJ, O'Donnell M, Forman SJ, Ries CA. Treatment of adult acute lymphoblastic leukemia with intensive cyclical chemotherapy: a follow-up of report. Blood 1991;78:2814-22.
8. Kantarjian H, O'Brien S, Beran M. Update of the hyper-CVAD program in newly diagnosed adult acute lymphocytic leukemia (ALL). Blood 1995;86:173a.

9. Goldstein LJ. Clinical reversal of drug resistance. Curr Probl Cancer 1995; 19:65-124.

10. van der Heyden S, Gheuens E, DeBruijn E, Van Oosterrom A, Maes R. P-glycoprotein: Clinical significance and methods of analysis. Crit Rev Clin Lab Sci 1995;32:221-64.

11. Malaeri R, Filipits M, Suchomel RW, Zochbauer S, Lechner K, Pirker R. Multidrug resistance in leukemias and its reversal. Leuk Lymphoma 1996;23:451-8.

12. Pall G, Spitaler M, Hofmann J, Thaler J, Ludescher C. Multidrug resistance in acute leukemia: a comparison of different diagnostic methods. Leukemia 1997;11:1067-72.

13. Nooter K, Stoter G. Molecular Mechanisms of multidrug resistance in cancer chemotherapy. Pathol Res Practice 1996;192:768-80.

14. Den Boer ML, Zwaan CM, Pieters R, Kazemier KM, Rottier MM, Flens MJ, et al. Optimal immunocytochemical and flow cytometric detection of P-gp, MRP and LRP in childhood acute lymphoblastic leukemia. Leukemia 1997;1:1078-85.

15. Guerci A, Merlin JL, Missoum N, Feldmann L, Marchal S, Witz F, et al. Predictive value for treatment outcome in acute myeloid leukemia of cellular daunorubicin accumulation and P-glycoprotein expression simultaneously determined by flow cytometry. Blood 1995;85 :2147-53.

16. Gruber A, Vitols S, Norgren S, Arestrom I, Peterson C, Bjorkholm M, et al. Quantitative determination of mdr1 gene expression in leukemic cells

from patients with acute leukemia. Br J Cancer 1992;66:266-72.

17. Campos L, Guyotat D, Archimbaud E, Calmard-Oriol P, Tsuruo T, Troncy J, et al. Clinical significance of multidrug resistance P-glycoprotein expression on acute nonlymphoblastic leukemia cells at diagnosis. Blood 1992;79:473-6.

18. Wood P, Burgess R, MacGregor A, Yin JA. P-glycoprotein expression on acute myeloid leukemia blast cells at diagnosis predicts response to chemotherapy and survival. Br J Haematol 1994;87:509-14.

19. Del Poeta G, Stasi R, Aronica G, Venditti A, Cox MC, Bruno A, et al. Clinical relevance of P-glycoprotein expression in de novo acute myeloid leukemia. Blood 1996;87:1997-2004.

20. Holmes J, Jacobs A, Carter G, Janowska-Wieczorek A, Padua RA. Multidrug resistance in haematopoietic cell lines, myelodysplastic syndromes and acute myeloblastic leukemia. Br J Haematol 1989;72:40-4.

21. Nussler V, Pelka-Fleischer R, Zwierzina H, Nerl C, Beckert B, Gieseler F, et al. P-glycoprotein expression in patients with acute leukemia-clinical relevance. Leukemia 1996; 10 Suppl 3:S23-S31.

22. Hart HM, Ganeshaguru K, Lyttelton MP, Prentice HG, Hoffbrand AV, Mehta AB. Flow cytometric assessment of multidrug resistance (MDR) phenotype in acute myeloid leukemia 1993;11:239-48.

23. Musto P, Melillo L, Lombardi G, Matera R, di Giorgio G, Carotenuto M. High risk of early resistance relapse for leukemic patients with presence of multidrug resistance associated P-glycoprotein positive cells in complete remission. Br J Haematol 1991;77:50-3.

24. te Boekhorst PA, Lowenberg B, van Kapel J, Nooter K, Sonneveld P. Multidrug resistance cells with high proliferative capacity determine response to therapy in acute myeloid leukemia. Leukemia 1996;9:1025-31.

25. Pogliani EM, Belotti D, Rivolta GF, Maffe PF, Corneo G. Anthracycline drugs and MDR expression in human leukemia. Cytotechnology 1996;19:229-35.

26. Willman CL. The prognostic significance of the expression and function of multidrug resistance transporter proteins in acute myeloid leukemia: studies of the Southwest Oncology Group Leukemia Research Program. Semin Hematol 1997;34(4 Suppl 5):25-33.

27. Marie JP, Bastie JN, Coloma F, Faussat Suberville AM, Delmer A, Rio B, et al. Cyclosporin A as a modifier agent in the salvage treatment of acute leukemia(AL). Leukemia 1993;7:821-4.

28. Leyland-Jones B, Dalton W, Fisher GA, Sikic BI. Reversal of multidrug resistance to cancer chemotherapy. Cancer 1993;72(11 Suppl):3484-8.

29. Ford JM, Yang JM, Hait WN. P-glycoprotein-mediated multidrug resistance: experimental and clinical strategies for its reversal. Cancer Treatment Res 1996;87:3-38.

30. Chen CJ, Chin JE, Ueda K, Clark DP, Pastan I, Gottesman MM, et al. Internal duplication and homology with bacterial transport proteins in the mdr1(P-glycoprotein) gene from multidrug-resistance human cells. Cell 1986;47:381-9.

31. Eisenberg D, Schwarz E, Komaromy M, Wall R. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. J Mol Biol 1984;179:125-42.

32. Chambers TC, Pohl J, Raynor RL, Kuo JF. Identification of specific sites in human P-glycoprotein phosphorylated by protein kinase C. J Biol Chem 1993;268:4592-5.

33. Rosenberg MF, Callaghan R, Ford RC, Higgins CF. Structure of the multidrug resistance P-glycoprotein to 2.5 nm resolution determined by electron microscopy and image analysis. J Biol Chem 1997;272:10685-94.

34. Georges E, Tsuruo T, Ling V. Topology of P-glycoprotein as determined by epitope mapping of MRK16 monoclonal antibody. J Biol Chem 1993 ;268:1792-8.

35. Yoshimura A, Kuwazuru Y, Sumizawa T, Ichikawa M, Ikeda S, Uda T, et al. Cytoplasmic orientation and two-domain structure of the multidrug transporter, P-glycoprotein, demonstrated with sequence-specific antibodies. J Biol Chem 1989;264:16282-91.

36. Senior AE, Gadsby DC. ATP hydrolysis cycles and mechanism in P-glycoprotein and CFTR. Semin Cancer Biol 1997;8:143-50.

37. Ambudkar SV, Lelong IH, Zhang J, Cardarelli CO, Gottesman MM, Pastan I. Partial purification and reconstitution of the human multidrug-resistance pump: characterization of the drug-stimulatable ATP hydrolysis. Proc Natl Acad Sci USA 1992;89:8472-6.

38. Sarkadi B, Price EM, Boucher RC, Germann UA, Scarborough GA. Expression of the human multidrug resistance cDNA in insect cells

generates a high activity drug-stimulated membrane ATPase. J Biol Chem 1992;267:4854-8.

39. Loo TW, Clark DM. Inhibition of oxidative cross-linking between engineered cysteine residues at positions 332 in predicted transmembrane segments (TM) 6 and 975 in predicted TM12 of human P-glycoprotein by drug substrates. J Biol Chem 1996;271:27482-7.

40. Sonneveld N, Shapiro AB, Goormaghtigh E, Ling V, Ruysschaert JM. Secondary and tertiary structure changes of reconstituted P-glycoprotein. A Fourier transform attenuated total reflection infrared spectroscopy analysis. J Biol Chem 1996;271:24617-24.

41. Kornfeld R, Kornfeld S. Assembly of asparagine-linked oligosaccharides. Annu Rev Biochem 1985;54:631-64.

42. Schinkel AH, Kemp S, Dolle M, Rudenko G, Wagenaar E. N-glycosylation and deletion mutations of the human MDR1 P-glycoprotein. J Biol Chem 1993;268:7474-81.

43. Chambers TC, Pohl J, Glass DB, Kuo JF. Phosphorylation by protein kinase C and cyclic AMP-dependent protein kinase of synthetic peptides derived from the linker region of human P-glycoprotein. Biochem J 1994;299(Pt 1):309-15.

44. Goodfellow HR, Sardini A, Ruetz S, Callaghan R, Gros P, McNaughton PA, et al. Protein kinase C-mediated phosphorylation does not regulate drug transport by the human multidrug resistance P-glycoprotein. J Biol Chem 1996;271:13668-74.

45. Hardy SP, Goodfellow HR, Valverde MA, Gill DR, Sepulveda V, Higgins CF. Protein kinase C-mediated phosphorylation of the human multidrug resistance P-glycoprotein regulates cell volume-activated chloride channels. EMBO J 1995;14:68-75.

46. Riordan JR, Romments JM, Kerem B, Alon n, Rozmahel R, Grzelczak Z, et al. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 1989;245:1066-73.

47. Kast C, Canfield V, Levenson R, Gros P. Membrane topology of P-glycoprotein as determined by epitope insertion: transmembrane organization of the N-terminal domain of mdr3. Biochemistry 1995 ;34:4402-11.

48. Kast C, Canfield V, Levenson R, Gros P. Transmembrane organization of mouse P-glycoprotein determined by epitope insertion and immunofluorescence. J Biol Chem 1996;271:9240-8.

49. Loo TW, Clark DM. Membrane topology of a cysteine-less mutant of human P-glycoprotein. J Biol Chem 1995;270:843-8.

50. Zhang X, Collins KI, Greenberger LM. Functional evidence that transmembrane 12 and the loop between transmembrane 11 and 12 form part of the drug-binding domain in P-glycoprotein encoded by MDR1. J Biol Chem 1995;270:5441-8.

51. Higgins CF, Gottesman MM. Is the multidrug transporter a flippase? Trend Biochem Sci 1992;17:18-21.

52. Holmoya L, Hollo Z, German UA, Pastan I, gottesman MM, Sarkadi B. Fluorescent cellular indicators are extruded by the multidrug

resistance protein. J Biol Chem 1993;268:21493-6.

53. Higgins CF. ABC transporters: from microorganisms to man. Annu rev Cell Biol 1992;8:67-113.

54. Schinkel AH. The physiological function of drug-transporting P-glycoproteins. Semin Cancer Biol 1997;8:161-70.

55. Endicott JA, Ling V. The biochemistry of P-glycoprotein-mediated multidrug resistance. Ann Rev Biochem 1989;58:137-71.

56. Loo TW, Clark DM. Functional consequences of phenylalanine mutations in the predicted transmembrane domain of P-glycoprotein. J Biol Chem 1993;268:19965-72.

57. Loo TW, Clark DM. Functional consequences of glycine mutations in the predicted cytoplasmic loops of P-glycoprotein. J Biol Chem 1994 ;26:7243-8.

58. Bruggemann EP, Currier SJ, Gottesman MM, Pastan I. Characterization of the azidopine and vinblastine binding site of P-glycoprotein. J Biol Chem 1992;267:21020-6.

59. Greenberger LM, Lisanti CJ, Silva JT, Horwitz SB. Domain mapping of the photoaffinity drug-binding sites in P-glycoprotein encoded by mouse mdr1b. J Biol Chem 1991;266:20744-51.

60. Lelong IH, Padmanabhan R, Lovelace E, Pastan I, Gottessman MM. ATP and GTP as alternative energy sources for vinblastine transport by P-170 in KB-V1 plasma membrane vesicles. FEBS Lett 1992;304:256-60.

61.Hamada H, Tsuruo T. Purification of the 170- to 180-kilodalton membrane glycoprotein associated with multidrug resistance. 170- to 180-

kilodalton membrane glycoprotein is an ATPase. J Biol Chem 1988 ;263:1454-8.

62. Horio M, Gottesman MM, Pastan I. ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. Proc Natl Acad Sci USA 1988;85:3580-4.

63. Tsuji A, Tamai I, Sakata A, Tenda Y, Terasaki T. Restricted transport of cyclosporin A across the blood-brain barrier by a multidrug transporter, P-glycoprotein. Biochem Pharmacol [Online] 1993 Sep;46(6):1096-9. Abstract from TOP's Medline on the Web 1993-94: Unique identifier 94030063

64.Gros P,Raymond M, Bell J, Housman D. Cloning and characterization of a second member of the mouse mdr gene family. Mol Cell Biol 1988 Jul;8:2770-8.

65. Lincke CR, Smith JJ, van der Velde-Koerts T, Borst P. St5ructure of the human MDR3 gene and physical mapping of the human MDR locus. J Biol Chem 1991;266:5303-10.

66. Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y. Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res 1981;41:1967-72.

67. Slater LM, Sweet P, Stupecky M, Gupta S. Cyclosporin A reverses vincristine and daunorubicin resistance in acute lymphocytic leukemia in vitro. J Clin Invest 1986;77:1405-8.

68. Twentyman PR. Modification of cytotoxic drug resistance by non-immunosuppressive cyclosporins. Br J Cancer 1988;57:254-8.

69. Yang CP, DePinho SG, Greenberger LM, Arceci RJ, Horwitz SB. Progesterone interacts with P-glycoprotein in multidrug-resistance cells and in the endometrium of gravid uterus. J Biol Chem 1989;264:782-8.

70. Safa AR. Photoaffinity labeling of P-glycoprotein in multidrug-resistant cells. Cancer Invest 1993;11:46-56.

71. Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 1993;62:385-427.

72. Feruson LR, Baguley BC. Multidrug resistance and mutagenesis. Mutat Res [Online] 1993 Jan;285(1):79-90. Abstract from TOP's Medline on the Web 1993-94: Unique identifier 93116729

73. Fardel O, Lecureur V, Guillouzo A. The P-glycoprotein multidrug transporter. Gen Pharmacol 1996;27:1283-91.

74. Beaulieu E, Demeule M, Ghitescu L, Beliveau R. P-glycoprotein is strongly expressed in the luminal membranes of the endothelium of the blood vessels in the brain. Biochem J 1997;326(Pt 2):539-44.

75. Schinkel AH, Wagenaar E, Mol CA, van Deemter L. P-glycoprotein in the blood -brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. J Cline Invest 1996;97:2517-24.

76. Arceci RJ, Croop JM, Horwitz SB, Housman D. The gene encoding multidrug resistance is induced and expressed at high levels during pregnancy in the secretory epithelium of the uterus. Proc Natl Acad Sci USA 1988;85 :4350-4.

77. valverde MA, Diaz M, Sepulveda FV, Gill DR, Hyde SC, Higgins CF. Volume-regulated chloride channels associated with the human multidrug-resistance P-glycoprotein. Nature 1992;355:830-3.

78. Zhang JJ, Jacob TJ. ATP-activated chloride channel inhibited by an antibody to P -glycoprotein. Am J Physiol 1994;267(4 Pt 1):C1095-102.

79. Valverde MA, Bond TD, Hardy SP, Taylor JC, Higgins CF, Altamirano J, et al. The multidrug resistance P-glycoprotein modulates cell regulatory volume decrease. EMBO J 1996;15:4460-8.

80. Gill Dr, Hyde SC, Higgins CF, Valverde MA, Mintenig GM, Sepulveda FV. Separation of drug transport and chloride channel function of the human multidrug resistance P-glycoprotein. Cell 1992:71:23-32.

81. Wang JC, Beauregard P, Soamboonsrup P, Neame PB. Monoclonal antibodies in the management of acute leukemia. Am J Hematol 1995;50:188-99.

82. Willingham MC, Richert ND, Cornwell MM, Tsuruo T, Hamada H, Gottesman MM, et al. Immunohistochemical localization of P170 at the plasma membrane of multidrug-resistance human cells. J Histochem Cytochem 1987;35:1451-6.

83. Scheper RJ, Bulte JW,Brakkee JG, Quak JJ, van der Schoot E, Balm AJ, et al. Monoclonal antibody JSB-1 detects a highly conserved epitope on the P-glycoprotein associated with multidrug resistance. Int J Cancer 1988 ;42:389-94.

84. Poloni F, Romagnoli G, Cianfriglia M, Felici F. Isolation of antigenic mimics of MDR1- P-glycoprotein by phase-displayed peptide libraries. Int J Cancer 1995;61:727-31.

85. Arceci RJ, Stieglitz K, Bras J, Schinkel A, Baas F, Croop J. Monoclonal antibody to an external epitope of the human mdr1 P-glycoprotein. Cancer Res 1993;53:310-7.

86. Chin-Yee I, Alshammari S, Anderson L, Kadri M, Keeney M. Comparison of two methods to detect P-glycoprotein in patients with chronic lymphocytic leukaemia. Clin Lab Haematol 1996;18:99-104.

87. Chu TM, Kawinski E, Lin TH. Characterization of a new monoclonal antibody F4 detecting cell surface epitope and P-glycoprotein in drug-resistant human tumor cell lines. Hybridoma [Online] 1993 Aug;12(4):417-29. Abstract from TOP's Medline on the Web 1993-94: Unique identifier 94063953

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

89. Georges E, Bradley G, Gariepy J, Ling V. Detection of P-glycoprotein isoforms by gene-specific monoclonal antibodies. Proc Natl Acad Sci USA 1990 ;87:152-6.

90. Cordon-cardo C, O'Brien JP, Boccia J, Casals D, Bertino JR, Melamed MR. Expression of the multidrug resistance gene product P-glycoprotein in human normal and tumor tissues. J Histochem Cytochem 1990;3:1277-87.

91. Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, Willingham MC. Immunohistochemical localization in normal tissues of different epitopes in the multidrug transport protein P170: evidence for localization in brain

capillaries and crossreactivity of one antibody with a muscle protein. J Histochem Cytochem 1989;37:159-64.

92. Del Poeta G, Venditti A, Aronica G, Stasi R, Cox MC, Buccisano F, et al. P-glycoprotein expression in de novo acute myeloid leukemia. Leuk Lymphoma 1997;27:257-74.

93. Del Poeta G, Venditti A, Stasi R, Aronica G, Cox MC, Buccisano F, et al. P-glycoprotein and terminal transferase expression identify prognostic subsets within cytogenetic risk classes in acute myeloid leukemia. Leuk Res 1999;23:451-65.

94. Rao VV, Anthony DC, Piwnica-Worms D. Multidrug resistance P-glycoprotein monoclonal antibody JSB-1 crossreacts with pyruvate carboxylase. J Histochem Cytochem 1995;43:1187-92.

95. Mechetner EB, Roninson IB. Efficient inhibition of P-glycoprotein-mediated multidrug resistance with a monoclonal antibody. Proc Natl Acad Sci USA 1992;89:5824-8.

96. Mechetner EB, Schott B, Morse BS, Stein WD, Druley T, Davis KA, et al. P-glycoprotein function involves conformational transformations detectable by differential immunoreactivity. Proc Natl Acad Sci USA 1997;94 :12908-13.

97. Hamada H, Tsuuo T. Functional role for the 170- to 180-kDa glycoprotein specific to drug-resistant tumor cells as revealed by monoclonal antibodies. Proc Natl Acad Sci USA 1986;83:7785-9.

98. Okochi E, Iwahashi T, Tsuruo T. Monoclonal antibodies specific for P-glycoprotein. Leukemia 1997;11:1119-23.

99. Heike Y, Tsuruo T. Antibodies in the study of multiple drug resistance. Cytotechnology 1993;12:91-107.

100. Hamada H, Miura K, Ariyoshi K, Heike Y, Sato S, Kameyama K, et al. Mouse-human chimeric antibody against the multidrug transporter P-glycoprotein. Cancer Res 1990;50:3167-71.

101. Rao VV, Anthono DC, Piwnica-Worms D. MDR1 gene-specific monoclonal antibody C494 cross-reacts with pyruvate carboxylase. Cancer Res 1994 ;54:1536-41.

102. Cenciarelli C, Currier SJ, Willingham MC, Thiebaut F, Germann UA, Rutherford AV, et al. Characterization by somatic cell genetics of a monoclonal antibody to the MDR1 gene product(P-glycoprotein): determination of P-glycoprotein expression in multi-drug-resistant KB and CEM cell variants. Int J Cancer 1991;47:533-43.

103. Rittman-Grauer LS, Yong MA, Sanders V, Mackensen DG. Reversal of vinca alkaloid resistance by anti-P-glycoprotein monoclonal antibody HYB-241 in a human tumor xenograft. Cancer Res 1992;52:1810-6.

104. Garberoglio C, Dudas M, Casper ES, Bertino J, Cordon-Cardo C. Expression of P-glycoprotein in normal muscle cells and myogenic tumors. Arch Pathol Lab Med 1992;116:1055-61.

105. Hyde SC, Emsley P, Hartshorn MJ, Mimmack MM, Gileadi U, Pearce SR, et al. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature 1990;3:362-5.

106. mackman N, Nicaud JM, Gray L, Holland IB. Secretion of haemolysin by *Escherichia coli.* Curr Top Microbiol Immunol 1986;125:159-81.

107. Krogstad DJ, Gluzman IY, Kyle DE, Oduola AM, Martin SK, Milhous WK, et al. Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. Science 1987;238:1283-5.

108. Martin SK, Oduola AM, Milhous WK. Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. Science 1987;235:899-901.

109. McGrath JP, Varshavsky A. The yeast STE6 gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein. Nature 1989;340 :400-4.

110. Tang-Wai DF, Kajiji S, DiCapua F, de Graaf D, Roninson IB, Gros P. Human (MDR1) and mouse (mdr1, mdr3) P-glycoprotein can be distinguished by their respective drug resistance profiles and sensitivity to modulators. Biochemistry 1995;34:32-9.

111. Ng WF, Sarangi F, Zastawny RL, Veinot-Drebot L, Ling V. Identification of members of the P-glycoprotein multigene family. Mol Cell Biol 1989 ;9:1224-32.

112. Chin JE, Soffir R, Noonan KE, Roninson IB. Structure and expression of the human MDR (P-glycoprotein) gene family. Mol Cell Biol 1989;9:3808-20.

113. van der Bliek AM, Kooiman PM, Schneider C, Borst P. Sequence of mdr3 cDNA encoding a human P-glycoprotein. Gene 1988;71:401-11.

114. Roninson IB, Chin JE, Choi KG, Gros P, Housman DE, Fojo A, et al. Isolation of human mdr DNA sequences amplified in multidrug-resistant KB carcinoma cells. Proc Natl Acad Sci USA 1986;83:4583-42.

115. Endicott JA, Juranka PF, Sarangi F, Gerlach JH, Deuchars KL, Ling V. Simultaneous expression of two P-glycoprotein genes in drug-sensitive Chinese hamster ovary cells. Mol Cell Biol 1987;7:4075-81.

116. Raymond M, Gros P. Mammalian multidrug-resistance gene: correlation of exon organization with structural domains and duplication of an ancestral gene. Proc Natl Acad Sci USA 1989;86:6488-92.

117. Ernest S, Rajaraman S, Megyesi J, Bello-Reuss EN. Expression of MDR (multidrug resistance) gene and its protein in normal human kidney. Nephron 1997;77:284-9.

118. Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, Willingham MC. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proc Natl Acad Sci USA 1987 ;84:7735-8.

119. Sugawara I. Expression and functions of P-glycoprotein (mdr1 gene product) in normal and malignant tissues. Acta Pathol Jpn [Online] 1990 Aug;40 (8):545-53. Abstract from TOP's Medline on the Web 1990-92: Unique identifier 91050556

120. Fojo AT, Ueda K, Slamon DJ, Poplack DG, Gottesman MM, Pastan I. Expression of a multidrug-resistance gene in human tumors and tissues. Proc Natl Acad Sci USA 1987;84:265-9.

121. Dalton WS, Grogan TM, Meltzer PS, Scheper RJ, Durie BG, Taylor CW, et al. Drug-resistance in multiple myeloma and non-Hodgkin's lymphoma: detection of P-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. J Clin Oncol 1989;7:415-24.

122. Goldstein LJ, Galski H, Fojo A, Willingham M, Lai SL, Gazdar A, et al. Expression of a multidrug resistance gene in human cancers. J Natl Cancer Inst 1989;81:116-24.

123. Lum BL, Gosland MP, Kaubisch S, Sikic BI. Molecular targets in oncology: implication of the multidrug resistance gene. Pharmacotherapy Mar-;13:88-109.

124. Biedler JL, Riehm H. Cellular resistance to actinomycin D in Chinese hamster cells in vitro: cross-resistance, radioautographic, and cytogenetic studies. Cancer Res 1970;30:1174-84.

125. Silbermann MH, Boersma AW, Janssen AL, Scheper RJ, Herweijer H, Nooter K. Effects of cyclosporin A and verapamil on the intracellular daunomycin accumulation in Chinese hamster ovary cells with increasing levels of drug-resistance. Int J Cancer 1989;44:722-6.

126. Cornwell MM, Pastan I, Gottesman MM. Certain calcium channel blockers bind specifically to multidrug –resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. J Biol Chem 1987 ;26:2166-70.

127. Naito M, Tsuruo T. Competitive inhibition by verapamil of ATP-dependent high affinity vincristine binding to the plasma membrane of multidrug-resistant K562 cells without calcium ion involvement. Cancer Res 1989;49:1452-5.

128. Safa AR. Photoaffinity labeling of the multidrug-resistance-related P-glycoprotein with photoactive analogs of verapamil. Proc Natl Acad Sci USA 1988;85:7187-91.

129. Kajiji S, Dreslin JA, Grizzuti K, Gros P. Structurally distinct MDR modulators show specific patterns of reversal against P-glycoproteins bearing unique mutations at serine939/941. Biochemistry 1994;33:5041-8.

130. Cardarelli CO, Aksentijevich I, Pastan I, Gottesman MM. Differential effects of P-glycoprotein inhibitors on NIH3T3 cells transfected with wild-type (G185) or mutant (V185) multidrug transporters. Cancer Res 1995 ;55:1086-91.

131. Miller TP, Grogan TM, Dalton WS, Spier CM, Scheper RJ, Salmon SE. P-glycoprotein expression in malignant lymphoma and reversal of clinical drug resistance with chemotherapy plus high-dose verapamil. J Clin Oncol 1991;9:17-24.

132. Pennock GD, Dalton WS, Roeske WR, Appleton CP, Mosley K, Plezia P, et al. Systemic toxic effects associated with high-dose verapamil infusion and chemotherapy administration. J Natl Cancer Inst 1991;83:105-10.

133. Solary E, Witz B, Caillot D, Moreau P, Desablens B, Cahn JY, et al. Combination of quinine as a potential reversing agent with mitoxantrone and cytarabine for the treatment of acute leukemias: a randomized multicenter study. Blood 1996;88:1198-205.

134. Leveque D, Jehl F. P-glycoprotein and pharmacokinetics. Anticancer Res 1995 ;15:331-6.

135. Broxterman HJ, Lankelma J, Pinedo HM, Eekman CA, Wahrer DC, Ossenkoppele GJ, et al. Theoretical and practical considerations for the management of P-glycoprotein function I acute myeloid leukemia. Leukemia 1997 ;11:1110-8.

136. Leith CP, Kopecky KJ, Godwin JE, McConnell T, Slovak ML, Chen IM, et al. Acute myeloid leukemia in the elderly: assessment of multidrug resistance (MDR1) and cytogenetics distinguishes biologic subgroups with remarkably distinct responses to standard chemotherapy. A Southwest Oncology Group study. Blood 1997;89:3323-9.

137. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group.Ann Intern Med 1985;103:620-5.

138. Homolya L, Hollo M, Muller M, Mechetner EB, Sarkadi B. A new method for a quantitative assessment of P-glycoprotein-related multidrug resistance in tumor cells. Br J Cancer 1996;73:849-55.

139. Bhalla K, Hindenburg A, Taub RN, Grant S. Isolation and characterization of an anthracycline-resistant human leukemic cell line. Cancer Res 1985 ;45:3657-62.

140. Baas F, Jongsma AP, Broxterman HJ, Arceci RJ, Housman D, Scheffer GL, et al. Non-P-glycoprotein mediated mechanism for multidrug resistance precedes P-glycoprotein expression during in vitro selection for doxorubicin resistance in a human lung cancer cell line. Cancer Res 1990 ;50:5392-8.

141. Marquardt D, Center MS. Drug transport mechanisms in HL60 cells isolated for resistance to adriamycin: evidence for nuclear drug accumulation and redistribution in resistant cells. Cancer Res 1992;52:3157-63.

142. Versantvoort CH, Broxterman HJ, Pinedo HM, de Vries EG, Feller N, Kuiper CM, et al. Energy-dependent processes involved in reduced drug accumulation in multidrug-resistant human lung cancer cell lines without P-glycoprotein expression. Cancer Res 1992;52:17-23.

143. Ivy SP, Olshefski RS, Taylor BJ, Patel KM, Reaman GH. Correlation of P-glycoprotein expression and function in childhood acute leukemia: a children's cancer group study. Blood 1996;88:309-18.

144. Norgaard JM, Bukh A, Langkjer ST, Clausen N, Palshof T, Hokland P. MDR1 gene expression and drug resistance of AML cells. Br J Haematol 1998 ;100:534-40.

145. Willman CL. Immunophenotyping and cytogenetics in older adults with acute myeloid leukemia: significance of expression of the multidrug resistance gene-1 (MDR1). Leukemia 1996;10 Suppl 1:S33-5.

146. Pallis M, Turzanski J, Harrison G, Wheatley K, Langabeer S, Burnett AK, et al. Use of standardized flow cytometric determinations of multidrug resistance to analyse response to remission induction chemotherapy in patients with acute myeloblastic leukaemia. Br J Haematol 1999;104:307-12.

147. Gottesman MM, Pastan I. The multidrug transporter, a double-edged sword. J Biol Chem 1988;263:12163-5.

148. List AF, Spier CM. Multidru resistance in acute leukemia: a conserved physiologic function. Leuk Lymphoma 1992;8:9-14.

149. Lamy T, Goasguen JE, Mordelet E, Grulois I, Dauriac C, Drenou B, et al. P-glycoprotein (P-170) and CD34 expression in adult acute myeloid

leukemia (AML). Leukemia 1994;8:1879-83.

150. MacFarland A, Dawson AA, Pearson CK. Analysis of MDR1 and MDR3 multidrug resistance gene expresion and amplification in consecutive samples in patients with acute leukaemias. Leuk Lymphoma 1995;19 :135-40.

151. Martinez A, San Miguel JF, Valverde B, Barez A, Moro MJ, Garcia-Marcos MA, et al. Functional expression of MDR1 in acute myeloid leukemia: correlation with the clinical-biological, immunophenotypical, and prognostic disease characteristics. Ann Hematol 1997;75:81-6.

152. Pokatikorn W, Apibal S, Atichartakarn V, Sriurairatana S, Chiewsilp D, Thitikawin P, Timvipark C. Cytochemical and immunological phenotyping of acute nonlymphoblastic leukemia in Thai patients. J Med Assoc Thai 1996;79:708-14.

153. Chevillard S, Vielh P, Validire P, Marie JP, Faussat AM, Barbu V, et al. French multicentric evaluation of mdr1 gene expression by RT-PCR in leukemia and solid tumours. Standardization of RT-PCR and preliminary comparisons between RT-PCR and immunohistochemistry in solid tumours. French Network of the Drug Resistance Intergroup, and Drug Resistance Network of Assistance Publique-Hopitaux de Paris. Leukemia 1997 ;11:1095-106.

154. Paietta E, Andersen J, Racevskiis J, Gallagher R, Bennett J, Yunis J, et al. Significantly lower P-glycoprotein expression in acute promyelocytic leukemia than in other types of acute myeloid leukemia: immunological, molecular and functional analyses. Leukemia 1994;8:968-73.

155. Drach D, Zhao S, Drach J, Andreeff M. Low incidence of MDR1 expression in acute promyelocytic leukaemia. Br J Haematol 1995;90:369-74.

156. List AF, Spier C, Greer J, Wolff S, Hutter J, Dorr R, et al. Phase I/II trial of cyclosporine as a chemotherapy-resistance modifier in acute leukemia. J Clin Oncol 1993;1:1652-60.

157. Haber DA. Multidrug resistance (MDR) in leukemia: is it time to test? Blood 1992;79:295-8.

BIOGRAPHY

NAME	Mr.Kittipong Dhanuthai
DATE OF BIRTH	7 November 1966
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTIONS ATTENDED	Chulalongkorn University, 1985-1991: Doctor of Dental Surgery Mahidol University, 1997-2000: Master of Science (Clinical Pathology)
RESEARCH GRANT	Research supporting fund, Faculty of Medicine, Ramathibodi Hospital 1999
POSITION&OFFICE	1991-present, Department of Oral pathology, Faculty of Dentistry, Chulalongkorn University, Thailand Position: Instructor