PHYSICOCHEMICAL PROPERTIES AND EFFECTS OF IODINATED RADIOGRAPHIC CONTRAST MEDIA ON CELLULAR ENERGETIC STATE OF K562 AND ITS CORRESPONDING MULTIDRUG RESISTANT CELL LINES

NITAYA SNITWONGSE NA AYUDHYA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE MASTER DEGREE OF SCIENCE
IN BIOLOGICAL SCIENCE
GRADUATE SCHOOL
BURAPHA UNIVERSITY
OCTOBER 2003

ISBN 974-382-521-5

The thesis of Nitaya Snitwongse Na Ayudhya has been approved by the examining committee to be partial fulfillment of the requirements for the Master Degree of Science in Biological Science of Burapha University

Advisory Committee

.....Principal advisor

(Dr. Samlee Mankhetkorn)

.....Member

(Assistant Professor Dr. Wichet Leelamanit)

.....Member

(Dr. Wisatre Kongcharoensuntorn)

Examining Committee

.....Principal examiner

(Dr. Samlee Mankhetkorn)

.....Member

(Assistant Professor Dr. Wichet Leelamanit)

.....Member

(Dr. Wisatre Kongcharoensuntorn)

.....Member

(Professor Dr. Jean-Paul Jay- Gerin)

.....Member

(Dr. Rachian Kosanlavit)

.....Member

(Assistant Professor Dr. Pichan Sawangwong)

This thesis has been approved by the Graduate School to be partial fulfillment of the requirements for the Master Degree of Science in Biological Science of Burapha University.

.....Dean of Graduate School

(Assistant Professor Dr. Pratoom Muongmee)

13 October 2003

ACKNOWLEDGEMENTS

I would like to express my gratitude to the Laboratory of Physical Chemistry, Molecular and Cellular Biology (PCMCB), Faculty of Science, Burapha University, Thailand, where I had performed my studies and experiments, and most of them to Dr.Samlee Mankhetkorn, Ph.D., Director of the Lab. and also, my advisor. Everything that concerned my Master Degree study would not have been done, or even, never ever had been started without him. My thesis is partly his guidance, advice, expenses, encouragement and patience.

I appreciate the helps and cheerful supports of my colleagues in the Lab., Chatchanok Loetchutinat, Samarn Dechsupa, Piboon Reungpatthanaphong, Jintana Meesungnoen, Suchart Kotan, and Montree Tungjai.

I am grateful to Professor Jean-Paul Jay-Gerin, Department of Nuclear Medicine and Radiobiolody, Faculty of Medicine, Universite' de Sherbrooke, Canada, for his supervisory and invaluable advices during my training at Universite' de Sherbrooke and as well, for his acceptance to be the jury of my thesis.

I gratefully acknowledge Asst. Prof. Dr. Wichet Leelamanit, Ph.D., Dr. Wisatre Kongcharoensuntorn, Ph.D., Dr. Rachian Kosanlavit, Ph.D. and Asst. Prof. Dr. Pichan Sawangwong Ph.D. who accepted to be juries of my thesis and their discussions and advices during my thesis performing.

I am indebted to Sappasittiprasong Hospital for permission to do my M.Sc.study and to my colleagues at work for doing my jobs in Radiology Department during my study.

And finally, thanks to my beloved family, I am indefinitely indebted for their loves.

Nitaya Snitwongse Na Ayudhya

43911075: สาขาวิชา: วิทยาศาสตร์ชีวภาพ (ชีวฟิสิกส์); วท.ม. (วิทยาศาสตร์ชีวภาพ)
คำสำคัญ: การดื้อยาแบบ multidrug resistance (การดื้อต่อยาหลายขนาน)/สารทึบรังสี/
พี-ไกลโคโปรตีน/ ความต่างศักย์เมมเบรนไมโตคอนเดรีย

นิตยา สนิทวงศ์ ณ อยุธยา: คุณสมบัติเชิงเคมีฟิสิกส์และผลของสารทึบรังสีต่อระดับพลังงานของเซลล์ K562 ที่ไวและดื้อต่อยาหลายขนาน อาจารย์ผู้ควบคุมวิทยานิพนธ์: สำรี มั่นเขตต์กรน์, Ph.D., วิเชษฐ์ ลีลามานิตย์, Ph.D., วิสาตรี คงเจริญสุนทร, Ph.D. 54 หน้า.
ปี พ.ศ. 2546. ISBN 974-382-521-5

การดื้อยาของเซลล์มะเร็งแบบ multidrug resistance เป็นอุปสรรคในการรักษาผู้ป่วยโดยวิธีเคมีบำบัด ซึ่งเซลล์มะเร็งจะลดการตอบสนองต่อพิษของยาต้านมะเร็งทั้งชนิดที่เคยและไม่เคยใช้ในการรักษามาก่อน เนื่องจากมีการขับยาออกนอกเซลล์โดยโปรตีนขนส่ง เช่น P-glycoprotein (P-gp) มีความพยายามในการค้นหาสารประกอบหรือโมเลกุลที่มีผลต่อเซลล์ที่ดื้อยาให้มีการตอบสนองต่อการรักษาดีขึ้น งานวิจัยนี้ได้ทำการศึกษาสารทึบรังสีสามชนิดที่ใช้ในการประเมินอาการผู้ป่วยโรคมะเร็งในงานรังสีวินิจฉัย ได้แก่ สารทึบรังสีชนิดแตกตัวได้ (diatrizoate) และสารทึบรังสีชนิดไม่แตกตัว (iotrolan และ iopromide) ในเซลล์มะเร็งเม็ดเลือดแดงชนิดที่ไว (K562) และดื้อต่อยาadriamycin (K562/adr) ซึ่งมีการแสดงออกมากเกินพอของ P-gp เพื่อศึกษาคุณสมบัติในเชิงเคมีฟิสิกส์โดยการใช้ spectrophotometer พบว่าค่าการละลายในไขมันของ diatrizoate, iopromide และ iotrolan ซึ่งแสดงค่าโดย log P มีค่าเท่ากับ -1.11, -0.22 และ -1.23 ตามลำดับ ในการศึกษาความเป็นพิษในในระดับเซลล์ โดยวิธี colorimetric MTT assay พบว่า diatrizoate, iopromide และ iotrolan (0-3500 μM)ไม่มีผลต่อการเจริญเติบโตของเซลล์มะเร็งทั้ง2 ชนิด ในขณะที่เมื่อใช้บ่มร่วมกับยาต้านมะเร็ง daunorubicin (DNR) พบว่าสารทึบรังสีทั้งสามชนิดโดยเฉพาะ diatrizoate เพิ่มความเป็นพิษของ DNR โดยค่า IC_{50} ลดลงจาก 610.7 $\pm$ 74.5 nM เป็น 360.3 $\pm$ 108.9 nM เฉพาะในเซลล์ K562/adr การศึกษาผลของสารทึบรังสีต่อการเปลี่ยนแปลงระดับพลังงานของเซลล์ โดยติดตามการเรืองแสงของ rhodamine B พบว่า สารทึบรังสีทั้งสามชนิดสามารถเปลี่ยนแปลงความต่างศักย์เมมเบรนไมโตคอนเดรีย ($\Delta\Psi_m$) ในเซลล์ K562/adr โดยที่ diatrizoate สามารถเปลี่ยนแปลงได้มากที่สุดประมาณ 50 % (จาก -162 mV เป็น -86 mV) และเมื่อทำการศึกษาผลของ diatrizoate, iopromide และ iotrolan ต่อจลศาสตร์ของ P-gp ในเซลล์ K562/adr โดยการติดตามสัญญาณการเรืองแสงของ DNR เมื่อเปรียบเทียบโดยใช้พารามิเตอร์ k_a^i/k_a^0 พบว่า diatrizoate มีผลต่อจลศาสตร์ของ P-gp ในทางลดลงมากที่สุด จากการศึกษานี้แสดงให้เห็นว่าสารทึบรังสีทั้งสามชนิดมีความสามารถในการเปลี่ยนแปลง $\Delta\Psi_m$ และมีผลต่อการทำงานของ P-gp ในเซลล์ K562/adr โดยที่ความ

สามารถนี้ไม่ได้ขึ้นอยู่กับค่าการละลายในไขมันของโมเลกุล เนื่องจาก diatrizoate มีประสิทธิภาพมากที่สุดในจำนวนโมเลกุลทั้งสามชนิด คุณสมบัติเหล่านี้ทำให้มีความเป็นไปได้ในการที่จะนำ diatrizoate มาใช้ร่วมในการสร้างภาพเซลล์มะเร็งที่ดื้อต่อยา อีกทั้งการใช้ร่วมกับยารักษาโรคมะเร็งเพื่อลดการดื้อยาในการรักษาแบบเคมีบำบัด ซึ่งเหล่านี้ต้องทำการศึกษาต่อไป

43911075: MAJOR: BIOLOGICAL SCIENCE (BIOPHYSICS);
M.Sc. (BIOLOGICAL SCIENCE)
KEYWORDS: MULTIDRUG RESISTANCE/CONTRAST MEDIA/
P-GLYCOPROTEIN/MITOCHONDRIAL MEMBRANE
POTENTIAL

NITAYA SNITWONGSE NA A YUDHYA: PHYSICOCHEMICAL PROPERTIES AND EFFECTS OF IODINATED RADIOGRAPHIC CONTRAST MEDIA ON CELLULAR ENERGETIC STATE OF K562 AND ITS CORRESPONDING MULTIDRUG RESISTANT CELL LINES. THESIS ADVISORS: SAMLEE MANKHETKORN, Ph.D., WICHET LEELAMANIT, Ph.D., WISATRE KONGCHAROENSUNTORN, Ph.D. 54 P. 2003. ISBN 974-382-521-5

Multidrug resistance is a major obstacle in cancer chemotherapy because the cancer cells decrease their intracellular drug accumulation by some energy- dependent multidrug efflux pumps, known as multidrug transporters such as P-glycoprotein (P-gp). This study observed iodinated radiographic contrast media, diatrizoate: an ionic monomer, iopromide: a nonionic monomer and iotrolan: a nonionic dimer and their effects on cellular energetic state and on kinetics of P-gp in drug-sensitive K562 and drug resistant K562/adr cell lines. Their physicochemical properties studied by spectrophotometer, such as lipophilicity, expressed as log P were -1.11, -0.22 and -1.23, respectively. The cytotoxicity of contrast media was conducted by colorimetric MTT assay. It was found that contrast media (0-3500 μM) had no effect on both K562 and K562/adr cell viabilities, but in co-treatment with daunorubicin (DNR), diatrizoate decreased cell viability in K562/adr cells by decreasing IC_{50} of DNR from 610.7 ± 74.5 nM to 360.3 ± 108.9 nM. The change in cellular energetic state was studied at mitochondrial level using rhodamine B as a probe followed by spectrofluorometer in order to estimate mitochondrial membrane potential ($\Delta\Psi_m$). The result showed that 3500 μM diatrizoate decreased $\Delta\Psi_m$ by about 50% from 162.2 ± 0.3 mV to 86.9 ± 9.9 mV in K562/adr cells. The modulation on the kinetics of P-gp-mediated efflux of DNR by contrast media was studied by spectrofluorometer in MDR cells. It was found that diatrizoate reduced k_a^i/k_a^0 which is the ability of molecule to inhibit active P-gp-mediated efflux of DNR from 0 (no inhibition) t

0.65 ± 0.11. This effect of inhibition could be partially prevented in co-incubation with 20 nM concanamycin A or 10 μM cytochalasin B. In conclusion, contrast media, diatrizoate, iopromide and iotrolan could regulate cellular energetic state by changing mitochondrial membrane potential and modulate P-gp-mediated drug efflux at different degrees by being not dependent on lipophilicity of molecules. Among the three molecules, diatrizoate showed the best efficiency. It could be proposed for further studies that diatrizoate could be used as MDR identification or MDR imaging and also acted as MDR sensitizing agent in cancer treatments.

CONTENTS

Page

ABSTRACT iv

CONTENT viii

LIST OF TABLE x

LIST OF FIGURES xi

LIST OF ABBREVIATION xii

CHAPTER

1 INTRODUCTION 1

Multidrug resistance (MDR) 2

Restoration of cytotoxicity in MDR cells 5

Mitochondrial membrane potential ($\Delta\Psi_m$) 6

Iodinated radiographic contrast media 7

Objectives 12

2 MATERIALS AND METHODS 13

Drugs and chemicals 13

Cell lines and cell culture conditions 15

Cell counting 15

Physicochemical properties of iodinated radiographic contrast media 16

Cytotoxicity of contrast media 18

Co-treatment of daunorubicin and contrast media 19

The effect of contrast media on mitochondrial membrane potential ($\Delta\Psi_m$) 20

Modulation of kinetics of the P-glycoprotein- mediated efflux of daunorubicin by contrast media 22

3 RESULTS 26

Physicochemical properties of contrast media: Molar extinction coefficient, pKa and lipophilicity of contrast media 26

Cytotoxicity of contrast media and co-treatment of daunorubicin with contrast media 32

CHAPTER Page

The effect of contrast media on mitochondrial membrane potential ($\Delta\Psi_m$) 35

Modulation of kinetics of the P-glycoprotein- mediated efflux of daunorubicin by contrast media 38

4 DISCUSSION 42

5 CONCLUSION 46

REFERENCES 47

BIOGRAPHY 54

LIST OF TABLE

Table Page

1-1 Physicochemical properties of contrast media 10

LIST OF FIGURES

Figure Page

1-1 The structural and topological model of P-gp 3

1-2 The structures of contrast media 9

1-3 The characteristic absorption spectra of contrast media 11

2-1 Chemical structures of four compounds 14

2-2 A typical kinetics of the P-gp-mediated efflux of DNR 25

3-1 The molar extinction coefficients of contrast media 28

3-2 The absorption spectra of contrast media 29

3-3 The pKa of diatrizoate 30

3-4 Lipophilicity of contrast media 31

3-5 Cytotoxicity of contrast media and daunorubicin (DNR) in K562/adr cells 33

3-6 The IC_{50} of daunorubicin in the absence and presence of contrast media 34

3-7 The typical kinetics of the uptake of rhodamine B by K562/adr cells 36

3-8 The mitochondrial membrane potential ($\Delta\Psi_m$) in intact cells 37

3-9 The kinetic parameters to compare the active efflux of P-gp in K562/adr cell line in the absence or presence of contrast media 40

Inset; The accumulation of DNR that intercalated between base pairs of DNA of K562/adr cells in the absence or presence of contrast media 40

3-10 The effect of concanamycin A, or cytochalasin B on P-gp-mediated efflux in K562/adr cells 41

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ADR	Adriamycin (Doxorubicin)
ATP	Adenosine 5'-triposphate
CM	Contrast media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNR	Daunorubicin
EDTA	Ethylenediaminetetra acetic acid di-sodium salt
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
IC	Inhibition concentraion (cell growth inhibition)
IV	Intravenous
JC-1	5-5',6-6'-tetrachloro-1,1',3,3-tetraethylbenzimidazolcabocyanide iodide
LRP	Lung resistance related- protein
MDR	Multidrug resistance
MRP	Multidrug resistance associated -protein
MTT	Tetrazolium salt, 3-(4,5-Dimethyl-2-thaiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
P-gp	P-glycoprotein
Rho B	Rhodamine B
SDHase	Succinate dehydrogenase
UV	Ultraviolet
$\Delta\Psi_m$	Mitochondrial membrane potential

CHAPTER 1
INTRODUCTION

Despite the numerous medical researches for century, cancer is a major cause of death of people worldwide nowadays, with an estimated 6 million new cases per year. It brings invaluable losses to mankind including expenses, natural resources and spirits. Cancer can be treated in variety ways depending on the size of the tumor, its location, the type of cancer, and a host of other factors. One common way to treat cancer is drug therapy or chemotherapy (Wang et al., 1999), which has been successful in treating acute leukemia, Hodgkin's and malignant lymphoma, small cell lung cancer, bladder and testicular cancer, and other forms of cancer. Chemotherapy is used to kill cancer cells, while attempting to limit the damage to normal cells. Of the roughly 50 anticancer drugs, some can be used alone, and some have to be used in combination with other(s). Unfortunately, the minority of cases represented the long-term survival after chemotherapy, perhaps 5-10 % with current treatments (Gottesman & Pastan, 1993). Up to 90% of small cell lung cancer tumors respond to chemotherapy, but patients almost always relapse with the disease again (Cole et al., 1992). It appears that the cancer cells increase tolerance or resistance to the cytotoxicity of drugs. The resistance can be either inherent, in diseases that are poorly responsive at very first presentation of chemotherapy, or acquired, when recurrent disease with a resistance phenotype occurs following a good response to initial chemotherapy (Twentyman & Bleehen, 1992). This often concerns a variety of unrelated chemical compounds and is then named multidrug resistance (MDR), which has been widely focused by the researchers in most experiments since it is a major obstacle in treatments of patients undergoing chemotherapy. In addition, the experimental models can be used either *in vivo* or *in vitro* selectively and comparatively.

Multidrug resistance (MDR)

A central goal in the study of chemotherapy is to understand how the cancer cells become drug resistant and how to make them response to chemotherapeutic drug again. MDR or multidrug resistance is a well-characterized phenomenon, which the cancer cells develop resistance to a wide range of structurally and functionally diverse drugs and compounds, frequently associated with decreased drug accumulation on their intracellular target (Gottesman & Pastan, 1993; Skovsgaard, 1978). The mechanisms underlying MDR have been extensively studied in models of cultured drug-sensitive and drug-resistant cancer cell lines. Many experimental evidences support a variety of mechanisms for MDR, including changes in cellular sites of drug sequestration, decreases in drug-target affinity, synthesis of specific drug inhibitors within cells, altered or inappropriate targeting of proteins, accelerated removal or secretion of drugs, and modifications in detoxification and DNA repair pathways (Simon & Schindler, 1994; Volm & Mattern, 1996). Any or all of these cellular responses may separately or synergistically result in the MDR phenotype. In MDR cells, the characteristic alteration most often observed is an increased expression of some energy-dependent multidrug efflux pumps, known as multidrug transporters such as P-glycoprotein (P-gp) (Juliano & Ling, 1976), multidrug resistance-associated protein (MRP) (Cole et al., 1992), and more recently, the lung resistance protein (LRP) (Scheper et al., 1993) leading to a lower accumulation of cytotoxic drugs than in their corresponding parental cells.

1. P-glycoprotein

Among a number of MDR transporter proteins that have been observed to be over- expressed in MDR cells, the most pronounced one to be determined is P-glycoprotein or P-gp. Juliano and Ling had initially found this 170,000 daltons cell surface glycoprotein predominantly labeled entity in the cloned colchicine-resistant Chinese hamster ovary tissue culture cells, relative to their parental cells (Juliano et al., 1976). It appeared to be associated with these cells displaying altered drug permeability, therefore they had designated it as the P glycoprotein. The over-expression of P-gp, which then had been confirmed as an integral membrane glycoprotein (Riordan & Ling, 1979), modulated plasma membrane properties to

reduce drug accumulation. P-gp consists of 1280 amino acids and is encoded by *mdr* gene that includes *MDR1*, *MDR2* in humans and *mdr1*, *mdr2*, *mdr3* in rodents (Shustik, Dalton, & Gros, 1995; Sukhai & Piquette-Miller, 2000). Over-expression of the *mdr* gene products has been implicated as a primary cause of MDR in tumors. The MDR1 mRNA levels reflect the extent of over-expression of P-gp in MDR cancer cell lines (Meesungneon, Jay-Gerin, & Mankhetkorn, 2002; Ueda, Pastan, & Gottesman, 1987) and the level of expression of P-gp was shown to correlate with degree of drug resistance (Riordan & Ling, 1979). P-gp is strongly homologous to a family of ATP-binding cassette (ABC) protein membrane transporters, also known as the traffic ATPases (Higgins, 1992). A typical ABC transporter protein consists of four units; two membrane-bound domains, each with six transmembrane segments with glycosylated loops and two nucleotide-binding domains, which bind and hydrolyze ATP (Sharom, 1997), as shown in Figure 1-1.

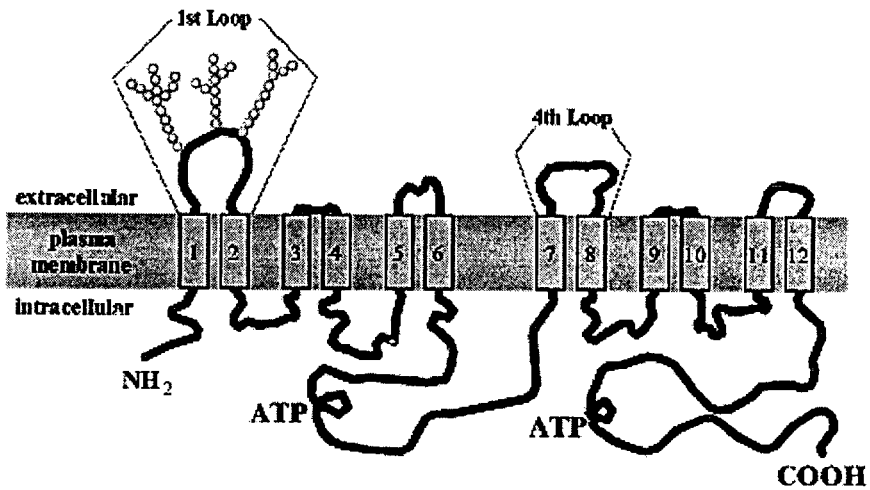


Figure 1-1 The structural and topological model of P-gp. The bars represent the 12 putative transmembrane helices numbered 1-12 starting from the NH_2-terminal end. The thick line represents both the intracellular and extracellular sequences in the P-glycoprotein. ATP binding sites are intracellular- present in each domain.

P-gp confers resistance against a wide spectrum of compounds that are hydrophobic, amphipathic natural product drugs including not only anticancer drugs (anthracyclines, Vinca alkaloids, epipodophyllotoxins, taxanes), but also other cytotoxic agents, linear and cyclic peptides, steroids, fluorescent dyes, and the γ-emitting radiopharmaceutical, ^{99m}Tc-sestamibi (Sharom, 1997). These compounds are chemically diverse, some of them may carry a positive charge at physiological pH and because all are hydrophobic, they enter cells by passive diffusion (Gottesman & Pastan, 1993).

2. MRP

In addition to P-gp-mediated MDR, there are many cultured drug selected-tumor cell lines that express MDR phenotype without demonstrating MDR1-overexpression. This led to the identification of another multidrug resistant-associated protein (MRP) in small cell lung cancer cell line (Cole et al., 1992). MRP, further designated as MRP1 is a 190,000 daltons protein encoded by the *mrp1* gene and is constituted by 1531 amino acids presenting N-linked glycosylation sites (Hipfner, Deely, & Cole, 1999; Teodori, Dei, Scapecchi, & Gualtieri, 2002). As P-gp, MRP1 is a member of the ATP- binding cassette (ABC) superfamily, so ATP hydrolysis provides the energy required for the translocation of substrates across membranes. The substrate specificity of both P-gp and MRP1 is partly overlapping and for instance, anticancer drugs, such as anthracyclines, vinca alkaloids and etoposide, are substrates of both transporters. Several findings indicated that MRP1 increased drug efflux by a GSH co- transport mechanism or after their conjugation to GSH (Lautier, Canitrot, Deely, & Cole, 1996), thus the depletion of intracellular GSH is capable of inhibiting MRP1-mediated transport but has no effect on P-gp-mediated transport.

3. LRP

More recently, a novel resistance-associated protein that functions as a putative drug efflux transporter like P-gp and MRP1 has been discovered by Scheper and colleagues (Scheper et al., 1993). The 110,000 daltons protein, which has been termed lung resistance-related protein (LRP) shares significantly amino acids identity with the M_r 104,000 rat major vault protein. Vaults are multisubunit ribonucleoproteins involved in nucleocytoplasmic transport: thus, it was suggested

that LRP may be involved in the intracellular transport of cytotoxic agents. LRP overexpression is associated with resistance to doxorubicine, vincristine, carboplatin, cisplatin and melphalan (Grandjean, Brémaud, Verdier, Robert, & Ratinaud, 2001; Izquierdo et al., 1996).

Restoration of cytotoxicity in MDR cells

The strategies to overcome MDR for some instances (Garnier-Suillerot, 1995; Mankhetkorn, Dubru, Hesschenbrouck, Fiallo, & Garnier-Suillerot, 1996; Shustik, Dalton, & Gros, 1995), include

(i) the use of high dose chemotherapy; this strategy was limited by the tolerability of normal cells,

(ii) the inhibition of encoding genes- expression,

(iii) the design and synthesis of non-cross-resistant analogues of MDR drugs; this strategy was very difficult because the multidrug transporter proteins had affinity for a wide range of structurally and functionally diverse drugs and compounds, and

(iv) the identification of selective and potential MDR-reversing agents, i.e., the chemosensitization of resistant cells, resulting in an increase in intracellular accumulation of cytotoxic drugs.

Efforts have been ongoing in several laboratories to understand the structural and functional basis of the inhibition of MDR transporter proteins, hereby will be emphasis on P-glycoprotein by chemosensitizers or modulators. Many modulators act as inhibitors of MDR transporter proteins by preventing specific recognition of the substrate, binding of ATP, ATP hydrolysis, or coupling of ATP hydrolysis to translocation of the substrate, either competitive or non- competitive binding (Ambudkar et al., 1999; Martin, Berridge, Higgins, & Callaghan, 1997). It is necessary that all the chemosensitizers or modulators are effective at binding transporter proteins, thereby inhibiting theirs function. Other possibilities as mechanisms for the inhibition have been suggested, such as alterations in the phosphorylation state of transporters, though, this explanation is less convincing than the competitive binding of transporter (Shustik, Dalton, & Gros, 1995).

Mitochondrial membrane potential ($\Delta\Psi_m$)

Over-expression of the MDR transporter proteins in resistant cells results in an increased requirement for ATP by the cells (Horio, Gottesman, & Pastan, 1988). The sources of most intracellular ATP are cytosolic glycolysis and mitochondrial energy production, which mainly by electron transport chain at inner membrane of mitochondria. In non dividing cells, mitochondria normally provide more than 90% of cellular ATP (Rolfe & Brown, 1997). The depletion of energy by sodium azide (Tsubaki & Yoshikawa, 1993), which is the inhibitor of cytochrome oxidase, the final enzyme in the mitochondrial electron transport chain results in drug accumulation in MDR cells similar to drug accumulation measured in their parental-sensitive cells (Meesungneon, Jay-Gerin, & Mankhetkorn, 2002). The electron transport pathway created a proton gradient across the inner membrane of mitochondria with the matrix, which is negative relative to the intermembrane space. As a consequence, there is energy available as well as the mitochondria membrane potential ($\Delta\Psi_m$). The mitochondrial membrane potential *in vitro* is about 180-200 mV, whereas, within living cells and organisms this potential is lower, usually about 130-150 mV (Murphy & Smith, 2000). The resistance tumor cells, compared with drug-sensitive cells have lower mitochondrial membrane potential (Harper et al., 2002). There are some compounds such as artemisinin and its derivatives (Reungpatthanaphong & Mankhetkorn, 2002) that exhibit chemosensitizer property by affecting energetic state of MDR cells at mitochondrial level. These compounds decrease the mitochondrial membrane potential leading to a decrease in intracellular ATP.

The difference in mitochondrial membrane potential and the existence of a specific MDR modulator is a crucial hint for MDR cell identification. There are fluorescent lipophilic cations such as rhodamine derivatives, JC-1 (Murphy & Smith, 2000) or the γ-emitting radiopharmaceutical, ^{99m}Tc-sestamibi (Vergote, Moretti, De Vries, & Garnier-Suillerot, 1998) that their cellular accumulation is dependent on the transmembrane potential. Thus, in the presence of MDR modulator, together with one of these probes, the MDR imaging can possibly be conducted.

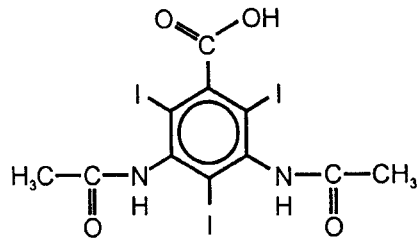
Iodinated radiographic contrast media

Iodinated radiographic contrast media are used in clinical practice as diagnostic pharmaceuticals. They are intravenously administered into human to indicate the site and size of pathology, including cancer staging. The most widely used contrast media are water soluble, fully substituted, tri-iodinated benzoic acid derivatives. The iodine in contrast media is covalently bound in the benzene ring so that the concentration of free iodine in contrast media is minute (Fanning, Manning, Buckley, & Redmond, 2002). They are commonly classified according to two characteristics; ionic versus nonionic and monomer versus dimer (Morris, 1993). The chemical structures of some contrast media are shown in Figure 1-2. The ionic contrast media can dissociate into monovalent anion and cation in aqueous solution, whereas nonionic ones that occurred by substituting a carboxylic acid (COOH) group with an amide ($CONH_2$) can not dissociate (Morcos & Thomsen, 2001). Most of contrast media, except nonionic dimer, are hyperosmolar with osmolality up to seven times more than the blood's osmolality (Morris & Katzberg, 1992). Some physicochemical properties of these contrast media are shown in Table 1.

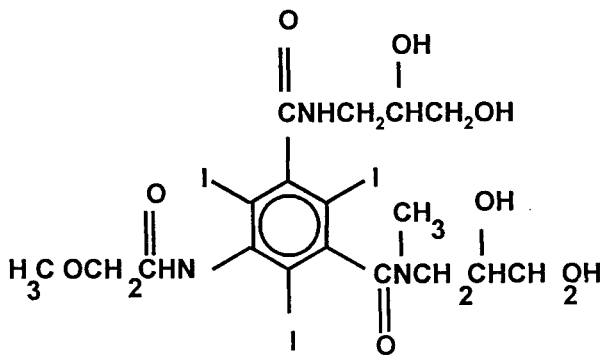
Tolerability of modern contrast media is very high that the huge amount (up to 100-200 g in total number) of drug can be injected as a bolus with a side effect coincidence of less than 5% (Krause, 1999). The administered contrast media into blood vessels (about 1-2 ml/ kg; ~ 300 mg of iodine per milliliter; 600-1500 mOsm) generated a concentration-dependent effect on endothelial cells. In case of ionic contrast media, the endothelial cell membrane potential depends on a large degree on the presence of a proper ion concentration. A change in the membrane potential may allow the anion portion to actually penetrate into the cell (Fisher, 1977). *In vivo*, about 3-4 mg of iodine per milliliter of ionic contrast media was measured in human blood and kidney tissue 40 min after CM injection. The half- life of nonionic contrast media in the plasma of dogs occurred about 70 min (Dascalu & Peer, 1994). It was found *in vitro* that the contrast media had effects on mitochondrial activity and reduced ATP level in LLC-PK1 and HRPTE cells (Hardiek, Katholi, Ramkumar, & Deitrick, 2001). Furthermore, some radio-contrast media such as iohexol, a monomer-nonionic contrast medium, could affect mrp2- and P-gp-mediated transport of fluorescein-

methotrexate (FL-MTX) and a fluorescent CSA derivative (NBD-CSA) in intact killifish renal proximal tubules (Masereeuw, Terlouw, Van Aubel, Russel, & Miller, 2000). These effects by contrast media make them interesting to get more insight into the property of these molecules and the possibility that they may cause biological changes in MDR cells.

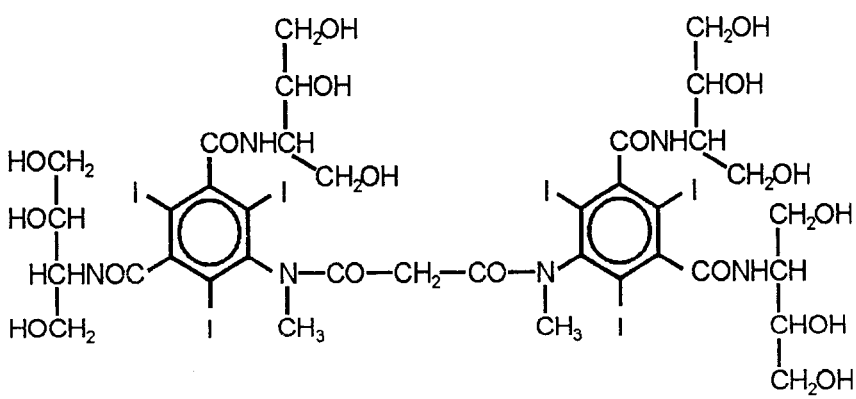
Contrast medium molecules absorb light in the far UV with maxima between 238-244 nm, as shown in Figure 1-3. This physicochemical property makes it practical to study the contrast media by spectrophotometry, which is very sensitive, direct and can be easily employed.



(a)



(b)



(c)

Figure 1-2 The structures of contrast media (a) diatrizoate; $C_{11}H_9I_3N_2O_4$ (b) iopromide; $C_{18}H_{24}I_3N_3O_8$ and (c) iotrolan; $C_{37}H_{48}I_6N_6O_{18}$.

Table 1-1 The contrast media used in this study and their physicochemical properties.

Generic name	Chemical name	MW	Iodine (mg/ml)	Osmolality (mOsm/kg H_2O) at 37°C
Ionic CM				
-Meglumine amidotrizoate [Angiografin ®] (monomer)	Diatrizoic acid or amidotrizoic acid; 3,5-bis-acetamido-2,4,6-triiodobenzoic	636	306	1522
Nonionic CM				
-Iopromide, [Ultravist ® (monomer)	N,N'-Bis(2,3-dihydroxypropyl)-2,4,6-triiodo-5-[(methoxyacetyl) amino]-N-methyl-1,3-benzenedicarboxamide	791.12	300	607
-Iotrolan [Isovist® (dimer)	5,5'(-[malonylbis (methylimino)] bis [N,N'-bis [2,3-dihydroxy-1-(hydroxy-methyl) propyl]-2,4,6-triiodoisophthalamide]	1626.24	300	320

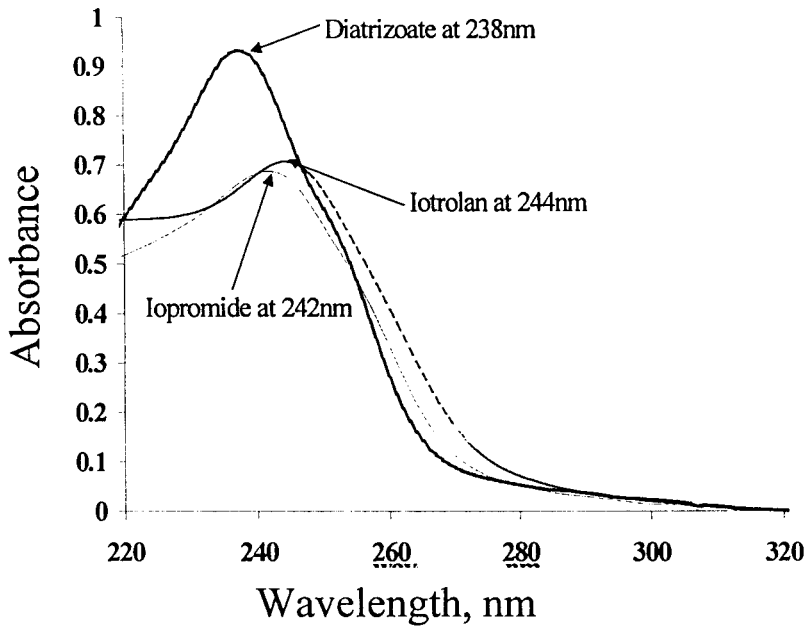


Figure 1-3 The characteristic absorption spectra of 2 $\times 10^{-5}$ g/ml of contrast media dissolved in 20 mM Tris-EDTA buffer solution, pH 6.8 and their maximum absorption peaks, followed by UV-visible spectrophotometer.

Objectives

The aims of this study are to determine:

1. The physicochemical properties of contrast media such as pK_a, partition coefficient that influence cellular transport, and their cytotoxicity.

2. The effects of contrast media on the mitochondrial membrane potential ($\Delta\Psi_m$) in K562 cell line, in comparison with its corresponding multidrug resistant cell line.

3. The aptitude of contrast media to modulate P-glycoprotein, a multidrug resistance transporter protein.

CHAPTER 2
MATERIALS AND METHODS

Drugs and chemicals

Adriamycin (Dox) and daunorubicin (DNR) (Figure 2-1a) were kindly provided by Professor Arlette Garnier-Suillerot, Laboratoire de Chimie Physique Biomoleculaire et Cellulaire, UFR Sante Medicine et Biologie Humaine, Bobigny, Université de Paris Nord, France. Iodinated radiographic contrast media; diatrizoate (Angiografin; Schering AG, Germany), iopromide (Ultravist; Schering AG, Germany) and iotrolan (Isovist; Schering AG, Germany) were kindly provided by Department of Radiology, Sappasittiprasong Hospital, Ubonratchatani, Thailand. Rhodamine B (Figure 2-1b) and tetrazolium salt (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)) were from Amresco. Concanamycin A (Figure 2-1c) and cytochalasin B (Figure 2-1d) were from Sigma Chemical Co. Deionized double distilled water was used throughout the experiments for solutions and buffers. Stock solutions of adriamycin (Dox) and daunorubicin (DNR) were prepared in sterilized water just before use and their concentrations were determined by UV-Visible Spectrophotometer, using a molar extinction coefficient of 11500 $M^{-1}cm^{-1}$ at 480 nm (Mankhetkorn, Dubru, Hesschenbrouck, Faillo, & Garnier-Suillerot, 1996). The dilutions of contrast media (diatrizoate, iopromide and iotrolan) were freshly prepared before used in water from known concentrations of these compounds in the commercially available solutions, stated in the manufacturer's information. Stock solution of MTT was prepared by dissolving 5 mg MTT/ml (12 mM) in HEPES-Na^+ buffer and filtered through a 0.22 μm filter then stored at 4 °C. A stock solution of cytochalasin B was prepared in DMSO (dimethyl sulphoxide) and stored at less than (-20 °C).

HEPES-Na^+ buffer consisted of 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.25 at 37 °C.

HEPES-K^+ buffer consisted of 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), 3.5 mM NaCl, 132 mM KC', 1 mM $CaCl_2$, and 0.5

mM $MgCl_2$, pH 7.25 at 37°C.

The absorption spectra were recorded on a Hewlett Packard G1103A spectrophotometer and the fluorescence spectra were recorded on a Perkin Elmer LS 50 B spectrofluorometer.

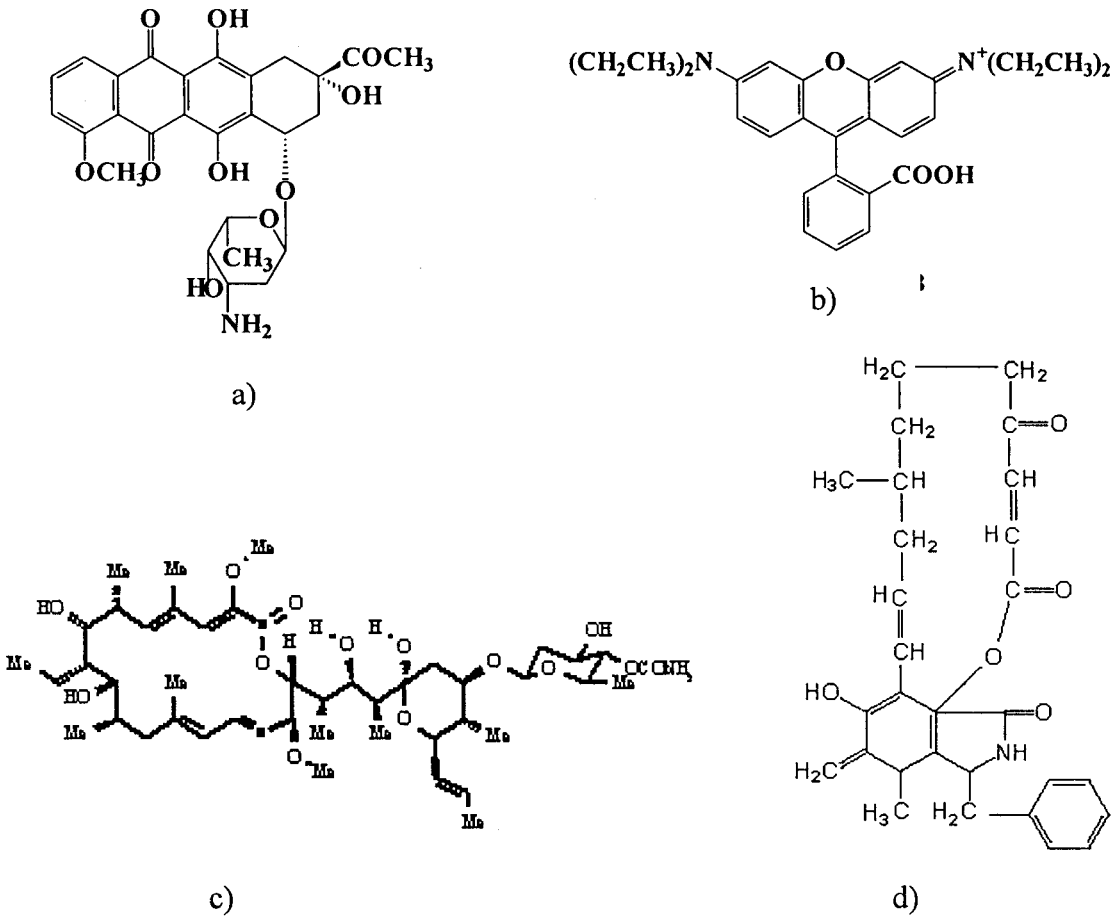


Figure 2-1 Chemical structures of a) daunorubicin, b) rhodamine B, c) Concanamycin A, and d) Cytochalasin B.

Cell lines and cell culture conditions

The adriamycin-sensitive erythromylogenous leukemic cell line; K562, and its P-glycoprotein-overexpressing K562/adr (Mankhetkorn et al., 1996) were kindly provided by Professor Arlette Garnier-Suillerot, Laboratoire de Chimie Physique Biomoleculaire et Cellulaire, UFR Sante Medicine et Biologie Humaine, Bobigny, Université de Paris Nord, France. These cells were routinely cultured in RPMI 1640 medium supplemented with 1% penicillin-streptomycin (Sigma Chemical Co.) and 10% fetal bovine serum (Gibco Biocult Ltd.) at 37 °C in a humidified incubator at 95% air and 5% CO_2. The resistant K562/adr cells were cultured with 100 nM doxorubicin two weeks before the experiments. The cultures were initiated at a density of 1×10^5 cells/ml, grew exponentially to about $8 - 10 \times 10^5$ cells/ml in 3 days. For the assays, the cultures were initiated at 5×10^5 cells/ml to have cells in the exponential growth phase and were used 24 hours later after reaching a density of approximately $8\text{-}10 \times 10^5$ cells/ml. Cell viability was assessed by Trypan blue exclusion. The number of cells was determined by a haemocytometer.

Cell counting

1. Total cell counting

Total cell counting was performed by a haemocytometer (Bright Line Improved Neubauer, 0.1 mm deep, American Optical, Buffalo, NY, USA). The cell suspension was transferred to both chambers of the haemocytometer by carefully touching the edges of cover-slip with a pipette tip and allowing each chamber to be filled by capillary action without overfilling or underfilling the chambers. Since each chamber of the haemocytometer represents a volume of 0.1 mm^3 or 10^{-4} cm^3 (width × length × depth = $1 \times 1 \times 0.1$ mm^3) and 1 cm^3 is equivalent to 1 ml, the subsequent cell concentration per ml was determined by the following equation:

Cells per ml = the average cell count per square $\times 10^4$

2. Viable cell, non-viable cell counting

Trypan blue was used for viable cell counting based on a dye exclusion principle that viable cells were not stained with this dye, whereas non-viable cells were. Cell suspensions were mixed with equal volumes of blue-colored trypan blue

solution resulting in colorless viable cells and blue-colored non-viable cells. Then viable cells and non-viable cells were counted by a haemocytometer as described previously. The concentration of viable cells and non-viable cells per ml could be calculated by:

Viable cells per ml = the average count of colorless cells per square $\times 10^4 \times 2$

Non-viable cells per ml = the average count of blue cells per square $\times 10^4 \times 2$

Physiocochemical properties of iodinated radiographic contrast media

1. Molar extinction coefficient (ε) of contrast media in 1-butanol and buffer

The absorbance of light by a sample depends on the concentration of absorbers, the path length of the light through the sample, and the molar extinction coefficient, as formulated by Beer:

$$A = \varepsilon cl \qquad [1.1]$$

where A = absorbance

c = concentration of absorbing species in mol L^{-1} (M)

l = path length in cm.

ε = molar extinction coefficient in L mol^{-1} cm^{-1}

Therefore, it was necessary to determine molar extinction coefficient (ε) in quantitative contrast media analysis in spectrophotometry. Using dried contrast media that collected after nitrogen blow, diatrizoate, iopromide and iotrolan were weighed. Then 500 µl of 1-butanol was added, 1-hour shaked and centrifuged. Four hundreds and seventy µl of supernatant was collected and measured by UV-Visible spectrophotometer in a 1-cm quartz cuvette containing 2 ml 1-butanol at 238 nm, 242 nm, and 244 nm of diatrizoate, iopromide and iotrolan, respectively. The 30 µl of residue was dried under nitrogen blow, weighed and dissolved in 1 ml HEPES Na^+ buffer. The absorbance at 238 nm, 242 nm, and 244 nm of diatrizoate, iopromide and iotrolan were spectrophotometrically measured in a 1 cm quartz cuvette containing 2 ml HEPES Na^+buffer. Consequently, molar extinction coefficient (ε) of contrast media both; in 1-butanol ($\varepsilon_{butanol}$) and in HEPES Na^+ buffer (ε_{buffer}) could be calculated

by equation;

$$\varepsilon = A/cl \qquad [1.2]$$

2. pK_a and partition coefficient of iodinated radiographic contrast media

By shake-flask method and spectrophotometric method, partition coefficient and pK_a of contrast media could be determined adding 20 µl of 0.1 M of diatrizoate, iopromide, or iotrolan into the mixed solvent of 0.5 ml 1-butanol and 0.5 ml tris-EDTA buffer (20 mM tris with 0.1 mM EDTA) of various pH, shaked well thoroughly for 60 minutes. After centrifugation (7000 rpm 10 minutes), 20 µl of 1-butanol phase was added into 2 ml 1-butanol in 1 cm quartz cuvette, the concentrations of contrast media were spectrophotometrically measured.

Because concentration of contrast medium in 1-butanol was much less than concentration of contrast medium in aqueous buffer, the concentration of contrast media in aqueous buffer (C_{buffer}) equaled to the difference between the initiated contrast medium concentration (C_{total}) and the concentration of contrast media in 1-butanol (C_{butaol});

$$C_{buffer} = C_{total} - C_{butanol} \qquad [2.1]$$

Lipophilicity of a contrast media could be measured as a partition coefficient between an organic phase, which was simulated by 1-butanol and aqueous phase, which simulated by buffer, the calculation of the partition coefficient was carried cut with the following equation:

$$\text{Partition coefficient (P)} = \frac{C_{butanol}}{C_{buffer}} \qquad [2.2]$$

where $C_{butanol}$ = the concentration of contrast media in 1-butanol phase

C_{buffer} = the concentration of contrast media in buffer

However, the measured value of P depended on ionization of the molecule and thus respected the pK_a and pH of buffer (charged molecules were less lipo-soluble than neutral molecules).

The pK_a of a compound is the pH where the concentration of charged molecules equals to the concentration of uncharged molecules, which refers to the pH where the ratio of the concentration of charged molecules to the concentration of non-

uncharged molecules equal to 1. The calculation of pK_a was carried out with the following equation:

$$pKa = pH \text{ that } \frac{C_{butanol}}{C_{buffer}} = 1 \quad [2.3]$$

This could be done by plotting ($C_{butanol}$ / (C_{buffer}) versus pH.

The % fraction of ionization = $1/(1+10^{pH-pKa})$ [2.4]

Eventually, measured P was corrected the effect of ionization by this equation;

$$P_{real} = P_{pH}*(1+10^{pKa-pH}) \quad [2.5]$$

where P_{real} = partition coefficient that is independent to pH

P_{pH}, or P_{app} = measured P at a particular pH

In general, partition coefficient (P) is reported as log P.

Cytotoxicity of contrast media

The viability of cells was determined using colorimetric MTT- reduction assay with some modification (Twentyman & Luscombe, 1987). This assay involves the ability of viable cells to convert a soluble tetrazolium salt, MTT, into insoluble formazan crystals by succinate dehydrogenase (SDHase) a component of complex II in mitochondria. The absorbance spectrum at 560 nm of formazan solution in dimethyl sulfoxide (DMSO) was determined by spectrophotometer. The absorbance at 560 nm of formazan solution was proportional to the concentration of cells (Meesungnoen, 2001).

Cells (concentration of 5×10^4 cells/ml) were incubated in RPMI-1640 medium supplemented with 10 % fetal bovine serum in the presence of various concentrations of contrast media (diatrizoate, iopromide, or iotrolan) at 37 °C in a humidified incubator with 95% air and 5% CO_2. After 72 hours, 2 mM final concentration MTT was added to the cell suspension and then further incubated for 4 hours. The percentage of cell growth inhibition (%IC) was determined using the following expression:

$$\%IC = [(Abs_{560}(control)_{t=72} - Abs_{560}(control)_{t=0}) - (Abs_{560}(CM)_{t=72} - Abs_{560}(control)_{t=0})] / [(Abs_{560}(control)_{t=72} - Abs_{560}(control)_{t=0}] \times 100$$

where Abs_{560} (control) is the absorbance value at 560 nm of MTT formazan solution in DMSO of cells that were incubated in RPMI-1640 medium without contrast medium for 72 hours and further incubated in 2 mM MTT for 4 hours.

Abs_{560}(CM) is the absorbance value at 560 nm of MTT formazan solution in DMSO of cells that were incubated in RPMI-1640 medium in the presence of various contrast medium concentrations for 72 hours and further incubated with MTT for 4 hours.

Co-treatment of daunorubicin and contrast media

Cells (5×10^4 cells/ml) were incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum in the presence of various concentrations of daunorubicin and a fixed concentration of 500, 2000 and 3500 μM contrast media (diatrizoate, iopromide, or iotrolan). The experiments were performed and calculated using colorimetric MTT- reduction assay as described previously.

The IC_{50} was determined by plotting the percentage of cell growth inhibition (%IC) versus the daunorubicin concentration: IC_{50} is the daunorubicin concentration that inhibits cell growth by 50% when measured at 72 h.

Consequently, the abilities of contrast media to reverse MDR phenotype of K562/adr cells to daunorubicin, in comparison with their parental sensitive cells were observed. The efficacy of contrast media (δ) in increasing the efficacy of daunorubicin was obtained by the following equation:

$$\delta = [IC_{50(R)} - IC_{50(RCM)}] / [IC_{50(R)} - IC_{50(S)}]$$

where $IC_{50(R)}$ was the concentration of daunorubicin that inhibited 50% of MDR cell growth, $IC_{50(RCM)}$ was the concentration of drug that inhibited 50% of MDR cell growth in the presence of contrast media, and $IC_{50(S)}$ is the concentration of drug that inhibited 50% of drug-sensitive cell growth.

The efficiency to reverse MDR phenotype or δ was equal to 0 when the MDR cells were treated with daunorubicin alone and δ was equal to 1 when the contrast media reverse 100 % of the MDR phenotype resulting in the same $IC_{50(RCM)}$ as that of drug sensitive cell line.

The effect of contrast media on mitochondrial membrane potential ($\Delta\Psi m$)

The mitochondrial membrane potential ($\Delta\Psi_m$) was measured using a non-invasive functional study (Reungpatthanaphong, Dechsupa, Meesungnoen, Loetchutinat, & Mankhetkorn, 2003), which could be used to determine and to monitor a spontaneous change in mitochondrial functions in drug-sensitive and drug-resistant cells. The method was simple and direct and could be easily employed using a standard spectrofluorometer. Rhodamine B was used as probe to estimate $\Delta\Psi_m$ in drug-sensitive and particularly, in drug-resistant cells because its rate of uptake by cells was four times higher than the rate of P-glycoprotein-mediated efflux and it was a very poor substrate of P-glycoprotein (Russell, Scaduto, & Lee, 1999). The accumulation of rhodamine B in cells followed the Nernstain distribution, but the plasma membrane potential did not contribute to the rhodamine B uptake by the cells. The estimation of $\Delta\Psi_m$ was done using Nernst equation:

$$C^o_m/C^o_i = 10^{(\Delta\Psi mF/2.303RT)}$$

where C^o_m is the concentration of mitochondrial matrix rhodamine B.

C^o_i is the concentration of cytosolic rhodamine B at steady state.

F is the Faraday constant in coulombs.mole^{-1}, R is a gas constant in joules. degree^{-1}.mole^{-1}, and T is the absolute temperature in Kelvin.

The mitochondrial matrix rhodamine B concentration could be determined as follows.

Cells (2×10^6 cells) were incubated with various concentrations of contrast media; diatrizoate, iopromide and iotrolan in 2 ml of HEPES- Na^+ buffer with 40 nM rhodamine B in 1 cm quartz cuvette and vigorously stirred at 37 °C. The rhodamine B fluorescence intensity (F) at 582 nm (excited at 553 nm) was monitored as a function of time. The addition of 200 μM MTT were performed within 10 minutes of incubation, yielding a progressive decrease in rhodamine B fluorescence intensity.

To simplify the system, cells were divided into three compartments: extracellular, cytoplasmic and mitochondrial, respectively. At steady state, rhodamine B equilibrated between the extracellular (C^o_e), cytoplasmic (C^o_i) and mitochondrial (C^o_m) compartments. The progressive decrease in rhodamine B fluorescence intensity was due to the translocation of rhodamine B molecules from outside to inside

mitochondria. Indeed, the reduction of MTT to produce formazan, a rhodamine B quencher, was located exclusively in the mitochondrial matrix, therefore only the rhodamine B located in this compartment was quenched. In this process, the limiting step is the passage of rhodamine B through the mitochondrial membrane because it is tighter than the plasma membrane. Under these conditions, the kinetics of uptake into the mitochondrial can be written as:

$$V_{rhoB} = PC^{o}_{i}$$

where P is a permeability coefficient which depends on rhodamine B and on the membrane of mitochondria. When 1 mole leaves the extracellular medium to go to cytosol and then to the mitochondria at $\Delta\Psi_m$, at the steady state the fluorescent intensity F_e is equal to F_i, and the variation of fluorescence per mole is:

$$\delta F = F_e - F_m$$

where F_e and F_m are the rhodamine B fluorescence intensity in the extracellular and mitochondrial compartments, respectively. When MTT is added to the cells, the F_m becomes F_{mtt}, and during Δt, n moles of rhodamine B move from the extracellular medium to the mitochondrial compartment, yielding a modification F of the fluorescence signal:

$$n = V_{rhoB}\,\Delta t$$

which can be written as:

$$n = PC^{o}_{i}\Delta t$$

$$\Delta F = n\delta F$$

$$\Delta F = PC^{o}_{i}\Delta t\delta F$$

$$\Delta F/\Delta t = PC^{o}_{i}(F_e - F_{mtt})$$

P does not depend on the $\Delta\Psi_m$, and the sign of $\Delta F/\Delta t$ does not depend on C^{o}_{i}. The accumulation of rhodamine B in the mitochondrial matrix is augmented in a $\Delta\Psi_m$-driven manner, which is predicted by the Nernst equation. During a very small Δt (50 seconds) after the addition of MTT, $\Delta F/\Delta t$ was determined by $(d(F)/dt)_{mtt}$, the slope of the tangent of the F = f(t), corresponding to V_i, the initial rate of the decrease of rhodamine B fluorescent intensity:

$$V_i = PC^{o}_{i}\,(F_e - F_{mtt})$$

In 50 seconds, the change in extracellular rhodamine B concentration is negligible compared with that before the addition of MTT, however the $\Delta F/\Delta t$ is easily determined. This signifies that the V_i should reflect the $(\Delta\Psi_m)_{mtt}$ value which is nearest to the in situ, $\Delta\Psi_m$. This method has its foundation in the quantification of the Nernstain distribution of dye across the mitochondrial membrane; V_i is largely empirical in design, representing the mitochondrial dye concentration. V_i can be used to estimate measurement of the $\Delta\Psi_m$:

$$C^o_m/C^o_i = 10^{(\Delta\Psi mF/2.303RT)}$$

$$\Delta\Psi_m = -61.51 \log V_i - 258.46$$

where $\Delta\Psi_m$ is the mitochondrial membrane potential in mV, RT/F is 26mV at 37°C, C^o_i is 40 nM and V_i is in $nM.s^{-1}$.

Using this technique, it is possible to monitor the alteration of mitochondrial function in intact cells because the cells can be incubated with the drug without compromising cell viability.

Modulation of kinetics of the P-glycoprotein- mediated efflux of daunorubicin by contrast media

Cells (2×10^6 cells) were incubated in 2 ml HEPES-K^+ buffer in the absence of glucose under continuous stirring at 37 °C in 1-cm quartz cuvette. The extracellular pH (pH_e) of 7.25 was chosen all the experiments to be equal to the pH of cytoplasm since the cytosolic pH in K562/adr cells was shown to be within the range of 7.2-7.3 (Frezard & Garnier-Suillerot, 1991). Initially, the cells were depleted energy by adding 10 mM NaN_3, an inhibitor of cytochrome oxidase in mitochondrial electron transport chain for 30 min, leading to depletion of ATP in these cells about 90 % (Kimmich, Randles, & Brand, 1975).The cells remained viable throughout the experiment as checked by trypan blue exclusion. Daunorubicin (DNR) was added to cell suspension at concentration of 1 μM. The fluorescence intensity of DNR at 590 nm (excited at 480 nm) was spectrofluorometrically followed as a function of time. A decrease in fluorescence intensity was observed during incubation with cells, due to quenching of fluorescence after the intercalation of DNR between the base pairs of DNA, as shown in Figure 2-2. At the first steady state, the intracellular free drug

concentration C'_i was equal to the free drug concentration in the extracellular medium (C_e) when pH_i equals pH_e. Then 5mM glucose was added at $t = t_{glu}$, after the sequential addition of various concentrations of contrast media. Under this condition, the active efflux of DNR was observed. The fluorescence intensity was F'_n and the concentration of drug intercalated between the base pairs of DNA in the nucleus (C'_n) was equal to:

$$C'_n = C_T \times (F_0 - F'_n) / F_0$$

where C_T is 1 μM. F_0 and F'_n are the fluorescence intensity at $t = 0$ and the fluorescence intensity at $t = t_{glu}$, respectively.

After addition of 5 mM of glucose, the fluorescence intensity increased. The slope of the tangent to the curve was $(dF/dt)_{tglu}$ and the kinetics of release of DNR (V_a) was:

$$V_a = (dF/dt)_{tglu} . (C_T/F_0) \text{ in } \mu M.s^{-1}$$

On the other hand, one can write in a first approximation that:

$$V_a = k_a.n.C'_i$$

where k_a is the active (P-glycoprotein- mediated) efflux coefficient of drug; n is the number of cell concentration.

As C'_i equals to C_e, and $C_e = C_T - C'_n$, k_a can be written as:

$$k_a = V_a / n((C_T - C'_n) \text{ in } s^{-1}$$

In the following, (k^i_a) and (k^0_a) stand for the P-glycoprotein -mediated efflux coefficient in the presence and in the absence of contrast media, respectively. The ability of contrast media to inhibit the P-glycoprotein active efflux of a drug can be determined using the ratio;

$$k^i_a / k^0_a$$

which is equal to 1 when there is no inhibition of active efflux, and to 0 when the P-glycoprotein active efflux is completely blocked.

At the new steady state, the fluorescence intensity was F_n. The concentration of drug intercalated between the base pairs of DNA in the nucleus under the active (P-glycoprotein -mediated) efflux (C_n) is equal to:

$$C_n = C_T ((F_0-F_n)/ F_0$$

where F_n is the fluorescence intensity at the steady state after 5 mM glucose

addition. After the addition of 0.08 % w/v saponin that leading to an equilibrium state, the fluorescence intensity was defined as F_N and the overall concentration of drug bound to the nucleus was $C_N = C_T ((F_0-F_N)/ F_0$.

The similar series experiments were also conducted in the presence of

a) concanamycin A, a family of macrolide antibiotics is a specific and potent inhibitor of vacuolar H^+-translocating ATPases (V-ATPases).

b) cytochalasin B, a fungal metabolite that acts as a microtubule inhibitor that inhibits cytokinesis by binding to high molecular weight complexes in the plasma membrane that have the ability to induce actin polymerization. It is a blocker of endocytosis.

Cells were incubated with 20 nM concanamycin A for 10 minutes, or 5 μg/ml (10 μM) cytochalasin B for 24 hours prior to 1 μM daunorubicin addition.

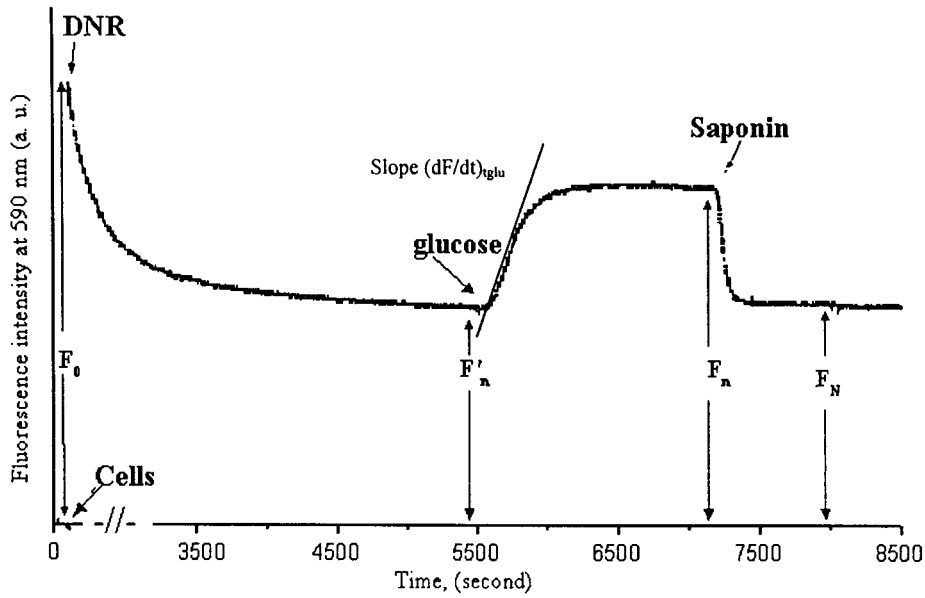


Figure 2-2 A typical kinetics of the P-gp-mediated efflux (V_a) of DNR. K562/adr cells (2×10^6 cells) were incubated with 1 μM DNR in 2 ml HEPES-K^+ buffer in the presence of 10 mM NaN_3 and in the absence of glucose. The fluorescence intensity at 590 nm (λ_{ex} = 480 nm) was recorded as a function of time. At the steady state, the fluorescence intensity was F'_n, then 5 mM glucose was added, after the sequential addition of various concentrations of contrast media, yielding the release of DNR. The slope of the tangent to the curve was $(dF(/dt)_{tglu}$. At the new steady state the fluorescence intensity was F_n. the addition of 4 μl of saponin 4% (w/v) yielded the equilibrium state defined as the fluorescence intensity F_N.

CHAPTER 3

RESULTS

Physicochemical properties of contrast media: Molar extinction coefficient, pK_a and lipophilicity of contrast media

All contrast media used were dissolved in 0.1 M NaCl and the solutions possessed the absorption spectra with the maximal absorbance at 238 nm, 242 nm and 244 nm in diatrizoate, iopromide and iotrolan, respectively. Figure 3-1 shows that the molar extinction coefficient (ε) of the three compounds in HEPES Na^+ and 1- butanol as a function of wavelengths. The ε of diatrizoate, iopromide and iotrolan in 1-butanol were 27000 ± 2300, 23700 ± 3500, and 12900 ± 0160 $mol^{-1}.L.cm^{-1}$, respectively, whereas, in HEPES Na^+ or tris-EDTA buffer were 28000 ± 5600, 24800 ± 4300, and 52400 ± 2100 $mol^{-1}.L.cm^{-1}$, respectively.

In order to get further insight into the interaction of contrast media with their cellular target, the lipophilicity of compounds should be determined. The shake flask method was applied using 1-butanol as organic phase and tris- EDTA buffer as aqueous phase. The lipophilicity of these compounds could be investigated by measuring their concentrations that partitioned in experimental solvents. Because the amphipathic molecules contain moieties that are polar (or charged) and non-polar (or uncharged), so they are able to protonate or deprotonate. As a consequence, the forms of existed molecules are dependent on pH of solution. In this study, the pH of tris-EDTA buffer was varied to determine the partition of charged and uncharged forms of diatrizoate, iopromide and iotrolan in solvents. Figure 3-2 demonstrates the absorption spectra of contrast media in 1-butanol phase with various pH of buffer. It was remarkable that the absorbance of, only diatrizoate, an ionic monomer was varied as a function of pH. The absorbance decreased when the pH of buffer increased because at the lower pH diatrizoate presented in neutral form more than at the higher pH, leading to the more concentration of diatrizoate partitioned in 1- butanol.

The pK_a of contrast media that is the pH of solution containing molecules of charged form equal to neutral form, could be found only in diatrizoate, was 2.3 (data

shown in Figure 3-3). Presumably, diatrizoate presented as anionic molecules at the most physiological pH. The partition coefficient (P) is the ratio of the solubility of the molecule in lipid (simulated by 1-butanol) and its solubility in biological fluid (simulated by tris-EDTA buffer at various pH). Since the measured value of partition coefficient was dependent on the ionization of molecules, and thus varied upon its pK_a and the pH of aqueous phase, the P or log P corrected with the fraction of ionization could be determined as indicated in Figure 3-4. There was only diatrizoate that needed to be eliminated the effect of the ionization of molecules. Eventually, the log P of diatrizoate, iopromide and iotrolan at the pH 7.25 were equal to -1.11, -0.22 and -1.23, respectively. In comparison, iotrolan was the least lipophilic substance, and iopromide was the most lipophilic substance.

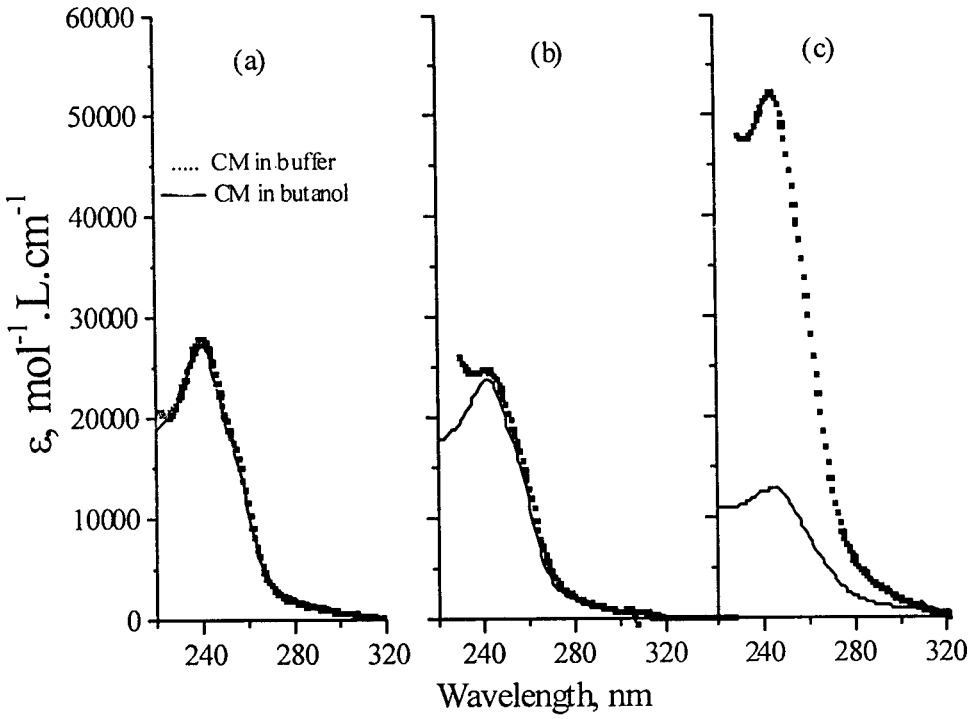


Figure 3-1 The spectra illustrated the molar extinction coefficients as a function of wavelengths in 1-butanol (solid line; __) and in HEPES Na^+ buffer pH 7.25 (dash line; ---). The spectra demonstrate the highest values at particular wavelengths: (a) diatrizoate at 238 nm, (b) iopromide at 242 nm and (c) iotrolan at 244 nm. The dried contrast media were weighed and dissolved in mixture 1:1 of 1-butanol and HEPES Na^+ buffer. The known concentrations of contrast media studied in each phase was spectrophotometrically determined, and the molar extinction coefficients (ε) was calculated by Beer's law; $A = \varepsilon cl$.

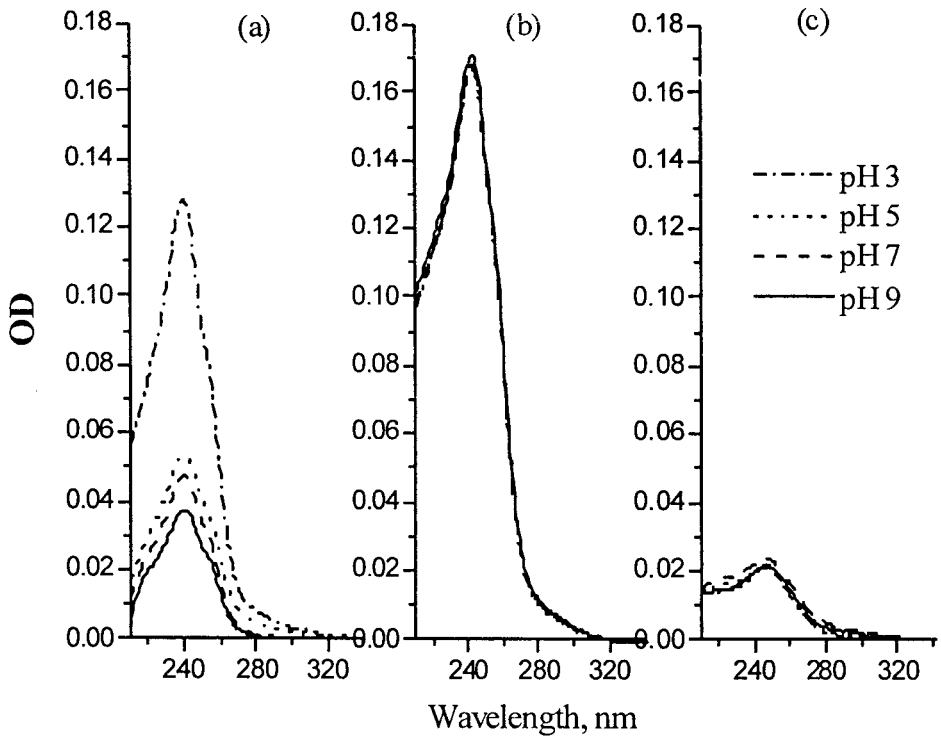


Figure 3-2 The absorption spectra of contrast media; (a) diatrizoate, (b) iopromide and (c) iotrolan, in 1-butanol phase that taken from mixture of 0.5 ml 1-butanol and 0.5 ml.tris-EDTA buffer at pH 3, 5, 7 and 9.

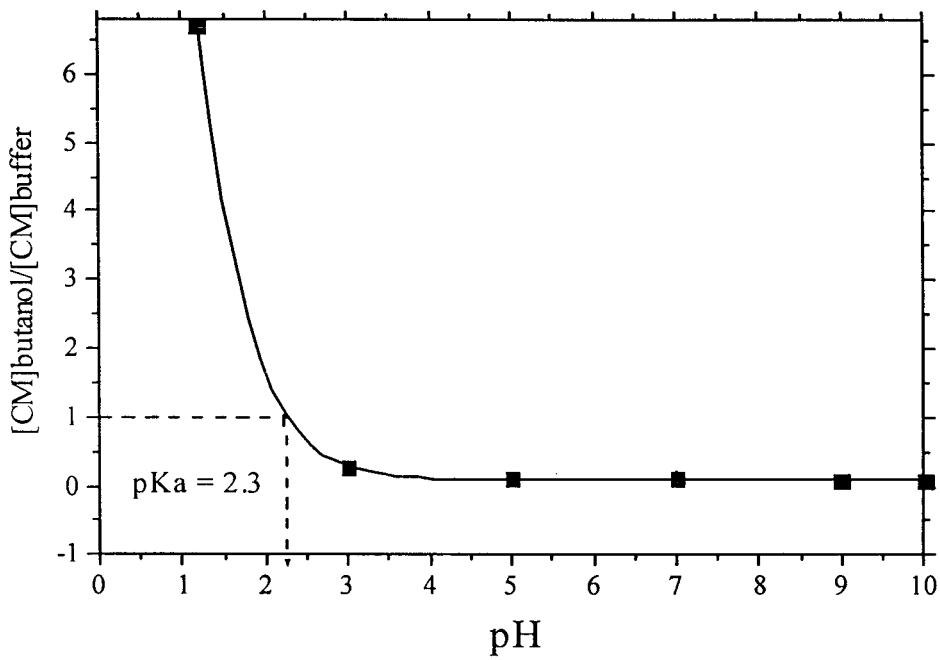


Figure 3-3 The pK_a which is the pH that concentration of diatrizoate in 1-butanol equals to concentration of diatrizoate in aqueous buffer is 2.3. This could not be found in iopromide and iotrolan (data not shown).

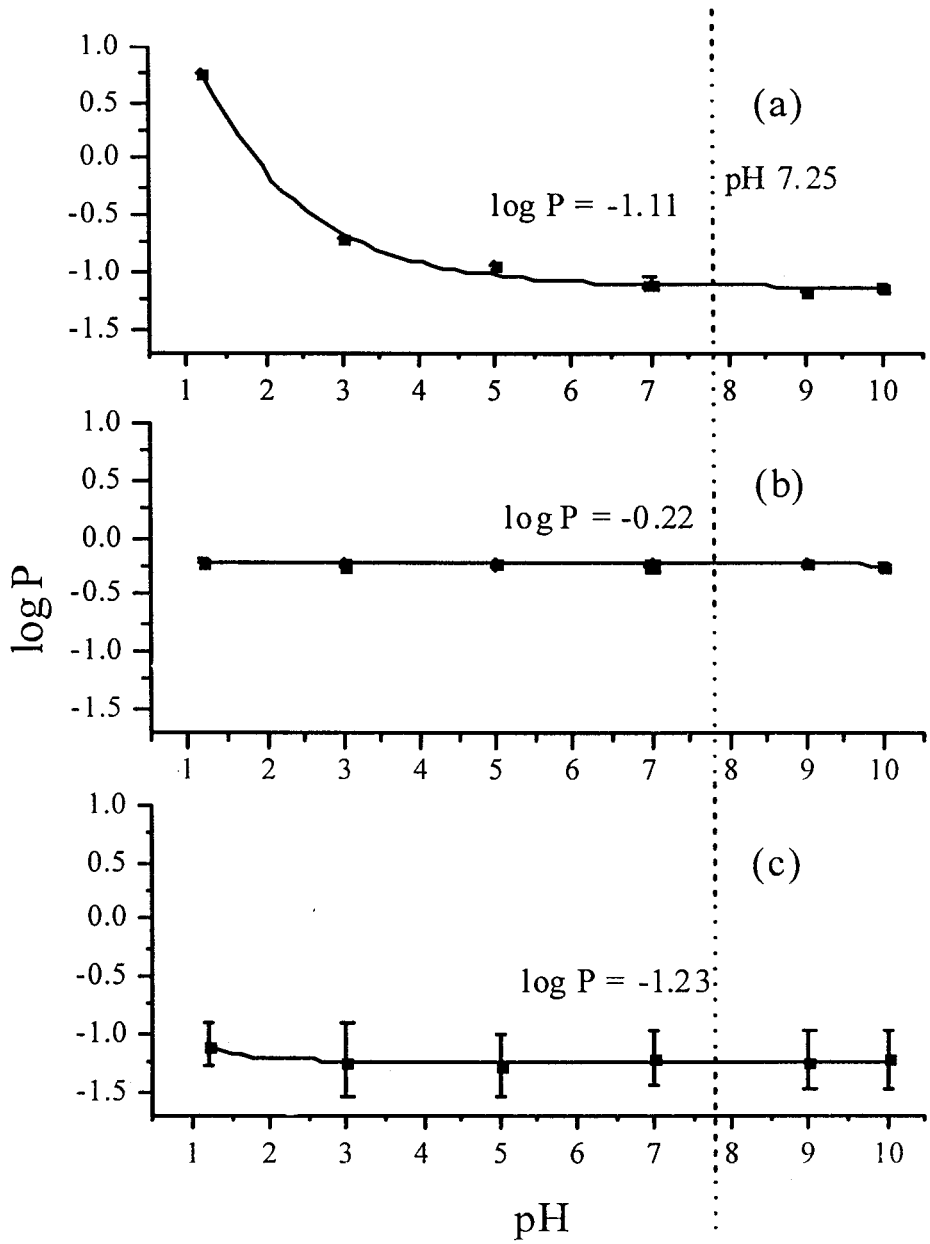


Figure 3-4 Correlation between the calculated 1-butanol/ tris-EDTA buffer partition coefficient (log P) and pH. The 2×10^{-3} M of contrast media in mixture 1:1 of 1-butanol and tris-EDTA buffer at various pH were spectrophotometrically determined to find out the concentrations of (a) diatrizoate, (b) iopromide and (c) iotrolan in each phase and at each pH. Log P was calculated by; $P = [CM]_{butanol}/[CM]_{buffer}$

Cytotoxicity of contrast media and co-treatment of daunorubicin with contrast media

The cytotoxicity of contrast media was determined by observing the percentage of cell growth inhibition (%IC). Figure 3-5a shows a typical result of the effects of contrast media on MDR, K562/adr viability. The concentrations of contrast media used; 0-3500 μM did not have effect on cell growth in both K562 and K562/adr cell line.

To study whether contrast media could alter the efficiency of MDR agent, the co-treatment studies using daunorubicin and contrast media were performed. Daunorubicin is a derivative of anthracyclines, which are known as anticancer drugs. They are MDR agents and their efficiencies depend on their intracellular concentrations. The cytotoxicity of daunorubicin (DNR) in K562/adr cells is mentioned in Figure 3-5b. IC_{50} of daunorubicin in K 562 and K562/adr cell line are 57.5 ± 0.1 nM and 610.8 ± 74.5 nM, respectively. Percent viabilities of K562/adr cells incubated with DNR were shown in Figure 11b. It was found that the co-treatment using DNR with various concentrations of diatrizoate, iopromide or iotrolan (500, 2000 and 3500 μM) did not affect the IC_{50} of DNR in the drug-sensitive K562 cell line, but in its corresponding MDR cell line; K562/adr, diatrizoate increased the efficacy of DNR when the similar series of experiments were performed (Figure 3-6). As a consequence, diatrizoate could efficiently reverse the MDR phenotype and resensitize the K562/adr cells to DNR. The capabilities of 500, 2000 and 3500 μm diatrizoate in increasing the efficacy of DNR (δ) were 0.11, 0.17 and 0.45, respectively.

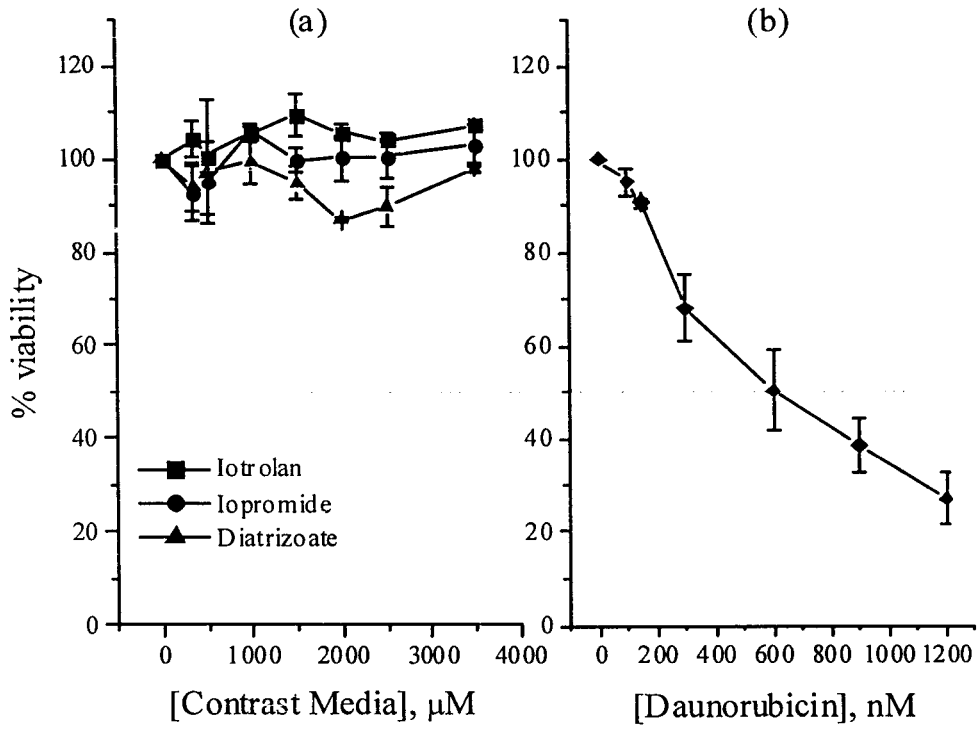


Figure 3-5 Cytotoxicity of contrast media and daunorubicin (DNR) in K562/adr cells. By incubating the cells with (a) contrast media; iotrolan (■), iopromide (●), or diatrizoate (▲), and (b) daunorubicin of various concentrations for 72 hours before adding MTT to study the cell proliferation or surviving cells that expressed in term of %viability. The dash line indicates the viability of cells that are reduced by 50 %. The control cells (no molecules added) exhibit % viability of 100. Each value is the mean ± SD of three independent experiments.

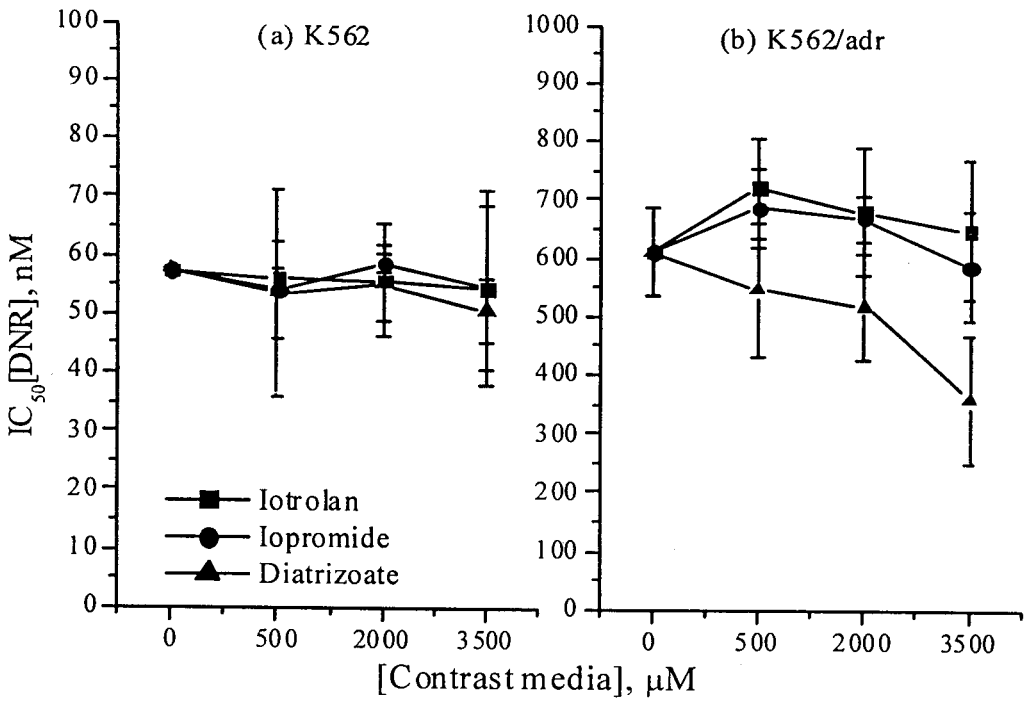


Figure 3-6 The IC_{50} of daunorubicin in the absence and presence of contrast media; iotrolan (■), iopromide (●), and diatrizoate (▲) in (a) K562 cell line and (b) K562/adr cell line. By colorimetric MTT reduction assay, 5×10^4 cells/ml were incubated with various concentrations of DNR and a fixed concentration of contrast media (500, 2000 or 3500 μM) in 24-well plates at 37°C for 72 hours. The cells were harvested after 4-hour of 2 mM MTT incubation. The formazan was spectrophotometically measured at 560 nm. Each value is the mean ± SD of three independent experiments.

The effect of contrast media on mitochondrial membrane potential ($\Delta\Psi_m$)

The mitochondrial function could be studied by following the change in mitochondrial membrane potential ($\Delta\Psi_m$). The $\Delta\Psi_m$ of K562 and drug- resistant K562/adr cells could be determined using rhodamine B, based on the observation that rhodamine B was accumulated within the mitochondrial matrix in accordance with the Nernst equation. After adding MTT to rhodamine B- loaded cells, the formazan, which was the product of the reduction between SDHase and MTT would quench the fluorescent intensity of rhodamine B that located in mitochondria matrix. The kinetic uptakes of rhodamine B by cells were shown in Figure 3-7. In this study, $\Delta\Psi_m$ of K562 and K562/adr were -166.2 ± 5.7 mV and -162.2 ± 0.3 mV, respectively. It was found that among the contrast media used, there was diatrizoate (350, 3500 μM) that apparently decreased $\Delta\Psi_m$ in K562/adr cells, whereas iopromide could do with less efficiency and iotrolan was the least to do. Otherwise, in K562 cell line; there were no differences between treated cells and control. The results were shown in Figure 3-8.

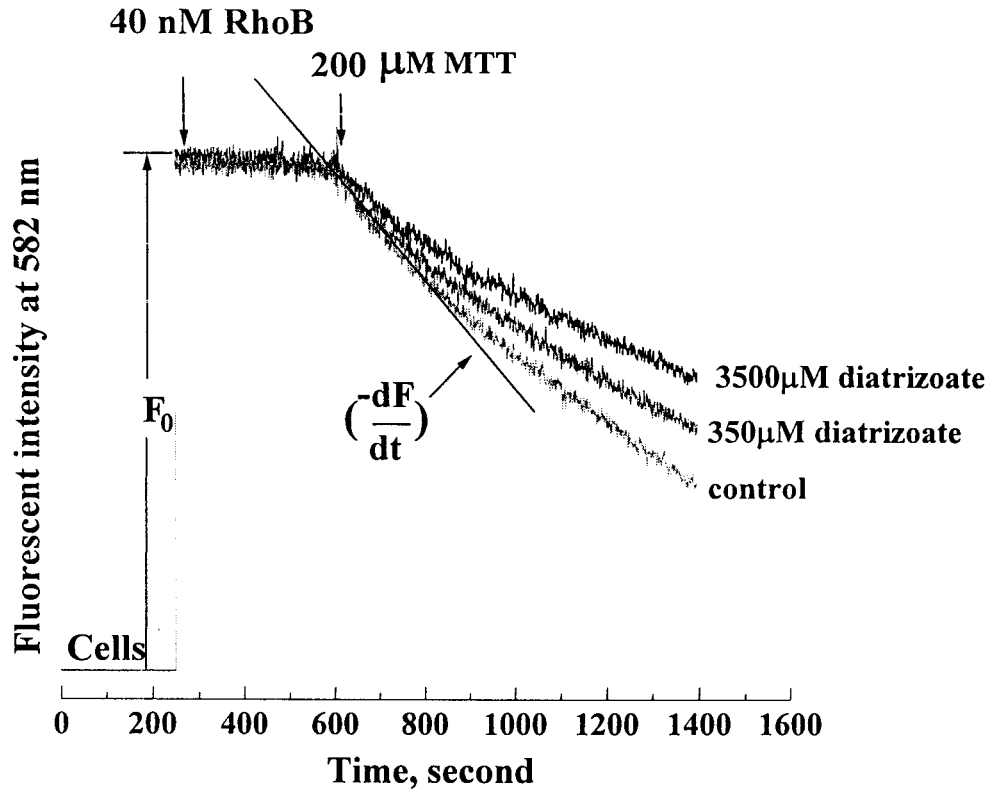


Figure 3-7 The typical kinetics of the uptake of rhodamine B by cells; Fluorescence intensity, F, at 582 nm (λ_{ex} = 553 nm) was recorded as a function of time. 2 $\times 10^6$ cells were incubated with various concentrations of diatrizote, iopromide or iotrolan in 2 ml of HEPES-Na^+ buffer at 37 °C and vigorously stirred. A small volume of rhodamineB stock was added to the solution, yielding a final concentration of 40 nM rhodamine B, and the corresponding fluorescence intensity F_0 was recorded for 10 min, and then 200 μM MTT were added. The slope of the tangent to the curve, F = f(t), after the addition of MTT was -dF/dt, and the initial rate of decrease of rhodamine B fluorescence was equal to:

$(V_i)_{CM} = (dF/dt)(C_T/F_0)$. $\Delta\Psi_m$ was determined using the expression: $\Delta\Psi_m = -61.51 \log V_i - 258.46$.

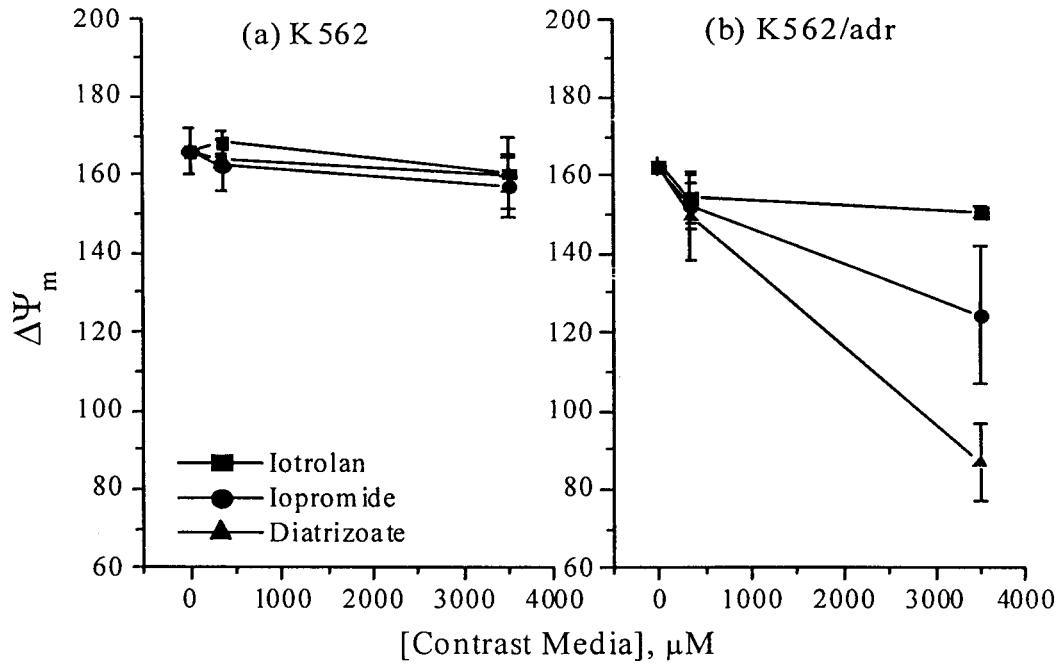


Figure 3-8 The mitochondrial membrane potential ($\Delta\Psi_m$) in intact cells (a) K562 and (b) K562/adr that resulted from following the kinetics of the uptake of rhodamine B by cells incubated with iotrolan (■), iopromide (●) and diatrizoate (▲) at concentration of 0, 350 and 3500 μM as described in Figure 3-7. The addition of 200 μM MTT into the experimental cells were done 10 minutes after the incubation.

Modulation of kinetics of the P-glycoprotein- mediated efflux of daunorubicin by contrast media

The effects of contrast media on the P-gp function could be determined by monitoring the efficiency of protein mediated efflux of daunorubicin in the absence and presence of contrast media. The kinetics of P-gp-mediated efflux of DNR could be investigated by following the fluorescence intensity of 1 μM daunorubicin (λ_{ex} = 480 nm, λ_{em}= 590 nm). The time required reaching the steady state of DNR accumulation in the energy- depleted cells was 90 minutes in K562/adr cells. The cells were viable throughout the experiments as being checked by trypan blue exclusion. The 5 mM glucose was added to initiate the active efflux and the net initial efflux represented the P-gp mediated active efflux only and V_a and the mean active efflux coefficients (k_a) were determined.

The k_a of DNR in K562/adr cells was 6.7 ± 0.6 (10^{-13}) $L.cell^{-1}.s^{-1}$. This value would be used as k_a^0 and to compare the ability of contrast media that modified the P-gp-mediated active efflux of DNR using the ratio; k_a^i / k_a^0; k_a^i was the mean active efflux coefficients in the presence of various concentrations (500, 1500, 2500 and 3500 μM) of diatrizoate, iopromide and iotrolan. The results in K562/adr cells were shown in Figure 3-9.

The contrast media were hyperdensity molecules that affected the cell morphology, which could be easily observed by light microscope. Cell shrinkage was immediately occurred after contrast media addition to the cells, and then they returned to normal morphology in a short time. This phenomenon could affect various cellular physiologies of cells such as acidifying the pH_i then followed by a correction of volume and pH_i, leading to modifications in protein synthesis (Dascular & Peer, 1994). In particular, the change in membrane conformation could influence the function of P-gp.

In order to verify the direct interaction of contrast media on P-gp where it took place in cytosolic site, concanamycin A and cytochalasin B were employed. It was found that the active efflux coefficient of P-gp, which was impaired by contrast media, was increased in the presence of 20 nM concanamycin A (a potent inhibitor of vacuolar H^+-translocating ATPases), or 10 μM cytochalasin B (a blocker of

endocytosis.) as shown in Figure 3-10a. The effect of 20 nM concanamycin A, or 10 μM cytochalasin B on the ability of contrast media that modified the P-gp-mediated active efflux of DNR using the ratio; k_a^i / k_a^0 were shown in Figure 3-10b.

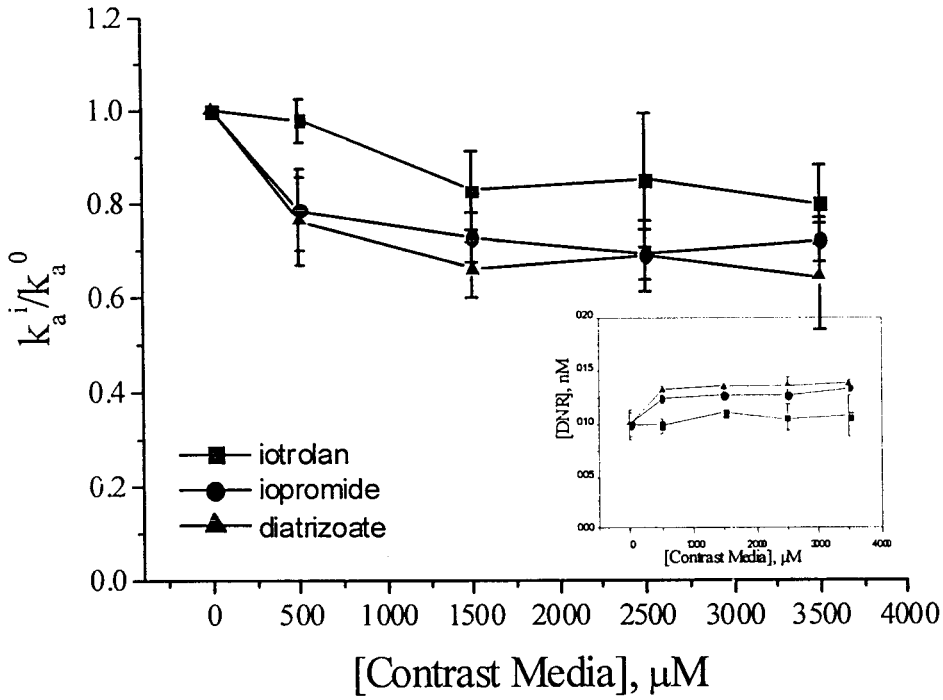


Figure 3-9 The kinetic parameters to compare the active efflux of P-gp in K562/adr cell line in the absence or presence of iotrolan (■), iopromide (●) and diatrizoate (▲) at various concentrations. The experiments were performed by following the fluorescence intensity of 1 μM DNR (λ_{ex} = 480 nm, λ_{em}= 590 nm) in 2 ×10^6 cells in 2 ml HEPES K^+buffer, pH 7.25 at 37 °C, which were depleted energy by adding 10 mM NaN_3 30 minutes before DNR addition. After the first steady state, 5 mM glucose was added before addition of contrast media then the active efflux coefficient of P-gp (k_a) was investigated. Each value represents the mean ± SD of three independent experiments.

Inset; The accumulation of DNR that intercalated between base pairs of DNA of K562/adr cells in the absence or presence of iotrolan (■), iopromide (●) and diatrizoate (▲) at various concentrations.

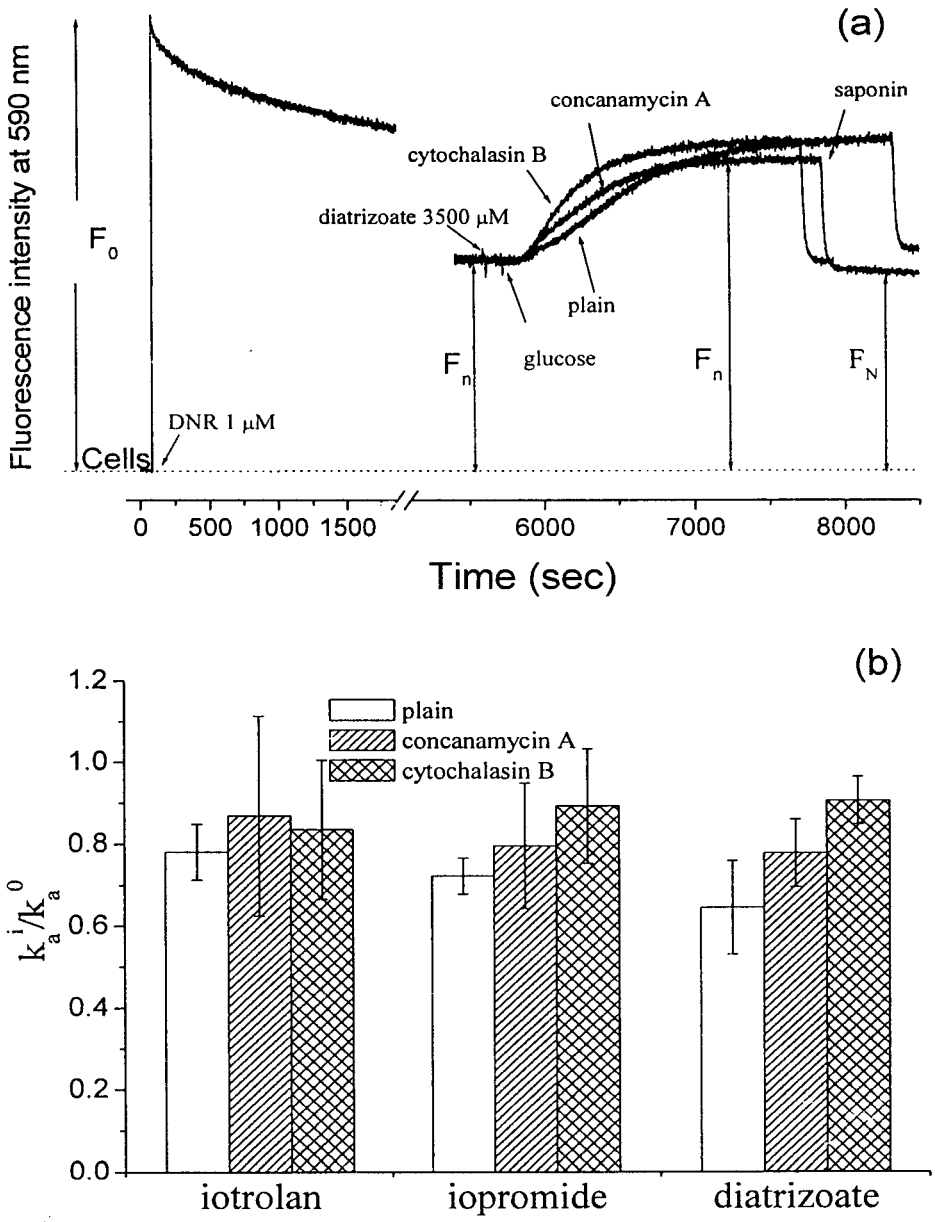


Figure 3-10 The effect of 20 nM concanamycin A, or 10 μM cytochalasin B on P-gp-mediated efflux in K562/adr cells, which was modulated by 3500 μM contrast media, a) the kinetic studies in the presence of diatrizoate without and with 20 nM concanamycin A or 10 μM cytochalasin B, and b) k_a^i/k_a^0 in the presence of CM without (□) and with 20 nM concanamycin A (▨)or 10 μM cytochalasin B (⊠)

CHAPTER 4
DISCUSSION

The study of physicochemical properties of molecules is very important in prediction of their *in vivo* performances and cellular interactions. The *in vitro* study model embraces permeability and solubility with qualifications related to pH and dissolution. In this work, the physicochemical properties of iodinated radiographic contrast media were determined to in terms of their cellular permeability or partition and solubility at physiological pH, which was the condition of MDR experimental model. The iodinated radiographic contrast media used in this study included diatrizoate (an ionic monomer), iopromide (a non-ionic monomer), and iotrolan (a non-ionic dimer). These molecules are intravenously administered in patients undergo intravenous pyelography (IVP) or computed tomography, which are the standard imaging modalities used to radiologically stage some cancers (Patz et al.,1999). The results in this work showed that they were very high water-soluble with very low lipophilicity as indicated by log P. Among the three molecules studied, iopromide was the most lipophilic and iotrolan was the least. This is corresponding to the findings in literature (Krause, Mikautz, Kollenkirchen, & Heimann, 1994). Chemical structure consideration of these molecules, at the physiological pH in living cells, diatrizoate exists as ionic molecule with one carboxyl group, which makes it hydrophilic, whereas iopromide and iotrolan have no charge but they are surrounded by hydroxyl groups. The more hydroxyl groups the higher hydrophilicity. In general, the molecules with these properties hardly partition with lipid bilayer nor passively diffuse through the biological membranes of living cells. They enter the cells by endocytosis.

The cytotoxicity of contrast media was as well determined by the cell growth inhibition dose (IC_{50}). At the concentrations used in the experiments, they did not affect the cell proliferation in K562 and its corresponding MDR cell line. These concentrations were comparable to and did not over-exceed the concentration administered in clinical uses (the average clinical concentration in blood circulation after IV. injection is about 2.5 mgI /ml; dose recommended by Schering AG, 1 ml of 300 mgI/ ml of contrast media per 1 kg body weight, whereas concentrations in this

study are 0.15 - 2.66 mgI /ml.). Although the contrast media themselves at the concentrations used in this study were not toxic to the cells, they (especially diatrizoate, an ionic monomer) were found, in co-treatment with daunorubicin, to enhance the efficiency of this anti-cancer drug to inhibit cell proliferation in the resistant K562/adr cell line. The difference between the K562/adr cell line and its parental cell line is that K562/adr cells are over-expressed by P-glycoprotein. It means that contrast media affected the function of P-glycoprotein, which was an active drug efflux transporter in this MDR cell line, therefore increasing the cytotoxicity of daunorubicin.

The study on the change of cellular energetic state was done by following the change in mitochondrial membrane potential ($\Delta\Psi_m$) of drug-sensitive and drug-resistant cells using rhodamine B. The mitochondrial membrane potential is essential for the production of ATP via oxidative phosphorylation. *In situ*, it is a sensitive indicator for the energetic state of the mitochondria and cells. It should be noted that even a slight decrease in the $\Delta\Psi_m$ could affected the cellular energetic state (Reungpatthanaphong & Mankhetkorn, 2002). Indeed, the three molecules of contrast media were able to decrease $\Delta\Psi_m$ in drug-resistant K562/adr cells, but not in drug sensitive K562 cells, with different degree. The aptitude to induce a decrease in $\Delta\Psi_m$ could be obviously seen in diatrizoate at high concentration, thus this reduced intracellular ATP. This was reasonably supported by the findings in literatures (Morgunov, Foster, & Hirsch, 1996; Soejima, Uozumi, Kanou, Fujiyama, & Masaki, 2003) that intracellular ATP could be reduced following the exposure of diatrizoate in animal kidney cells, which were also expressed some drug transporters such as mrp2 and P-gp (Masereeuw et al., 2000). However, the co-incubation with contrast media did not affect the cytotoxicity of daunorubicin in drug-sensitive K562 cells, in comparison with drug-resistant K562/adr cells, which need more ATP to assure their ATPase activity of protein membrane transporter, i.e. P-glycoprotein. The function of P-glycoprotein was lessened resulting in an increase in intracellular cytotoxic agent, leading to MDR cell death. (Broxterman, Pinedo, Kuiper, Schuurhuis, & Lankelma, 1989).

In vitro and *in vivo* MDR cell imaging is of uppermost importance for

successful cancer chemotherapy. In this work, it was demonstrated that diatrizoate could probably be used as modulator of $\Delta\Psi_m$ in combination with some mitochondrial probes such as, for *in vitro* studies, $DiOC_6$, JC-1 and rhodamine derivatives, and for *in vivo* studies, Tc-99m sestamibi. With an appropriate choice of probe, this modulation could be employed to distinguish the MDR cells or even to make the image of MDR cells, because only the MDR cells, herein K562/adr, would be affected by diatrizoate. This is the case, for example, of Tc-99m sestamibi, the γ-emitting probe that was administered in patients for localizing and imaging cancer in diagnostic nuclear medicine. Upon addition of diatrizoate, the chemo-resistant cancer cells would be recognized in low profile compared to the responsive cells.

The efficacies of diatrizoate, iopromide and iotrolan to inhibit the P-glycoprotein-mediated daunorubicin efflux in MDR cells were as well observed. This work had investigated the aptitude of these contrast media to inhibit the P-glycoprotein-mediated efflux by following the uptake of daunorubicin by K562/adr cells, demonstrated with the ratio k^i_a/k_a^0 as a function of contrast medium concentration added. Among the three molecules, diatrizoate had the highest capacity to inhibit the P-glycoprotein-mediated efflux. Furthermore, this was confirmed by treating experimental MDR cells with concanamycin A, and cytochalasin B. Since the active binding sites of protein membrane transporter, P-glycoprotein, were located intracellularly, any molecules or compounds that regulated P-glycoprotein had to do from inside of the cell. Considerately, the physicochemical properties of contrast media determined in this study and referred to the literature reviews (Dobrota, Powell, Holtz, Wallin, & Vik, 1995; Tervahartiala, Kivisaari, Kivisaari, Vehmas, & Virtanen, 1997), diatriziate, iopromide and iotrolan could uptake into cells by endocytosis where molecules were engulfed and retained in vacuoles, which represented a part of lysosomal compartment (Dobrota et al, 1995). Concanamycin A was used to inhibit the action of vacuolar H^+-translocating ATPases that presented on membrane of lysosome, but did not inhibit the P-glycoprotein-mediated efflux of drug (Leotchutnat, Priebe, & Garnier-Suillerot, 2001). In addition, cytochalasin B was used to inhibit a wide variety of cellular movements that is one of important mechanisms of endocytosis by interacting with microfilaments in the cells (Lin & Spudich, 1974).

These two compounds prevented contrast media to get inside the cell, leading to higher capability of P-glycoprotein-mediated efflux, when compared with the condition that contrast media were added alone. The efficacy of contrast media, especially diatrizoate, to impair the function of P-gp seemed very apparent but the mechanism(s) of this modulation still was not clear whether they were substrates of P-gp or they affected the energy consumption of P-gp as mentioned above.

In general, most studies have focused on the effects of contrast media on kidney cells since they were excreted by nephron units after intravenous administration into patients. This is the first study to show that they also affected cellular energetic state and the function of P-glycoprotein in MDR cancer cells.

CHAPTER 5
CONCLUSION

The physicochemical properties including light absorption, pK_a and lipophilicity, and their interactions with intact drug-sensitive and –resistant cells of iodinated radiographic contrast media used in diagnostic radiography; diatrizoate (an ionic monomer), iopromide (a nonionic monomer) and iotrolan (a non ionic dimer) were investigated. These contrast media are very hydrophilic compounds with relatively low cytotoxicity at the concentrations used that was comparable to the dosage administered in clinics. However, in the co-treatment with daunorubicin, an anticancer drug that was a substrate of P-glycoprotein, diatrizoate could enhance the efficacy of this anticancer drug in K562/adr cells. All the contrast media used affected the cellular energetic state only in MDR cells by decreasing $\Delta\Psi_m$ and inhibit P-glycoprotein function, leading to the restoration of intracellular drug concentration, consequently, the cytotoxicity was increased in the drug-resistant cells. The results provide a new possible application of contrast media other than usual utilization. In combination with appropriate mitochondrial probe that accumulated in the cells depending on $\Delta\Psi_m$, the contrast media could be used as a mitochondrial energetic state modulator that was specific to MDR cells (K562/adr cells). This study had led to some points of view that diatrizoate might be raised for further study as MDR identification in MDR imaging *in vitro* or *in vivo* with the help of some probes that their main target were in mitochondria and, eventually, a MDR chemo-sensitizing agent in cancer treatment.

REFERENCES

Ambudkar, S. V., Dey, S, Hrycyna, C. A., Ramachandra, M., Pastan, I., & Gottesman, M. M. (1998). Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annual Reviews of Pharmacological Toxicology, 39,* 361-398.

Bradley, G., Juranka, P. F., & Ling, V. (1998). Mechanism of multidrug resistance. *Biochimica et Biophysica Acta, 948,* 87-128.

Broxterman, H. J., Pinedo, H. M., Kuiper, C. M., Schuurhuis, G. J., & Lankelma, J. (1989). Glycolysis in P-Glycoprotein- overexpressing human tumor cell lines; Effects of resistance- modifying agents. *FEBS LETTERS, 247,* 405-410.

Cole, S. P. C., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C.E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M., & Deeley, R.G. (1992). Over-expression of a transport gene in a multidrug- resistant human lung cancer cell line. *Science, 258,* 1650-1654.

Dascula, A., & Peer, A. (1994). Effects of radiological contrast media on human endothelial and kidney cell lines: Intracellular pH and cytotoxicity. *Academic Radiology, 1,* 145-150.

Dobrota, M., Powell, C. J., Holtz, E., Wallin, A., & Vik, H. (1995). Biochemical and morphological effects of contrast media on the kidney. *Acta Radiologica, Supplement 399,* 196-203.

Eytan, G. D., Borgnia, M. J., Regev, R., & Assaraf, Y. G. (1994). Transport of polypeptide ionophores into proteoliposomes reconstituted with rat liver P-glycoprotein. *The Journal of Biological Chemistry, 269,* 26058-26065.

Fanning, N. F., Manning, B. J., Buckley, J., & Redmond, H. P. (2002). Iodinated contrast media induce neutrophil apoptosis through a mitochondrial and caspase mediated pathway. *The British Journal of Radiology, 75,* 861-873.

Frezard, F., & Garnier-Suillerot, A. (1991). Determination of the osmotic active drug concentration in the cytoplasm of anthracycline- resistant and sensitive K562 cells. *Biochimica et Biophysica Acta, 1091,* 29-35.

Fischer, H.W. (1977). Introduction to angiographic contrast agents. In R.E. Miller, & J. Skucas (Eds.), *Radiographic Contrast Agents* (pp. 329-335), Baltimore: University Park Press.

Garnier-Suillerot, A. (1995). Impaired accumulation of drug in multidrug resistant cells. What are the respective contributions of the kinetics of uptake and of P-glycoprotein- mediated efflux of drug? *Current Pharmaceutical Design, 1*, 69-82.

Gottesman, M. M., & Pastan, I. (1993). Biochemistry of multidrug resistance mediated by the multidrug transporter. *The Annual Reviews of Biochemistry, 62*, 385-427.

Grandjean, F., Brémaud, L., Verdier, M., Robert, J., & Ratinaud, M. H. (2001). Sequential gene expression of P-glycoprotein (P-gp), Multidrug resistance-associated protein (MRP) and lung resistance protein: functional activity of P-gp and MRP present in the doxorubicin- resistant human K562 cell lines. *Anti- Cancer Drugs, 12,* 247-258.

Hardiek, K., Katholi, R. E., Ramkumar, V., & Deitrick, C. (2001). Proximal tubule cell response to radiographic contrast media. *American Journal of Physiolgical Renal Physiology, 280*, F61-F70.

Harper, M. E., Antoniou, A., Villalobos-Menuey, E., Russo, A., Trauger, R., Vendemello, M., George, A., Bartholomew, R., Carlo, A., Shaikh, A., Kupperman, J., Newell, E. W., Bespalov, I. A., Wallace, S. S., Liu, Y., Rogers, J. R., Gibbs, G. L., Leahy, J. L., Camley, R. E., Melamede, R., & Newell, M. K. (2002). Characterization of a novel metabolic strategy used by drug-resistant tumor cells. *FASEB, 16*, 1550-1557.

Higgins, CF. (1992). ABC transporters: From microorganism to man. *Annual Reviews of Cellular Biology, 8*, 67-113.

Hipfner, D. R., Deeley, R. G., & Cole, S. P. C. (1999). Structural, mechanistic and clinical aspects of MRP1. *Biochimica et Biophysica Acta, 1461*, 359-376.

Horio, M., Gottesman, M. M., & Pastan, I. (1998). ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. *Proceeding of the National Academy of Sciences of the United States of America, 85,* 3580-3584.

Izquierdo, M. A., Shoemaker, R. H., Flens, M. J., Scheffer, G. L., Wu, L., Prather, T. R., & Scheper, R. J. (1996). Overlapping phenotypes of multidrug resistance among panals of human cancer- cell lines. *International Journal of Cancer, 65*, 230-237.

Juliano, RL., & Ling, V. (1976). A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica et Biophysica Acta, 455*, 152-162.

Kimmich, G., Randles, J., & Brand, J. (1975). Assay of picomole amounts of ATP, ADP and AMP using the luciferase enzyme system. *Analytical Biochemistry, 69*,187-192.

Krause, W. (1999). Delivery of diagnostic agents in computed tomography. *Advanced Drug Delivery Reviews, 37*, 159-173.

Krause, W., Miklautz, H., Kollenkirchen, U., & Heimann, G. (1994). Physicochemical parameters of X-ray contrast media. *Investigative Radiology, 29*, 72-80.

Lautier, D., Canitrot, Y., Deely, R. G., & Cole, S. P. C. (1996). Multidrug resistance mediated by the multidrug resistance protein (MRP) gene. *Biochemical Pharmacology, 52*, 967-977.

Lin, S., & Spudice, J. A. (1974). Biochemical studies on the mode of action of cytochalasin B. *The Journal of Biological Chemistry, 249*, 5778-5783.

Loetchutinat, C., Priebe, W., & Garnier-Suillerot, A. (2001). Drug sequestration in cytoplasmic organelles does not contribute to the diminished sensitivity of anthracyclines in multidrug resistant K562 cells. *European Journal of Biochemistry, 268*, 4459-4467.

Mankhetkorn, S., Dubru, F., Hesschenbrouck, J., Fiallo, M., & Garnier-Suillerot, A. (1996). Relation among the resistance factor, kinetics of uptake, and kinetics of the P-glycoprotein-mediated efflux of doxorubicin, daunorubicin, 8-(S)-fluoroidarubicin, and idarubicin, multidrug resistant K562 cells. *Molecular Pharmacology, 49*, 532-539.

Martin, C., Berridge, G., Higgins, C. F., & Callaghan, R. (1997). The multi-drug resistance reversal agent SR33557 and modulation of vinca alkaloid binding to P-glycoprotein by an allosteric interaction. *The British Journal of Pharmacology, 122,* 765-711.

Masereeuw, R., Terouw, S. A., Van Abel, R. A. M. H., Russel, F. G. M., & Miller, D. S. (2000). Endothelin B receptor-mediated regulation of ATP-driven drug secretion in renal proximal tubule. *Molecular Pharmacology, 57*, 59-67.

Meesungnoen, J. (2001). *Cellular multidrug resistance: mitochondrial energetic state, cellular drug distribution, and kinetics of P-glycoprotein-mediated efflux of pirarubicin in K562/adr cells with different resistance factors.* Master's thesis, Department of Biological Science, Graduate School, Burapha University.

Meesungneon, J., Jay-Gerin, J. P., & Mankhetkorn, S. (2002). Relation between MDR1 mRNA levels, resistance factor, and the efficacy of P-glycoprotein-mediated efflux of pirarubicin in multidrug resistant K562 sublines. *Canadian Journal of Physiological Pharmacology, 80,* 1054-1063.

Morcos, S. K., & Thomsen, H. S. (2001). Adverse reactions to iodinated contrast media. *European Radiology, 11,* 1267-1275.

Morgunov, N. S., Foster, S. G., & Hirsch, D. J. (1996). Contrast media induced changes in tubular electrophysiology and glomerular filtration rate in mouse kidney. *Canadian Journal of Physiological Pharmacology, 75*, 988-995.

Morris, T. W. (1993). X-ray contrast media: where are we now, and where are we going? *Radiology, 188,* 11-16.

Morris, T. W., & Katzberg, R. W. (1992). Intravascular contrast media: Properties and general effects. In R. W. Katzberg, (Eds.), *The Contrast Media Manual* (pp. 1-18). Baltimore: Williams & Wilkins.

Murphy, M. P., & Smith, R. A. J. (2000). Drug delivery to mitochondria: the key to mitochondrial medicine. *Advanced Drug Delivery Reviews, 41,* 235-250.

Patz, E. F., Erasmus, J. J., McAdams, H. P., Connolly, J. E., Marom, E. M., Goodman, P. C., Ledger, R. A., Keogan, M. T., & Herndon, J. E. (1999). Lung cancer staging and management: comparison of contrast-enhanced and non-enhanced helical CT of thorax. *Radiology, 212*, 56-60.

Reungpatthanapong, P., & Mankhetkorn, S. (2002). Modulation of multidrug resistance by artemisinin, artesunate and dihydroartemisimin in K562/adr and GLC4/adr resistant cell lines. *Biological Pharmacology Bulletin, 25*, 1555-1561.

Reungpatthanapong, P., Dechsupa, S., Meesungnoen, J., Loetchutinat, C., & Mankhetkorn, S. (2003). Rhodamine B as a mitochondrial probe for measurement and monitoring of mitochondrialmembrane potential in drug-sensitive and -resistant cells. *Journal of Biochemical and Biophysical Methods, 1663*, 1-16.

Riordan, R. L., & Ling, V. (1979). Purification of P- glycoprotein from plasma membrane vesicles of Chinese hamster ovary cell mutants with reduced colchicine permeability. *The Journal of Biological Chemistry, 254,* 12701-12705.

Rolfe, D. F. S., & Brown, G. C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physics Reviews, 77,* 731-758.

Russell, C., Scaduto, J. R., & Lee, W. G. (1999). Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophysics Journal, 76*, 469-477.

Scheper, R. J., Broxterman, H. J., Scheffer, G. L., Kaaijik, P., Dalton, W. S., van Heijningen, T. H. M., van Kalken, C. K., Slovak, M. L., de Vries, E. G. E., van de Valk, P., Meijer, C. J. L. M., & Pinedo, H. M. (1993). Overexpression of a Mr 110000 vesicular protein in non-P-Glycoprotein-mediated multidrug resistance. *Cancer Research, 53*, 1475-1479.

Sharom, F. J. (1997). The P-glycoprotein efflux pump: How does it transport drug? *Journal of Membrane Biology, 160*, 161-175.

Simon, S. M., & Schindler, M. (1994). Cell biological mechanisms of multidrug resistance in tumors. *Proceeding of the National Academy of Sciences of the United States of America, 91,* 3497-3504.

Shustik, C., Dalton, W., & Gros, P. (1995). P-glycoprotein-mediated multidrug resistance in tumor cells: biochemistry, clinical relevance and modulation. *Molecular Aspects of Medicine, 16*, 1-78.

Skovsgaard, T. (1978). Mechanism of cross resistance between vincristine and daunorubicin in Ehrlich ascites tumor cells. *Cancer Research, 38*, 4722-4727.

Soejima, K., Uozumi, J., Kanou, T., Fujiyama, C., & Masaki, Z. (2003). Nonionic contrast media are less nephrotoxic than ionic contrast media to rat renal cortical slices. *Toxicology Letters, 143*, 17-25.

Sukhai, M., & Piquette-Miller, M. (2000). Regulation of the multidrug resistance genes by stress signals. *Journal of Pharmacological and Pharmaceutical Science, 3*, 268-280.

Tamai, I., & Safa, A. R. (1991). Azidopine noncompetitively interacts with vinblastine and cyclosporin A binding to P-glycoprotein in multidrug resistant cells. *The Journal of Biological Chemistry, 266*, 16796-16800.

Teodori, E., Dei, S., Scapecchi, S., & Gualtieri, F. (2002). The medicinal chemistry of multidrug resistance (MDR) reversing drugs. *Il Farmaco, 57*, 385-415.

Tervahartiala, P., Kivisaari, L., Kivisaari, R., Vehmas, T., & Virtanen, I. (1997). Structural changes in the renal proximal tubular cells induced by iodinated contrast media; Studies in dehydrated rats. *Nephron, 76*, 96-102.

Tsubaki, M., & Yoshikawa, S. (1993). Fourier-transform infra-red study of azide binding to the Fe3-CuB binuclear site of bovine cytochrome oxidase. *Biochemistry, 32*, 174-182.

Twentyman, P. R., & Bleehan, N. M. (1992). Cytotoxic drug resistance: molecular basis and clinical significance. *The British Journal of Radiology, Supplement*, 24, 84-88.

Twentyman, P. R., & Luscombe, M. (1987).A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. *British Journal of Cancer, 56*, 279-285.

Ueda, K., Pastan, I., & Gottesman, MM. (1987). Isolation and sequence of the promoter region of the human MDR (PGP) gene. *The Journal of Biological Chemistry, 262*, 17432-17436.

Vergote, J., Moretti, J. L., De Vries, E. G. E., & Garnier-Suillrot, A. (1998).. Comparison of the kinetics of active efflux of ^{99m}Tc-MIBI in cells with P-glycoprotein-mediated and multidrug- resistance protein associated multidrug- resistance phenotypes. *European Journal of Biochemistry, 252,* 140-146.

Volm, M., & Mattern, J. (1996). Resistance mechanisms and their relation in lung cancer. *Critical Review of Oncogenesis, 7,* 227-244.

Wang, X., Krebs, L. J., Al-Nuri, M., Pudavar, H. E., Ghosal, S., Liebow, C., Nagy, A. A., Schally, A. V., & Prasad P. N. (1996). A chemically labeled cytotoxic agent: Two- photon fluorophore for optical tracking of cellular pathway in chemotherapy. *Proceeding of the National Academy of Sciences of the United States of America, 96,* 11081-11084.

BIOGRAPHY

Name	Mrs. Nitaya Snitwongse Na Ayudhya
Date of birth	December 15, 1964
Place of birth	Chantaburi, Thailand
Present address	19/26 Sukhasongkoh 1.1 Rd., Naimuang, Muang, Ubonratchatani, 34000
Position held	
1989- present	Radiological technologist, Sappasittiprasong Hospital
Education	
1983-1987	Bachlor of Science (Radiological Technology) Chiangmai University
2000-2003	Master of Science (Biological Science) Burapha University