CHAPTER IV

Modulation of Multidrug Resistance by Artemisinin, Artesunate and Dihydroartemisinin in K562/adr and GLC4/adr Resistant Cell Lines

Published in
Biological & Pharmaceutical bulletin (2002)
Volume 25(12): 1555—1561

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Modulation of Multidrug Resistance by Artemisinin, Artesunate and Dihydroartemisinin in K562/adr and GLC4/adr Resistant Cell Lines

Paiboon Reungpatthanaphong and Samlee Mankhetkorn*

** Laboratory of Physical Chemistry, Molecular and Cellular Biology, Faculty of Science, Burapha University; Bangsaen, Chonburi 20131, Thailand.*

Abstract

Overcoming MDR (multidrug resistance) phenomena is a crucial aspect of cancer chemotherapy research. Artemisinin and its derivatives have been found to inhibit the proliferation of cancer cells in the μM range. They poorly inhibited the function of P–glycoprotein and did not inhibit the function of MRP1–protein. The concentrations required to inhibit by 50% the function of P–glycoprotein are 110 ± 5μM. Artemisinin, artesunate and dihydroartemisinin efficiently decreased the mitochondrial membrane potential, leading to a decrease in intracellular ATP in all cell lines tested: by 30 to 50% at 5 μM. Artemisinin, artesunate and dihydroartemisinin increased cytotoxicity of pirarubicin and doxorubicin in P–glycoprotein–overexpressing K562/*adr*, and in MRP1–overexpressing GLC4/*adr*, with the $\delta_{0.5}$ ranging from 200 to 860 nM, but not in their corresponding drug-sensitive cell lines. In this range of concentrations these compounds did not decrease the function of P–glycoprotein, suggesting a mechanism by which the drugs did not reverse MDR phenomenon at the P–glycoprotein level but at the mitochondrial level.

Key words multidrug resistance (MDR) reversing agent; P–glycoprotein; MRP1–protein; artemisinin; dihydroartemisinin; artesunate

* **Corresponding author** *: Tel : 66 38 745900 ext 3098; Fax : 66 38 745199*
E-mail address : samlee@bucc4.ac.th

1. INTRODUCTION

Multidrug resistance (MDR) is a principle cause of failure in human drug treatment. MDR phenomena are intensively studied in anti-cancer, anti-bacterial, anti-paludism, and human immunodeficiency virus–1 (Bradley *et al.*, 1988; Borst & Ouellette, 1995; Levy, 1992; van Veen & Könings, 1997; Saves & Masson, 1998). The MDR phenotype is characterized by a low free intracellular cytotoxic drug concentration in comparison with their corresponding drug sensitive cells. This is frequently associated with protein membrane transporters, such as P–glycoprotein and MRP1–protein, which ATP–dependently extrude cytotoxic agents out of cells, thus reducing their efficacy. To overcome MDR phenomena, the direct interaction between inhibitors and proteins has been researched by many groups. A variety of small molecules, such as

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

verapamil, dihydropyridines, forskolin and cyclosporin were found to bind to P–glycoprotein and inhibit its ability to pump out antitumor drugs. (Tsuro *et al.*, 1981; Slater *et al.*, 1986; Tasaka *et al.*, 2001; Wadler & Wiernik,1988). All compounds cited are difficult to use *in vivo* due to their toxicity. As a consequence, the clinical utility of non-toxic derivatives of these and other molecules as chemosensitizing agents has been extensively studied (Teodori *et al.*, 2002; Lehne, 2000). It was reported that in MDR *plasmodium falciparum* strains, the *pfmdr-1* gene and *pgh-1* were characterized, which has about 54% homology to *mdr-1* gene of MDR cancer cells (Karcz & Cowman, 1991; Foote *et al.*, 1989; Hasson *et al.*, 1992; Reed *et al.*, 2000). However, its function is still not clear. Recently, it was reported that qinghaosu is an effective compound against MDR *plasmodium falciparum* strains, (Kain, 1995; Olliaro *et al.*, 2001) but studies regarding its effect on protein transporters have not been performed, neither in erythrocytes infected by MDR *plasmodium falciparum* strains nor in cancer MDR cells. Qinghaosu drugs such as artemisinin and artesunate are now in widespread use, particularly in Southast Asia, and are used as a prophylaxis in China (Wongsrichanalai *et al.*, 2002; Labbe *et al.*, 2001).

In this study we have demonstrated that artemisinin, artesunate and dihydroartemisinin poorly inhibited P–glycoprotein and did not inhibit MRP1 protein function in multidrug resistant K562/*adr* cells, overexpression P–glycoprotein, or in GLC4/*adr* cells overexpression of MRP1–protein, respectively. However, they increased in cytotoxic effect induced by pirarubicin or doxorubicin only in MDR cell lines. We also demonstrated that these qinghaosu modulate mitochondrial function, leading to a decrease in intracellular ATP content in all cell lines tested.

2. MATERIALS AND METHODS

2.1 Cell Culture and Cytotoxicity Assay

The erythromyelogenous leukemic, K562, and its P–glycoprotein–overexpressing K562/*adr*,(Lozio & Lozio, 1975; Tsuro *et al.*, 1986) and the human small cell lung cancer, GLC4 and its MRP1-overexpressing GLC4/ *adr*, (Muller *et al.*, 1994; Versantvoort *et al.*, 1995; Zijlstra *et al.*, 1987) were kindly provided by Professor Arlette Garnier–Suillerot, Laboratoire de Chimie Physique, Biomoleculaire et Cellulaire, UFR Sante Medecine Biologie Humaine, Universite de Paris Nord, France. These cells were routinely cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco Biocult, Ltd.). The resistant K562/*adr* and GLC4/*adr* cells were cultured with 100 nM doxorubicin two weeks before the experiments. For the assays, a culture was initiated at 5×10^5 cells per ml to have cells in the exponential growth phase; the cells were used 24 h later, when the culture had grown to approximately 8×10^5 cells per ml. Cell viability was assessed by Trypan blue exclusion. The number of cells was determined by hemocytometer.

The cytotoxicity assay was performed as follows: 5×10^4 cells per ml were incubated in the presence of various concentrations of artemisinin, artesunate or dihydroartemisinin. Co–treatment using pirarubicin and qinghaosu or doxorubicin and qinghaosu were performed. Cells (5×10^4 per ml) were incubated in the

Copyright © by Chiang Mai University
All rights reserved

presence of various pirarubicin or doxorubicin concentrations and a fixed concentration of qinghaosu ranging from 20 to 1000 nM. The viability of cells was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT)–reduction assay. The IC_{50} was determined by plotting the percentage of cell growth inhibition *versus* the pirarubicin concentration: IC_{50} is the pirarubicin or doxorubicin concentration that inhibits cell growth by 50% when measured at 72 h. A resistance factor (RF) was defined as the IC_{50} of resistant cells divided by the IC_{50} of the sensitive cells.

2.2 Drugs and chemicals

Rhodamine B and Tetrazolium salt (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)) were from Amresco. Cyclosporin A (CsA) was from Sandoz. Artemisinin, ATP (K^+ salt), and luciferin–luciferase were from Sigma. Artesunate was kindly provided by Professor Ruchanee Udomsangpetch, Faculty of Science, Mahidol University, Thailand. Dihydroartemisinin was synthesized and provided by Professor Yodhathai Thebtaranonth, Department of Chemistry, Faculty of Science, Mahidol University, Thailand. Purified doxo-rubicin and pirarubicin (4-Q-tetra-hydropyrayl-doxorubicin) were kindly provided by laboratoire Roger Bellon (France). Deionized double distilled water was used throughout the experiments for solutions and buffers.

Experiments were performed at 37 °C using a HEPES–Na^+ medium consisting of 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose, 20 mM HEPES, pH 7.25.

A stock solution of 0.012 M MTT was prepared in HEPES–Na^+ buffered solution, filtered through a 0.22 μm filter, and stored at 4 °C. Solutions of qinghaosu and pirarubicin and doxorubicin were freshly prepared before being used. The concentration of anthracyclines was spectrophotometric-ally determined using $\varepsilon_{\lambda=480\ nm}$ equal to 11,500 $M^{-1}.cm^{-1}$ (Shimadzu UV2501–PC).

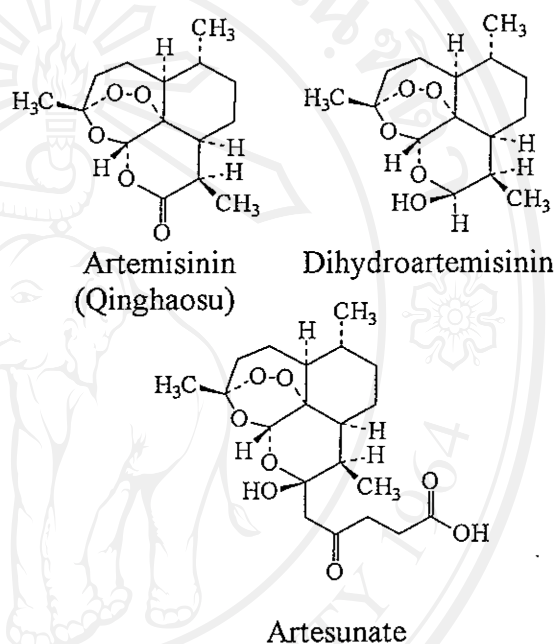

Fig 1. Chemical Structure of Qinghaosu

2.3 Cellular drug accumulation

The rationale and validation of our experimental set–up for measuring the kinetics of the active transport of anthracyclines from tumor cells has been extensively described (Frezard & Garnier-Suillerot, 1991; Mankhetkorn *et al.*, 1999; Frezard & Garnier–Suillerot, 1991).

Briefly, 2×10^6 cells were incubated in 2 ml of HEPES–Na^+ in 1 cm quartz cuvettes, vigorously stirred at 37 °C, then 1 μM anthracycline was added to the cells. The fluorescence intensity of anthracycline at 590 nm

Copyright© by Chiang Mai University
All rights reserved

(excited at 480 nm) was followed as a function of time (Perkin–Elmer model LS 50B spectrofluorometer). A decrease in fluorescence intensity was observed during incubation with cells, due to quenching of fluorescence after the intercalation of anthracycline between the base pairs of DNA.

The drug influx was measured by the decrease in fluorescence intensity that occurred during incubation with the cells. The initial rate of uptake (V_+) (passive influx) was determined from the equation

$$V_+ = |(dF / dt)_{t=0}| \cdot (C_T / F_0)$$

where $|(dF / dt)_{t=0}|$ and F_0 are the slope (in absolute value) of the tangent of the curve $F = f(t)$ and the fluorescence intensity at t = 0, respectively, and C_T is 1 μM. The initial rate rate of uptake can be written as

$$V_+ = k_+ \cdot n\,(C_e)_{t=0}$$

where k_+ is the mean influx coefficient for the drug, and $(C_e)_{t=0}$, the extracellular free drug concentration at t = 0, is equal to C_T. After a stable fluorescent signal was obtained, the fluoresence intensity was termed F_n. At the steady state, the kinetics of P–glycoprotein– and MRP1–mediated efflux of anthracycline derivatives is

$$V_a = V_+ + V_-$$

where V_+ and V_- are the kinetics of passive influx and efflux of a drug, respectively. In this expression, $V_+ = k_+.n.C_e$ and $V_- = k_- \cdot n.C_i$ where C_e and C_i are the extracellular and intracellular free drug concentration at the steady state, respectively; n is the number of cell concentration; and k_+ and k_- are the passive coefficients of influx and efflux, respectively. Assuming that k_+ is equal to k_-, it follows that

$$V_a = k_+ \cdot n \cdot (C_e - C_i)$$

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

$$V_a = k_a \cdot n \cdot C_i$$

where k_a is the active (P–gp– or MRP1–mediated) efflux coefficient for the drug. k_a can be written as

$$k_a = k_+ \cdot n \cdot (C_e - C_i) / C_i$$

where $C_e = C_T - C_n$ and $C_i = C_E / (C_N \cdot C_n)$.

At the steady state, the addition of MDR modulators verapamil or qinghaosu yielded a decrease in fluorescence intensity to reach a new steady state F^i_n. The overall intracellular anthracycline concentration was expressed in the absence of modulator, $C_n = C_T(F_0 - F_n) / F_0$ and in the presence of modulators, $C^i_n = C_T(F_0 - F^i_n) / F_0$. At the end of the experiments, cell viability was assessed using trypan blue exclusion.

In the following, (k_a^i) and (k_a^0) stand for the P–gp– and MRP1–mediated active efflux of drug in the presence and absence of qinghaosu, respectively. The ability of qinghaosu to inhibit the P–gp–and MRP1–mediated efflux of a drug can be determined using the ratio

$$k_a^i / k_a^0$$

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

2.4 Determination of mitochondrial membrane potential ($\Delta\Psi_m$)

The mitochondrial membrane potential ($\Delta\Psi_m$) was measured using a non–invasive functional study, which can be used to determine and to monitor

Copyright © by Chiang Mai University
All rights reserved

a spontaneous change in mitochondrial function in drug-sensitive and drug–resistant cells (Reungpatthanaphong & Mankhetkorn, 2002). The method is simple and direct and can be easily employed using a standard spectrofluorometer. Rhodamine B was used as a probe to estimate $\Delta\Psi_m$ in drug–sensitive and, particularly, in drug-resistant cells because its rate of uptake by cell is four times higher than the rate of P–glycoprotein–mediated efflux and it is not a substrate of MRP1 protein (Reungpatthanaphong & Mankhetkorn 2002). The accumulation of rhodamine B in cells follows Nernstain distribution, but the plasma membrane potential does not contribute to rhodamine B uptake by cells. The estimation of $\Delta\Psi_m$ was done by using Nernst equation:

$$C^{o}_{m} / C^{o}_{i} = 10^{(\Delta\Psi m\, F/2.303RT)}$$

Where C^{o}_{m} is the mitochondrial matrix rhodamine B concentration, C^{o}_{i} is the cytosolic rhodamine B concentration at steady state, and F, R and T have their usual meanings.

The mitochondrial matrix rhodamine B concentration can be determined as follows. Cells (2×10^{6}) were incubated 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 (F) at 582 nm (excited at 553 nm) was monitored as a function of time. After 20 minutes of incubation, 200 μM MTT was added to the solution, yielding a progressive decrease in rhodamine B fluorescence.

To simplify the system, cells were divided into three compartments: extracellular, cytoplasmic and mitochondrial, respectively. At steady state, rhodamine B was equilibrated between the extracellular (C_e^{o}), cytoplasmic (C_i^{o}) and mitochondrial compartments (C_m^{o}). 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 matrix can be written as:

$$V_{\text{rhoB}} = PC_i^{o}$$

where P is a permeability coefficient which depends on rhodamine B and on the membrane of the 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 fluorescence intensity F_e is equal to F_i, and the variation of the fluorescence per mole is:

$$\delta F = F_e - F_m$$

where F_e, F_i and F_m are the rhodamine B fluorescence intensity in the extracellular, cytoplasmic 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 ΔF of the fluorescent signal:

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

Copyright by Chiang Mai University
All rights reserved

which can be written as:

$$n = PC_i^{\circ}\Delta t$$
$$\Delta F = n\delta F$$
$$\Delta F = PC_i^{\circ}\Delta t\delta F$$
$$\Delta F/\Delta t = PC_i^{\circ}(F_e - F_{mtt})$$

P does not depend on the $\Delta\Psi_m$, and the sign of $\Delta F/\Delta t$ does not depend on C_i^o. The accumulation of rhodamine B in the mitochondrial matrix is augmented in a $\Delta\Psi_m$–driven maner 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 $F = f(t)$, as demonstrated in Figure 2, corresponding to V_i, the initial rate of the decrease of rhodamine B fluorescence intensity:

$$V_i = PC_i^{\circ}(F_e - F_{mtt})$$

In 50 seconds, the change in extracellular rhodamine B concentration is negligible compared with that before the addition of MTT, but 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 $\Delta\Psi_m$:

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

where V_i is in $nM.s^{-1}$.

To determine the $\Delta\Psi_m$ change, which is due to the effect of qinghaosu, after 20 minutes of cell incubation with 40 nM rhodamine B the sequential addition of various concentrations of qinghaosu and 200 µM MTT was performed. 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.

2.5 Measurement of ATP in cell extracts

Quantitative determination of ATP was performed by a chemiluminescent technique. (Kimmich *et al.*, 1975). Briefly, luciferase produces light by ATP–dependent oxidation of luciferin enhances the emission maximum at 560 nm at pH 7.8. Cells (5×10^5) were incubated in the presence or absence of qinghaosu or oligomycin at 37 °C for 10 minutes to 1 hour. The cells were pelleted and then permeabilized by 500 µl lysis solution containing 0.1% Triton X–100, 20 mM Tris, 0.1 mM EDTA, and 5 mM $MgSO_4$. The intensity of light was recorded by Perkin–Elmer LS 50B spectrofluorometer after the addition of 50 µl luciferin–luciferase (20 mg/ml).

3. RESULTS AND DISCUSSION

3.1 Co-treatment using pirarubicin and qinghaosu or doxorubicin and qinghaosu

Artemisinin, artesunate or dihydroartemisinin (see Figure 1 for their chemical structures) alone exhibited anticancer properties in a micromolar concentration range, with the same efficacy in MDR cells and their corresponding sensitive cell lines (Table 1). Artesunate and dihydroartemisinin showed 15–fold greater efficacy than artemisinin in K562 cells and 60–fold greater efficacy in GLC4

Copyright by Chiang Mai University
All rights reserved

cells. Anthracyclines are well known as MDR agents. The efficacy of anthracycline drugs depends on the concentration of molecules localized in their intracellular targets (Mankhetkorn, *et al.*, 1996). The concentrations of pirarubicin and doxorubicin that inhibit 50 % of cell growth are indicated in Table 1.

Table 1. Pattern of Cytotoxicity of Artemisinin, Artesunate and Dihydroartemisinin

Compounds	IC_{50} (K562), μM	R.F.	IC_{50} (GLC4), μM	R.F.
Artemisinin	15 ± 5	1.9	34 ± 6	0.9
Artesunate	1 ± 0.3	1	0.6 ± 0.3	1.5
Dihydroartemisinin	1 ± 0.2	1.4	0.5 ± 0.2	1
Pirarubicin	0.017 ± 0.006	23.5	0.022 ± 0.007	3.3
Doxorubicin	0.020 ± 0.006	118	0.023 ± 0.004	39.6

* IC_{50} (K562) and IC_{50}(GLC4) are the drug concentrations required to inhibit 50% of sensitive cell growth. The resistance factor value was calculated as resistant cell IC_{50}/sensitive cell IC_{50}. The value represents the mean ± standard deviation of triplicate experiments.

Table 2. MDR reversing pattern of artemisinin, artesunate and dihydroartemisinin.

Compounds	$\delta_{0.5}$, μM (pirarubicin)		$\delta_{0.5}$, μM (doxorubicin)	
	K562/*adr*	GLC4/*adr*	K562/*adr*	GLC4/*adr*
Artemisinin	0.86	0.18	0.8	0.87
Artesunate	0.083	0.065	0.19	0.19
Dihydroartemisinin	0.16	0.056	0.17	0.17

* $\delta_{0.5}$ is the artemisinin, artesunate or dihydroartemisinin concentration required to decrease resistant cell IC_{50} by 50 % when the MDR cells were treated with pirarubicin or doxorubicin.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

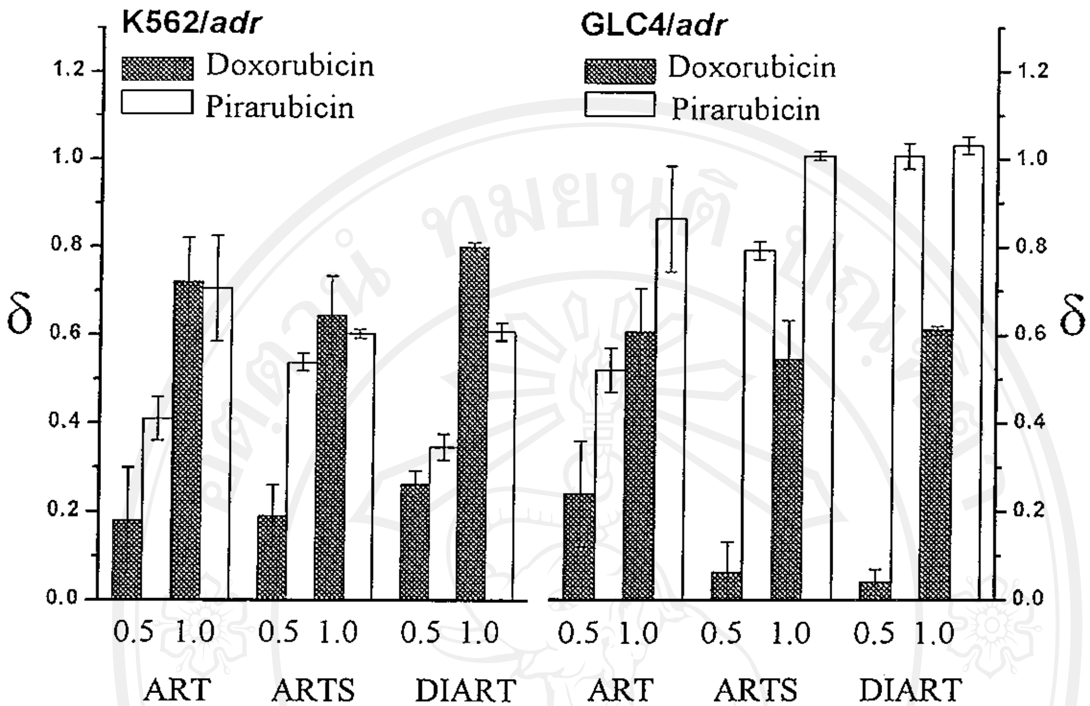

Figure 2. The efficacy (δ) of molecules to enhance the cytotoxicity of pirarubicin and doxorubicin for K562/*adr* and GLC4/*adr*. δ was calculated as described in the text. Values are presented as mean ± SD (n=3).

Co–treatment using pirarubicin or doxorubicin with various concentrations of artemisinin, artesunate or dihydroartemisinin did not affect the IC_{50} for both of the drug–sensitive cell lines. But it obviously increased the efficacy of both pirarubicin and doxorubicin when a similar series of experiments was performed with their corresponding MDR cell lines. These results suggest that each of the three molecules can efficiently reverse the MDR phenomena. The efficacy of qinghaosu (δ) in increasing the efficacy of pirarubicin or doxorubicin on MDR cells was calculated by the following expression:

$$\delta = \frac{[IC_{50}(R) - IC_{50}(RQ)]}{[IC_{50}(R) - IC_{50}(S)]}$$

where $IC_{50}(R)$ is the concentration of drug that inhibits 50% of MDR cell growth, $IC_{50}(RQ)$ is the concentration of drug that inhibits 50% of MDR cell growth in the presence of qinghaosu, and $IC_{50}(S)$ is the concentration of drug that inhibits 50% of drug–sensitive cell growth.

The efficacy of artemisinin, artesunate, and dihydroartemisinin in reversing MDR phenomena and/or resensitizing the MDR cells to pirarubicin and doxorubicin was indicated in Figure 2. The δ varied from 0 to 1, where δ = 0 when the MDR cells are treated with pirarubicin alone or doxorubicin alone and δ = 1 when artemisinin, artesunate or dihydroartemisinin can reverse 100 % of MDR phenomena, resulting in the same $IC_{50}(RQ)$ as that of drug–sensitive cell lines. The qinghaosu concentration

Copyright © by Chiang Mai University
All rights reserved

which increases the efficacy of pirarubicin or doxorubicin by 50% is defined as $\delta_{0.5}$. Artesunate and dihydroartemisinin were more effective than artemisinin as MDR reversing agents (Table 2). We would like to stress that artemisinin, artesunate and dihydroartemisinin are more effective in increasing the cytotoxicity of pirarubicin than doxorubicin in GLC4/*adr*. In the case of pirarubicin, only 200 nM artesunate and dihydroartemisinin can cause a reversal of 100% in GLC4/*adr* and 80% in K562/*adr* cell.

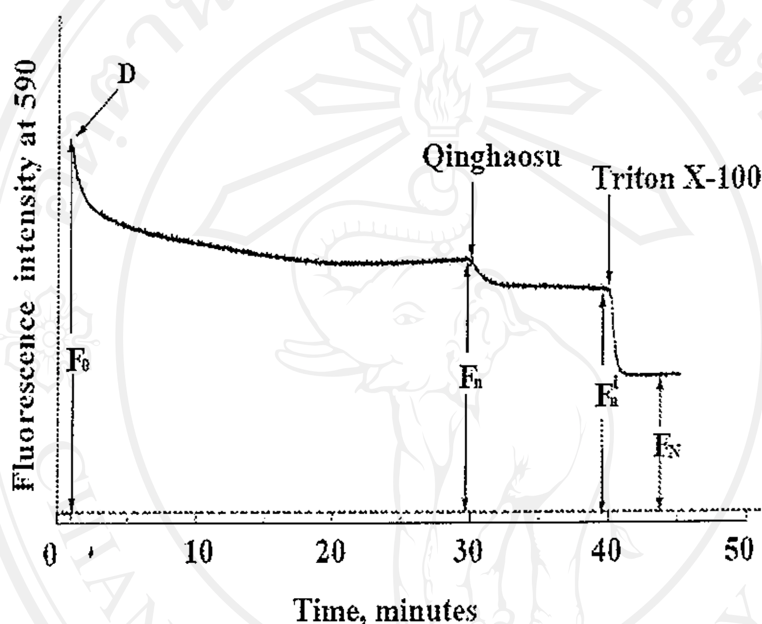

Figure 3. Effect of qinghaosu on pirarubicin (THP) accumulation in K562/*adr* cells. Cells (2×10^6 cells) were suspended in HEPES–Na^+ buffer at pH 7.25, 37 ^oC in a cuvette under vigorous stirring. At t = 0, 10^{-4} M stock THP solution was added to the cells, yielding a C_T = 1 μM THP solution. The fluorescence intensity was then F_0. Once the steady state was reached, the fluorescence intensity was F_n and the concentration of drug intercalated between the base pairs in the nucleus was $C_n = C_T (F_0 - F_n)/ F_0$. When the steady state was reached, qinghaosu was added, leading to a new steady state in which the fluorescence intensity was $F_n^{\,i}$, and the concentration of drug intercalated the base pairs in the nucleus was $C_n^{\,i} = C_T(F_0 - F_n^{\,i})/ F_0$. The addition of 0.02% Triton X–100 yielded an equilibrium state. The overall concentration C_N of drug intercalated between the base pairs in the nucleus was then $C_N = C_T(F_0 - F_N)/F_0$.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

3.2 Influence of artemisinin, artesunate, and dihydroartemisinin on cellular drug accumulation

In order to gain further insight into the mode of action of these molecules and how they increase the efficacy of pirarubicin and doxorubicin in MDR cell lines, we investigated the aptitude of these molecules to inhibit the kinetics of P-glycoprotein and MRP1-protein-mediated efflux. The uptake of pirarubicin or doxorubicin was performed in both MDR cell lines. A typical experiment is shown in Figure 3. The ability of qinghaosu to inhibit the P–gp–and MRP1–mediated efflux of a drug, with the ratio k_a^i / k_a^0 as a function of qinghaosu concentration added, was demonstrated in Figure 4. Artemisinin, artesunate and dihydroartemisinin poorly inhibited the P–glycoprotein–mediated pirarubicin efflux compared to cyclosporin A and verapamil. It should be noted that the percentage of cell death was significantly increased when the concentrations of drugs used were higher than 150 µM. The efficacy of drugs to inhibit the P–glycoprotein–mediated pirarubicin efflux was determined. The concentrations of drug required to inhibit by 50% the functionality of P- glycoprotein were 115 µM, 110 µM, 120 µM, 1 µM and 2 µM for artemisinin, artesunate, dihydroartemisinin, cyclosporin A, and verapamil, respectively. None of them were able to inhibit the functionality of MRP1–protein.

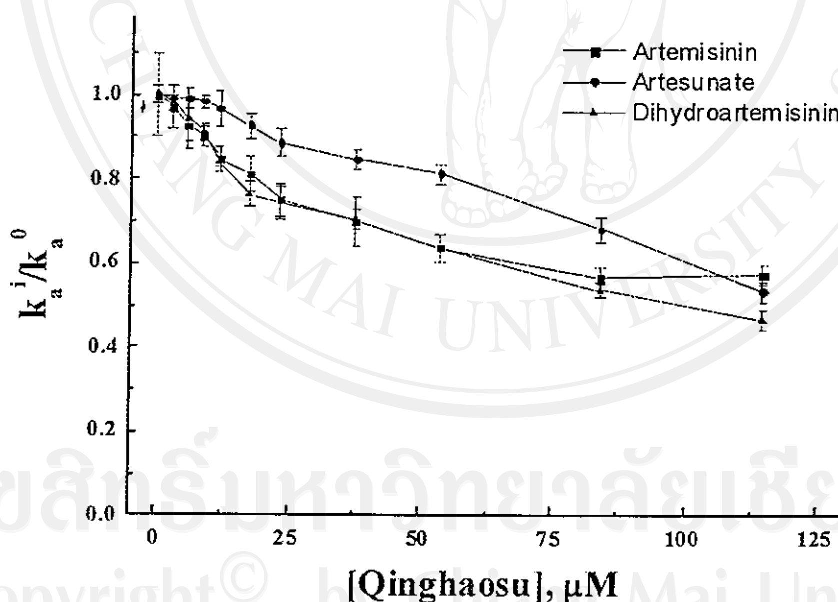

Figure 4. Efficacy of artemisinin (□), artesunate (o) and dihydroartemisinin (Δ) to inhibit the P–glycoprotein–mediated THP efflux. The ratio k_a^i/k_a^o is plotted as a function of the qinghaosu concentration added. Values were presented as mean ± SD (n=3).

Copyright© by Chiang Mai University
All rights reserved

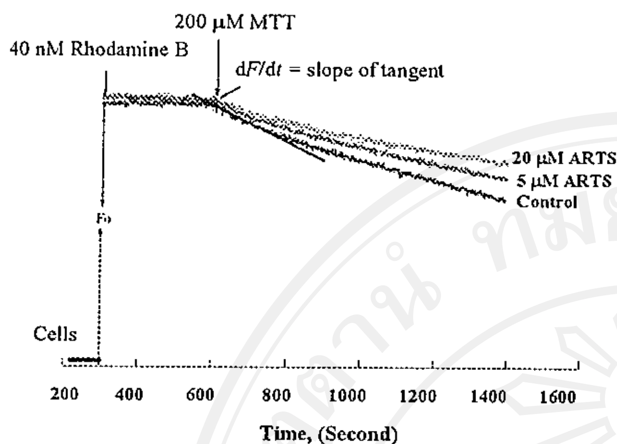

Figure 5. (a) Typical kinetics of the uptake of rhodamine B by cells. Fluorescence intensity, F, at 582 nm (λ_{ex} = 553 nm) was recorded as a function of time. 2×10^6 cells were suspended in 2 ml of HEPES–Na^+ buffer at 37 °C and vigorously stirred. A small volume of rhodamine B stock was added to the solution, yielding a final concentration of 40 nM rhodamine B, and the corresponding fluorescence intensity F_0 was recorded for 20 minutes, then various concentrations of qinghaosu and 200 μM MTT were sequentially 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: $(V_i)_{art} = (dF/dt)(C_T/F_0)$. $\Delta\Psi_m$ was determined using the expression: $\Delta\Psi_m = -61.51 \log V_i - 258.46$.

3.3Effect of artemisinin, artesunate, and dihydroartemisinin on mitochondrial function

It should be noted that the three molecules affected the cytotoxicity of pirarubicin and doxorubicin, even at very low concentrations, compared to that required to inhibit by 50% the functionality of P–glycoprotein or by 50% cell growth. This suggests another mechanism to explain their ability in reversing the MDR phenotype. The mitochondrial function can be studied by following the change in $\Delta\Psi_m$. It is also well established that a slight decrease in the $\Delta\Psi_m$, even in a very narrow window, can affect the cellular energetic state, leading to cell death. As mentioned in Materials and Methods, the $\Delta\Psi_m$ of drug-sensitive and drug–resistant cells can be determined using rhodamine B. A typical experiment is shown in figure 5. Indeed, the three molecules were able to decrease the $\Delta\Psi_m$ in all cell lines used in our experiments. The aptitude of molecules to induce a decrease in the $\Delta\Psi_m$ (Figure 6), as well as a decrease in intracellular ATP concentration, was quantitatively measured (Figure 7).

The three molecules were found to significantly decrease both $\Delta\Psi_m$ and intracellular ATP concentration in the four cell lines in a dose–dependent manner. However, artemisinin, artesunate and dihydroartemisinin did not affect the cytotoxicity of pirarubicin or doxorubicin in sensitive cell lines.

These results suggest that in drug–sensitive cell lines, in comparison with drug-resistant cell lines which need more ATP concentration to assure their ATPase activity, the decrease in intracellular ATP does not produce a deleterious effect, as it does in drug–resistant cell lines. It was clearly shown by electron microscope that in NB2a neuroblastoma cells, (Fishwick *et al.*, 1998) as well as in plasmodium infected erythrocytes, (Maeno *et al.*, 1993) dihydroartemisinin (2 μM) caused damage to mitochondrial cristae and the endoplasmic reticulum, but affected neither the cellular cytoskeleton nor the plasma membrane

The same authors proposed that artemisinin and its derivatives react

Copyright© by Chiang Mai University
All rights reserved

with mitochondria and the endoplasmic reticulum. In addition, these drugs are now in widespread use, particularly in Southast Asia, and are used as a prophylaxis in China. And the results of various clinical trials suggest that artemisinin derivatives are relatively safe drugs (Sidu *et al.*, 1998; Ashton *et al.*, 1998). Our results clearly show for the first time that artemisinin and its derivatives are very potent compounds that can be used in combination with anticancer drugs to overcome MDR phenomena.

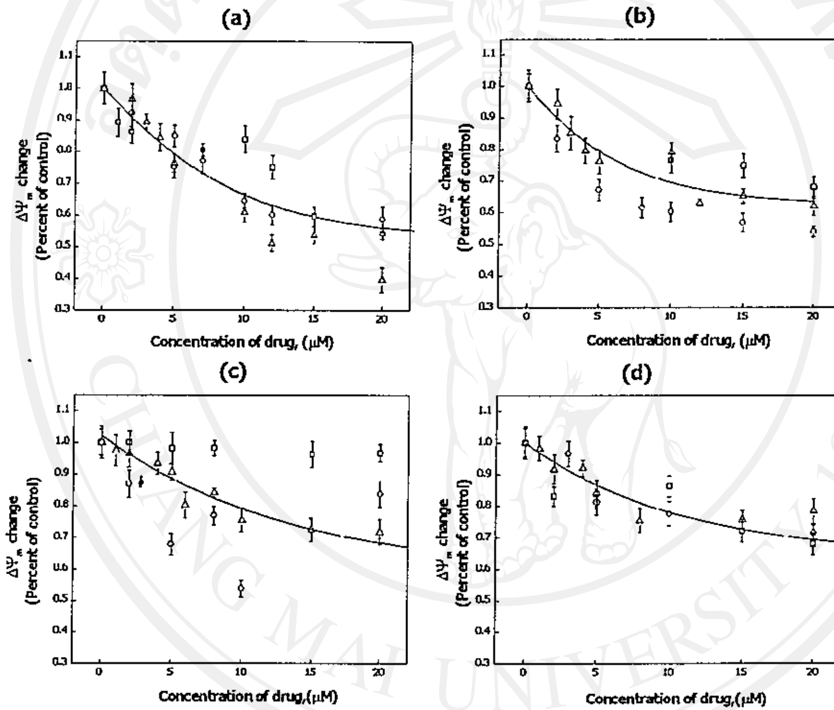

Figure 6. Variation of $\Delta\Psi_m$ (% of control) as a function of artemisinin (□), artesunate (o) and dihydroartemisinin (Δ) concentration in (a) K562, (b) K562/*adr*, (c) GLC4 and (d) GLC4/*adr* cells. The results were obtained from the series of experiments described in figure 6, and the $\Delta\Psi_m$ values are presented as mean ± SD (n= 3).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright by Chiang Mai University
All rights reserved

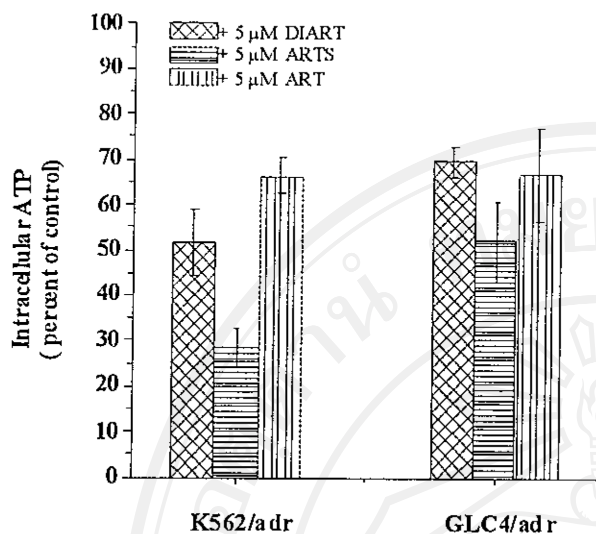

Figure 7: Intracellular ATP levels and the effect of 5 µM artemisinin, artesunate or dihydroartemisinin on intracellular ATP levels of K562/*adr* and GLC4/*adr* cells (see materials and methods). Values are presented as mean ± SD (n=3).

ACKNOWLEDGEMENTS:

This study was partly supported by the Royal Thai Government and the Royal Golden Jubilee program (RGJ). P.R. thanks the Royal Golden Jubilee Ph.D. program for financial support.

4. REFERENCES

Ashton, M., Hai, T.N., Sy, N.D., Huong, D.X., Houng, N.V., Nieu, N. T. & Cong, L.D. (1998). Artemisinin pharmacokinetics is time–dependent during repeated oral administration in healthy male adults. *Drug Metabolism and Disposition: the Biological Fate of Chemicals*, 26(1), 25–27.

Borst, P. & Ouellette, M. (1995). New mechanisms of drug resistance in parasitic protozoa. *Annual Review of Microbiology*, 49, 427–460.

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

Fishwick, J., Edvards, G., Ward, S.A. & Mclean, W.G. (1998). Morphological and immunocytochemical effects of dihydroartemisinin on differentiating NB2a neuroblastoma cells. *Neurooxicology*, 19, 393–404.

Foote, S.J., Thompson, J.K., Cowman, A.F. & Kemp, D.J. (1989). Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falciparum. Cell*, 57, 921–930.

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.

Frezard, F. & Garnier–Suillerot, A. (1991) Comparison of the membrane transport of anthracycline derivatives in drug–resistant and drug–sensitive K562 cells. *European Journal of Biochemistry*, 196, 483–491.

Hasson, A.M., Bjorkman, A, Lindgren, A. & Ashton, M. (1992). Post kalaazar dermal leishmaniasis in the Sudan: clinical features, pathology and treatment. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 86, 365–367.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Kain, K.C. (1995). Chemotherapy and prevention of drug–resistant malaria. *Wilderness & Environmental Medicine*, 6, 307–324.

Karcz, S. & Cowman, A.F. (1991). Similarities and differences between the multidrug resistance phenotype of mammalian tumor cells and chloroquine resistance in *Plasmodium falciparum*. *Experimental Parasitology*, 73, 233–240.

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

Labbe, A.C., Loutfy, M.R. & Kain, K.C. (2001). Recent Advances in the Prophylaxis and Treatment of Malaria. *Current Infectious Disease Reports*, 3, 68–76.

Levy, S.B. (1992). Active efflux mechanisms for antimicrobial resistance. *Antimicrobial Agents and Chemotherapy*, 36, 695–703.

Lehne G. (2000) P-glycoprotein as a drug target in the treatment of multidrug resistant cancer. *Current Drug Targets*, 1, 85–99.

Lozio, C.B. & Lozio, B.B. (1975). Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome. *Blood*, 45, 321–334.

Maeno, Y., Toyoshima, T., Fujioka, H., Ito, Y., Meshnick, S., Benakis, A., Milhous, W.K. & Aikawa, M. (1993). Morphologic effects of artemisinin in *Plasmodium falciparum. The American Journal of Tropical Medicine and Hygiene*, 49,485–491.

Mankhetkorn, S., Dubru, F., Hesschenbrouck, J., Fiallo, M. & Garnier–Suilletrot, 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 in multidrug–resistant K562 cells. *Molecular Pharmacology*, 49,532–539.

Mankhetkorn, S., Teodori, E. & Garnier–Suillerot, A. (1999). Partial inhibition of the P–glycoprotein–mediated transport of anthracy-clines in viable resistant K562 cells after irradiation in the presence of a verapamil analogue. *Chemico-Biological Interactions*, 121, 125–140.

Muller, M., Meijer, C., Zaman, G.J.R., Borst, P., Scheper, R.J., Mulder, N.H., de Vries, E.G.E. & Jansen, P.L.M. (1994). Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP–dependent glutathione S–conjugate transport. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 13033–13037.

Olliaro, P., Taylor, W.R. & Rigal, J. (2001). Controlling malaria: challenges and solutions. *Tropical Medicine & International Health*, 6, 922–927.

Reed, M.B., Saliba, K.J., Caruana, S.R., Kirk, K. & Cowman, A. F. (2000). Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature* (London), 403, 906–909.

Reungpatthanaphong, P., Dechsupa, S., Meesungnoen, J., Loetchutinat, C. & Mankhetkorn, S. (2003). Rhodamine B as a mitochondrial probe for measurement and monitoring of mitochondrial membrane potential in drug–

Copyright © by Chiang Mai University
All rights reserved

sensitive and –resistant cells. *Journal of Biochemical and Biophysical Methods*, 57(1), 1–16.

Saves, I. & Masson, J.M. (1998). Mechanisms of resistance to xeno-biotics in human therapy. *Cellular and Molecular Life Sciences*, 54, 405–426.

Sidu, J.S., Ashton, M., Houng, N.V., Karlsson, M.O., Sy, N.D. & Cong, L.D. (1998). Artemisinin population pharmacokinetics in children and adults with uncomplicated falciparum malaria. *British Journal of Clinical Pharmacology*, 45, 347–354.

Slater, L.M., Sweet, P., Stupecky, M. & Gupta, S. (1986). Verapamil potentialtion of VP–16–213 in acute lymphatic leukemia and reversal of pleiotropic drug resistance. *The Journal of Clinical Investigation*, 77, 1405–1408.

Tasaka, S., Ohmori, H., Gomi, N., Iino, M., Machido, T., Kiue, A., Naito, S. & Kuwano, M. (2001). Synthesis and structure–activity analysis of novel dihydropyridine derivatives to overcome multidrug resistance. *Bioorganic & Medicinal Chemistry Letters*, 11, 275–277.

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

Tsuro, T., Iida, H., Kawabata, H., Oh-Hara, T., Hamada, H. & Utakoji, T. (1986). Characteristics of resistance to adriamycin in human myelogenous leukemia K562 resistant to adriamycin and in isolated clones. *Japanese Journal of Cancer Research*, 7, 682–692.

Tsuro, T., Iida, H., Tsukagoshi, S. & Sakurai, Y. (1981). Overcoming of vincristine resistance in P388 leuke-mia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Research*, 41, 1967–1972.

van Veen, H.W. & Konings, W.N. (1997). Multidrug transporters from bacteria to man: similarities in structure and function. *Seminars in Cancer Biology*, 8, 183–191.

Versantvoort, C.H.M., Withoff, S., Broxterman, H.J., Kuiper, C.M., Scheper, R.J., Mulder, N.H. & de Vries, E.G.E. (1995). Resistance-associated factors in human small-cell lung-carcinoma GLC4 sublines with increasing adriamycin resistance. *International journal of cancer*, 61, 375–380.

Wadler S. & Wiernik P.H. (1988) Partial reversal of doxorubicin resistance by forskolin and 1,9-dideoxy forskolin in murine sarcoma S180 variants. *Cancer Research*, 48, 539–543.

Wongsrichanalai, C., Pickard, A.L., Wernsdorfer, W.H., Meshnick, S.R. (2002). Epidemiology of drug-resistant malaria. *The Lancet Infectious Diseases*, 2, 209–218.

Zijlstra, J.G., de Vries E.G.E. & Mulder N.H. (1987). Multifactorial drug resistance in an adriamycin-resistant human small cell lung carcinoma cell line. *Cancer Research*, 47, 1780–1784.

Copyright© by Chiang Mai University
All rights reserved