CHAPTER I

GENERAL INTRODUCTION

1. Cellular drug resistance

Resistance to anticancer drug is the leading cause of failure in the treatment of systemic human cancer (Larsen *et al.*, 2000). Since such chemo-resistance typically exhibits simultaneous resistance to a number of structurally and functionally unrelated chemotherapeutic agents, the term multidrug resistance (MDR) has been introduced to describe the phenomenon characterized by the ability of cancer cells to resist drug treatment. A large number of studies have been devoted to elucidate the mechanisms leading to MDR, often compared the behaviors of drug-sensitive with MDR cancer cells. MDR phenomenon is the results of a variety of mechanisms by the following:

1.1 Reduction in activity of DNA topoisomerase

DNA topoisomerase involve in the process of DNA replication. This enzyme is a therapeutic target for anticancer drugs in rapidly dividing cancer cells. For example, doxorubicin (DOX), a member of anthracycline drugs, targets topoisomerase II alters DNA topology by causing transient double strand break. (Tewey *et al.,* 1984; Liu, 1989; Ross *et al.*, 1984). Anthracyclins stabilize the transient covalent complex between DNA and topoisomerase II, by blocking rejoin of DNA strands, resulting in the death of drug sensitive cancer cells (Drake *et al.,* 1987). However, MDR cancer cells become resistant to topoisomerase inhibitors such as DOX probably due to either the under-expression of topoisomerase

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II or topoisomerase II gene mutation (Altenberg *et al.*, 1993). However, the exact mechanism of this down regulation is poorly understood.

1.2 Down-regulation of apoptosis

Whether a cell pass to cell cycle or undergoes apoptosis is depended on a complex interplay of genes and proteins that exert a regulatory role on cellular events. Anticancer drugs typically induce apoptosis; however MDR cells seem to develop a loss of genes required for apoptosis. It is known that B–cell lymphoma–2 (*bcl–2*) is a gene that plays a key role in the regulation of apoptosis pathways (Krishna & Mayer, 2000).

1.3 Changes in pH distribution in the cell

Mechanisms of such intracellular pH regulation among organelles was mainly explained by the progressive decreases of H^+ permeability from the endoplamic reticulum (ER) to the Golgi and the secretory granules: following by successive increase in the active H^+ pump density for acidification from the ER to secretory granules (Wu *et al.*, 2001).

In various MDR cell lines, such as MCF–7 breast carcinoma cells and HL–60 leukemia cells, the alteration of intracellular pH distribution with respect to their normal counterparts were reported. Intracellular pH in drug sensitive cells undergoes several changes compared to normal cells, including a more acidic cytoplasmic pH and a more alkaline vesicular pH. However, long-term exposure of such sensitive cell lines to anticancer drugs leads to the selection of the corresponding MDR cells that have reverted back to similar pH distributions as in non-cancer cells. Moreover, treatment of DOX–resistant cells with ionophores that disrupt vesicle acidification leads to the resensitization of the MDR phenotype, strongly suggesting a direct

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relationship between the intracellular pH distributions and MDR (Schindler *et al.*, 1996)

Most of all anticancer drugs are considered as weak–base, such the pH changes of these particular organelles can change the nature of the cellular drug distribution. These phenomenon render acidic compartments to work as sinks and result in sequestration of the weak base drugs into cytoplasmic organelles. Since most anticancer drugs target DNA or nuclear enzyme, their sequestration to acidic compartments will lead to a decrease in the drug–target interaction and thereby, decreased cytotoxicity. However, after these sinks are saturated by drug, it is expected that drug–target interaction will be dramatically increased. Drug accumulation in acidic compartments is transported to the cell surfaces by cellular membrane trafficking pathways, as same as the transport of many cellular lipids and proteins (Larsen *et al*, 2000). These are routes for delivering newly synthesized proteins to the plasma membrane or other cell components.

1.4 Over-expression of transporter proteins

It is well known that MDR alters expression of drug transporter proteins, which extrude a number of anticancer drugs out of the cell, thus decreasing their concentration at intracellular targets. Among drug transporter protein, P–glycoprotein (P–gp) and multidrug resistance associated protein (MRP) are members of the ATP–binding cassette family (ABC) of membrane transporters include at least 200 prokaryotic and eukaryotic proteins. While other transporters in the ABC family are relatively specific for their substrates (e.g. amino or sugar), MDR–ABC transporters including P–gp and MRP have exceptionally broad specificity for structurally dissimilar compounds. It is reported that these transporters act not only drug

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transporters but also also ion transporter/regulators. The expression of transporter proteins cause intracellular pH changes by ion transport or regulation while the functionality of the MDR proteins was not affected by pH changes (Marbeuf–Gueye *et al.*, 2000).

In contrast to P–gp and MRP, lung resistance-related protein (LRP) is found to be a non–ATP-dependent MDR protein. Recently, it was reported that, among P–gp, MRP and LRP, the mRNA synthesis of LRP was most correlated to MDR phenotype in intrinsically resistant human cancer cell lines (Laurencot *et al.*, 1997). The expression of plasma membrane caveolae is also reported to be increased in MDR cells (Lavie *et al.*, 2001).

1.4.1 P-glycoprotein

P–glycoprotein (P–gp), a member of the ATP–binding casstte (ABC) family is the first protein that showed to convey MDR to anticancer agent (Juliano & Ling, 1976). P–gp is a 1280 amino acid long (molecular weight 170 kDa) glycoprotein. It is founded in human, mouse, hamster, and many other mammalian cells. The genes that encode P–pg, are MDR1 and MDR2 in human tissues, or mdr1a, mdr2 and mdr1b in murine species. P–gp constitutes a very important part of the plasma membrane, accounting for 20% of the total plasma membrane proteins in Chinese hamster cells selected with DOX (Sognier *et al.*, 1994). P–gp is found not only in cancer cells but also in brush border of proximal tubules of kidney, bile canalicular membrance of hepatocytes, apical membrane of mucosal cells, intestine and luminal membrane of endothelial cells at blood–tissue barrier sites (Relling, 1996)

The model structure of P–gp is shown in Figure 1. P–gp composes of twelve transmembrane domains (putative α-helicases) that are hydrophobic and span the

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membrane and two ATP binding domain that play a role in cleaving ATP (hydrolysis) to deliver the energy necessary for transporting drugs, ions and other substrates. Both ATP binding sites have approximately the same affinity and are functionally equivalent.

Most of the residues involved in substrate binding and transports are located within the transmembrane domains (Cool *et al.*, 2002), probably inferred that drugs are transporter via membrane. Therefore, the ability of drugs to intercalate into the phospholipid bilayer would preselect drugs to be transported from other cellular compounds that are not to be transported. Subsequently, the interaction between the preselected drug and the transporter would be a second determinant of specificity.

P–gp is involved in the transport of many different endogenous substrates including lipids and steroids, and pharmacological substrates including anthracyclines (e.g. DOX), vinca alkaloids (e.g. vincristine), podophyllotoxins (e.g. etoposide) and taxanes (*e.g.* taxol). Others substrates of P–gp are included verapamil, trifluoroperazine, quinine, pepstatin, valinomycin, gramicidin and cyclosporine (Terry & Olafur, 2002).

1.4.2 Multidrug resistance associated protein (MRP1)

In 1992, Cole *et al.* have characterized the second major membrane protein transporter called multidrug resistance associated protein (MRP) involved in MDR phenomena (Cole *et al.*, 1992). The MRP, a 190 Kilodaltons membrane protein, also belonged to ATP binding–cassette (ABC) superfamily of transmembrane transporters containing 1531 amino acids. It is only about 15% structural identity with P–gp. In contrast, mechanism by which this protein mediated drug to transport appears to be differ from P–glycoprotein's (Litman *et al.*, 2001). The substrate specificity seem to

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be more difficult to be defined whereas P–gp has the greatest affinity for binding its substrate due to large, hydrophobic cations. MRP appears to be the most effective protein for transporting organic anions. Cole *et al.* proposed that MRP1 increased drug efflux by co–transport of GSH but not in case of P–gp.

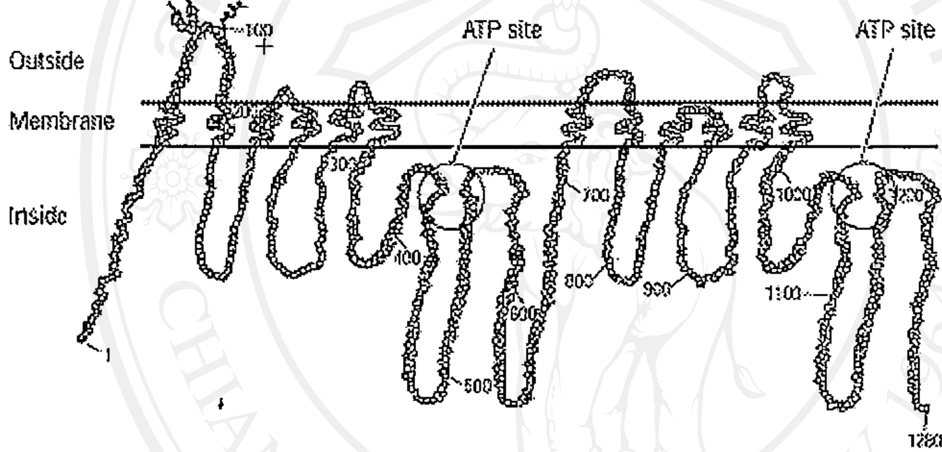

Figure 1. Topology of the human P–glycoprotein. The ATP–binding sites, based on sequence homology with bacterial transport systems, are circled and putative N–linked carbohydrates are shown as curly lines. This picture in modified from Germann, 1996.

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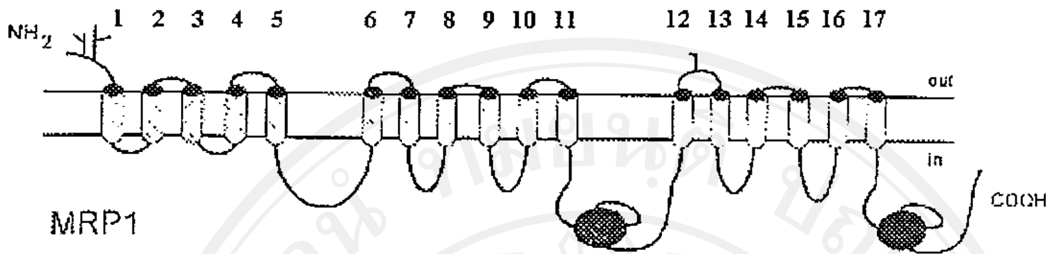

Figure 2. Model of Multidrug resistance associated protein (MRP1). This picture in modified from Litman *et al.*, 2001.

2. Cellular energetic states and Multidrug resistance phenotypes

Several studies revealed that MDR cells need more cellular ATP than that of their corresponding sensitive cells (Miccadei *et al.*, 1996; Dorward *et al.*, 1997; Nieminen *et al.*, 1994; Jia *et al.*, 1996). A strong evidence is demonstrated that ATP depletion in MDR cells, partially will block on P-glycoprotein and MRP1 pump activity that leading to an increase in cellular drug accumulation (Versantvoort, *et al.*, 1992; Mankhetkorn *et al.*, 1996). Indeed, how to understand the source of cellular ATP production and cellular energetic state of MDR cells is a crucial point to overcome MDR phenomena. In other words, we must get insight to study how the important role of mitochondria will play in the MDR phenotype since they supply the cellular ATP pool.

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3. ABC-transporters in parasites related to ABC-transporters in human cancer

Malaria, caused by the parasite Plasmodium, is the most prevalent tropical disease and is estimated to affect 300/500 million people and causes 2/3 million deaths annually. Of the four Plasmodium species that infect men, *Plasmodium falciparum* causes profound pathology and an overwhelming parasitemia resulting in high mortality rates in untreated cases. More than 41% of the world's population is at risk for this infection that is due to the rapid spread of drug-resistant protozoans (Olliaro *et al.*, 1996).

At least three ABC–transporter encoding genes have been identified in *P. falciparum*, two P–gp homologues *Phg1* (Foote *et al.*, 1989; Wilson *et al.*, 1989) and *Phg2* (Zalis *et al.*, 1993), and PfGCN20 (Bozdech *et al.*, 1996). The *pfmdr1* gene on chromosome 5 encoding *Phg1* was originally identified and amplified in chloroquine–resistant isolates (Foote *et al.*, 1989). This resistance corresponds to a reduction in cellular accumulation of these drugs, in particular their accessibility to haematin, the proposed target within the digestive vacuole (Bray *et al.*, 1999). Biochemical mechanisms for resistance are likely to be mediated by parasite-encoded drug transporters. Sidhu and colleagues reported the use of a powerful reverse genetics approach to assess the importance of several commonly occurring alleles of the *P. falciparum* that homologue to mammalian multidrug resistance (*mdr*) genes, *pfmdr*1 (Sidhu *et al.*, 2005).

In parasitic species, the *MDR* homologues represent good candidates for drug-resistance genes as it has been shown that *MDR* genes in mammals directly cause multidrug resistance. Overexpression of MDR–like genes in unicellular

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The organization of the thesis dissertation

The thesis provides on original of results and new methodology allows understanding the different in cellular mitochondrial and energetic state of drug–sensitive to drug-resistant cells. Such differences can be used as intracellular targets for overcoming MDR phenomenon. In particular, artemisinin, artesunate and dihydroartemisinin were demonstrated as anticancer agents particularly in drug–sensitive and drug-resistant cells. Overall of results provides five original research articles; the one was submitted and other four already published in the scientific journal specialized in the fields.

The thesis was edited and presented in article research formats, chronologically demonstrated from the establishment of a spectrofluorometric method for measuring the mitochondrial membrane potential, its application to determine the cellular energetic change during exposing to cytotoxic agents, the assessments of drug–sensitive and drug-resistant cells response to these compounds and two articles presented as appendix.

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