CHAPTER III

Spontaneous mitochondrial membrane potential change during apoptotic induction by quercetin in K562 and K562/*adr* cells

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Spontaneous mitochondrial membrane potential change during apoptotic induction by quercetin in K562 and K562/*adr* cells

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Abstract

Natural products from plants such as flavonoids are the potential drugs to overcome multidrug resistance (MDR) in cancer treatments. However, its modes of action are still unclear. In this study, the effects of quercetin on mitochondrial membrane potential ($\Delta\Psi_m$) change as well as quercetin's ability to induce apoptosis and inhibit Pgp-mediated efflux of ^{99m}Tc-MIBI in K562/*adr* cells were investigated. Quercetin exhibits cytotoxicity against erythroleukemic cells: IC_{50} are 11.0 ± 2.0 μM and 5.0 ± 0.4 μM for K562 and K562/*adr*, respectively. Quercetin induces cell death via apoptosis in both K562 and K562/*adr* cells and does not inhibit Pgp-mediated efflux of ^{99m}Tc-MIBI. Quercetin (10 μM, 3 h) and etoposide (100 μM, 24 h) induces similar levels of apoptosis in K562 and K562/*adr* cells. Quercetin induces an increase followed by a decrease in $|\Delta\Psi_m|$ value depending on its concentration. A decrease in the $|\Delta\Psi_m|$ value is associated with an increase in the percentage of early apoptotic cells. It is clearly shown that quercetin results in a spontaneous $\Delta\Psi_m$ change during apoptotic induction. Therefore, quercetin is potentially an apoptotic inducing agent by which reacts at the mitochondrial level.

Key words: Multidrug resistance (MDR); Quercetin; Apoptosis; ^{99m}Tc-Annexin V; Mitochondrial membrane potential ($\Delta\Psi_m$); ^{99m}Tc-MIBI

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INTRODUCTION

Over the past decades, researchers searching for new drugs to use in oncology have refocused on natural products. The flavonoid consists of various groups of natural, low molecular weight polyphenolic compounds, which can be found in all vascular plants (Middleton, 1996). Flavonoids exert a wide range of biological activities including antioxidant, anticarcinogenic, anti-proliferative, and antiviral actions (Aherne and O'Brien, 1999; Formica and Regelson, 1995; Csokay *et al.*, 1997). Moreover, flavonoids such as genistein, apigenin, and quercetin, are

also known as apoptotic inducers (Wang *et al.*, 1999; Yin *et al.*, 1999, Choi *et al.*, 2001) and MDR modulators (Conseil *et al.*, 1998; De Wet *et al.*, 2001). Multidrug resistance (MDR) of cancer cells is often associated with overexpression of the P-glycoprotein (Pgp) or the Multidrug Resistance associated Protein (MRP1). The plasma membrane transporters, Pgp and MRP1, extrude chemotherapeutic drugs by using ATP hydrolysis as an energy source (Tanigawara 2000; Borst *et al.*, 2000). Experimental approaches aiming to determine the direct interaction between flavonoids and Pgp were studied by many research groups. On one hand, Conseil *et al.* (1998) studied the fixation of flavonoids at vicinal ATP-binding site. They measured the resonance-energy transfer of tryptophan-intrinsic fluorescence of H6-NBD2, a highly soluble recombinant protein, from mouse Pgp and flavonoids (Conseil *et al.*, 1998). Similar results were obtained from the series of experiments dealing with the inhibition the photolabelling of ATP analogues on the ATP-binding site within the C-terminal nucleotide-binding domain of mouse Pgp using 30 flavonoids (De Wet *et al.*, 2001). De Wet *et al.* (2001) showed the structure-activity relationships of 30 flavonoids on their ability to bind to the vicinal ATP- and steroid-binding site. On the other hand, Phang *et al.* (1993) reported that flavonols (quercetin, kaempferol and galangin) were potent stimulators of the Pgp-mediated efflux of 7, 12-dimethylbenz (a)-anthracence in multidrug-resistant breast cancer cells (Phang *et al.*, 1993). Consistently with previously cited data, Critchfield *et al.* (1994) found that galangin, kaempferol and quercetin reduced [^{14}C] ADR accumulation and that phenomenon was blocked by verapamil, vinblastine and quinidine in HCT-15 colon cells (Critchfield *et al.*, 1994).

In this study, we report that quercetin is a cytotoxic agent, with a 2-fold potency greater efficacy in K562/*adr* than K562 cells. It does not inhibit Pgp-mediated efflux of ^{99m}Tc-MIBI, a myocardial perfusion imaging agent and a known substrate of Pgp and MRP1 (Hendrikse *et al.*, 1998; Del Vecchio *et al.*, 2000). Quercetin induces cellular apoptosis by changing the mitochondrial membrane potential ($\Delta\Psi_m$) in K562 and K562/*adr* was specially investigated.

MATERIALS AND METHODS

Drug preparation

Quercetin (Extrasynthèse) was prepared in ethanol at 10^{-3} M, and aliquots were frozen. We used a new aliquot for each experiment. Acridone carboxamide derivative (GG918), a Pgp inhibitor (GlaxoWellcome Inc., Research Triangle Park, N.C.), was dissolved in ethanol and kept in the dark at 4°C (Wallstab *et al.*, 1999; Muzzammil *et al.*, 2000).

All series of experiments were performed using Hepes/Na^+ isotonic buffer solution, pH 7.25 at 37° C, containing 20 mM Hepes plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5 mM glucose.

Radiolabeled compounds

The radiolabeled compounds ^{99m}Tc-MIBI and ^{99m}Tc-N_2S_2-AnnexinV (^{99m}Tc-AnnexinV) were synthesised using the one-step kit from DuPont Pharma (Cardiolite®) and from Malinckrotd, respectively. A 0.5 mL-aliquot of Cardiolite® was prepared according to the manufacturer's

instruction. The radiochemical purity was checked by thin-layer chromatography using ethanol (absolute) as the mobile phase and was always greater than 96%.

The AnnexinV kit was dissolved in 1 mL of NaCl (0.9%) containing $^{99m}TcO_4Na$ and incubated for 20 min at room temperature. The radiochemical purity was determined on a Sephadex G25m (PD10/Pharmacia) column, using NaCl 0.9% as the mobile phase. The radiochemical purity was around 92%. Generator equilibrium equations were used to calculate the absolute concentration of total $^{(99m+99)}$Tc-tracers (radioactive and cold) in the solution. Molarity was expressed in terms of total $^{(99m+99)}$Tc-tracers.

Cell culture and cytotoxicity assay

The erythromyelogenous leukemic K562 and its Pgp-overexpression K562/*adr* cells were cultured in RPMI 1640 medium containing L-glutamine and supplemented with 10% fetal calf serum and 1% penicillin /streptomycin (BioMedia) at 37 °C in 5% CO_2. The resistant K562/*adr* cells were cultured with 400 nM doxorubicin, 2 weeks before experiments. Cells were changed twice every week and maintained in exponential growth phase.

Cytotoxicity assay was performed as followed: cells were plated in a 6-well plate at initial density of 1×10^5 cells per ml, with 4.0 mL of medium per well and incubated in the presence of various quercetin concentrations. Number of cells was measured by coulter counter. Then the percentage of cell growth inhibition, %IC, was plotted against the flavonoid concentration. IC_{50} is the quercetin concentration that inhibits cell growth by 50% when measured at 72 h. The resistance factor (RF) was defined as the IC_{50} of resistant cells divided by the IC_{50} of sensitive cells.

Induction of apoptosis

Exponentially growing cells were seeded in flask-T25 at initial density at 2.5×10^5 per 10 mL of medium. After 24 h, 10μM quercetin was added and cells were further incubated at 37 °C for various times: 0, 1, 3, 6, 18, 24, 48 and 72 h. Cells were also incubated with increasing concentrations of quercetin ranging from 0 to10 μM and a fixed incubation time of 3h. The concentration of etoposide (Sigma) used to induce apoptosis was 100 μM demonstrated by Fukumi *et al.* (Fukumi *et al.*, 2000).

Cytofluorometric staining of the cells

For detection of apoptosis, the treated cells were centrifuged for 5 min, 1000×g, at room temperature (18-24 °C), resuspended, and washed once with 5 mL phosphate-buffered saline prior to staining with Annexin V (apoptosis detection kit (R&D Systems Inc., Minneapolis, Minn.)). Flow cytometry analysis was performed in a Coulter Epics XL-MCL (Coultronics France SA, Villepinte, Val d'Oise, France) and cells were evaluated on 5,000 events per sample. The data were represented in monoparametric histograms. Biparametric histograms were used to visualize cells distributed as a function of their signal intensity with respect to Annexin V-FITC and PI.

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

The mitochondrial membrane potential ($\Delta\Psi_m$) was measured using a non-invasive functional spectro-fluorometric method, which can be used to determine and to monitor a spontaneous change in mitochondrial

function in drug-sensitive and drug-resistant cells as prevoiusly described by Reungpatthanaphong *et al.* (Reungpatthanaphong *et al.*, 2003). Briefly, 2×10^6 cells were incubated in 2 mL of HEPES-Na^+ buffer with 40 nM rhodamine B (Sigma) in 1 cm quartz cuvettes and vigorously stirred at 37 °C. The rhodamine B fluorescence (F_0) read at 582 nm (excited at 553 nm) was monitored as a function of time. An accumulation of rhodamine B in cells follows the Nernstain distribution, but the plasma membrane potential does not contribute to rhodamine B uptake by cells. The estimation of $\Delta\Psi_m$ was done using Nernst equation (1):

$$C^o_m / C^o_i = 10^{(-\Delta\Psi m\, F/2.303RT)} \qquad (1)$$

where C_{om} is the mitochondrial matrix rhodamine B concentration, C_{oi} is the cytosolic rhodamine B concentration at steady state, and F, R and T have their usual meanings.

C_{om} was determined by adding 200μM MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide; Sigma Singapore Science Park II, Singapore) to the solution yielding a progressive decrease in rhodamine B fluorescence. 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 in rhodamine B fluorescence was equal to:

$$V_i = \frac{dF}{dt} \times \frac{C_T}{F_0} \qquad (2)$$

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 $\Delta\Psi_m$:

$$\Delta\Psi_m = -61.51 \log V_i - 258.46 \qquad (3)$$

Determination of cellular uptake of ^{99m}Tc-tracers

All experimental samples were run in triplicate and most of the experiments were repeated 2 or 3 times. Cells, sensitive or resistant, were counted and suspended in Hepes/Na^+ buffer solution (pH = 7.25 at 37°C). To minimize non-specific ^{99m}Tc-MIBI binding to plastic tubes, they were pre-saturated for 1h with a phosphate buffered saline (PBS) solution at pH 7.4, containing 1% fetal calf serum, washed twice with PBS buffer.

After 30 min incubation with ^{99m}Tc-AnnexinV (1.85 MBq/tube) or after 1h incubation with 1 nM ^{99m}Tc-MIBI (7.4 MBq/tube), tubes containing 10^6 cells in 1 mL were centrifuged at 2800×g for 3 min, and the pellets were carefully resuspended and quickly washed twice with 1.8 mL of ice-cold PBS. The radioactivity in the tubes containing the pellet was counted in an auto gamma-well counter (LKB Wallac, 1261 Multigamma). After incubation with ^{99m}Tc-MIBI, cell viability was assessed by Trypan blue dye exclusion and compared with that of the control cells in each experiment. Cell viability was similarly found in unexposure and after exposure to ^{99m}Tc-MIBI cells, and was always superior to 95%. The ^{99m}Tc-MIBI accumulation in dead cells was always negligible.

RESULTS

Cytotoxicity of quercetin against K562 and K562/adr cells

Quercetin (for chemical structure see Fig. 1) exhibits cytotoxicity against K562 and K562/*adr* cell with IC_{50} (means ± SD) values equal to 11.0 ± 2.0 μM and 5.0 ± 0.4 μM for K562 and K562/*adr* cells, respectively. It should be noted that quercetin exhibits more cytotoxicity in MDR cell than its corresponding drug-sensitive cell. The resistance factor value (RF) is equal to 0.45.

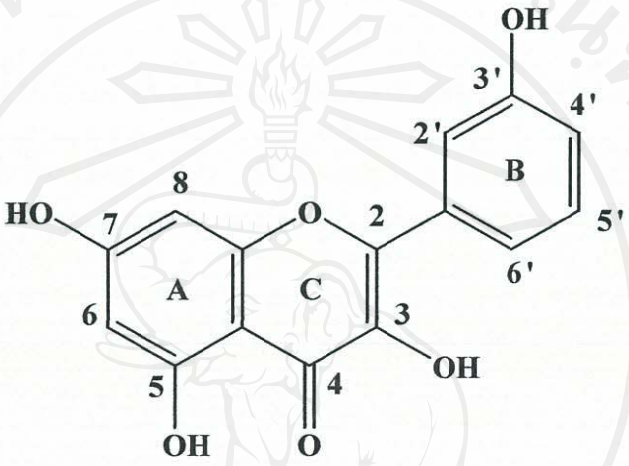

Figure 1. Chemical structure of quercetin

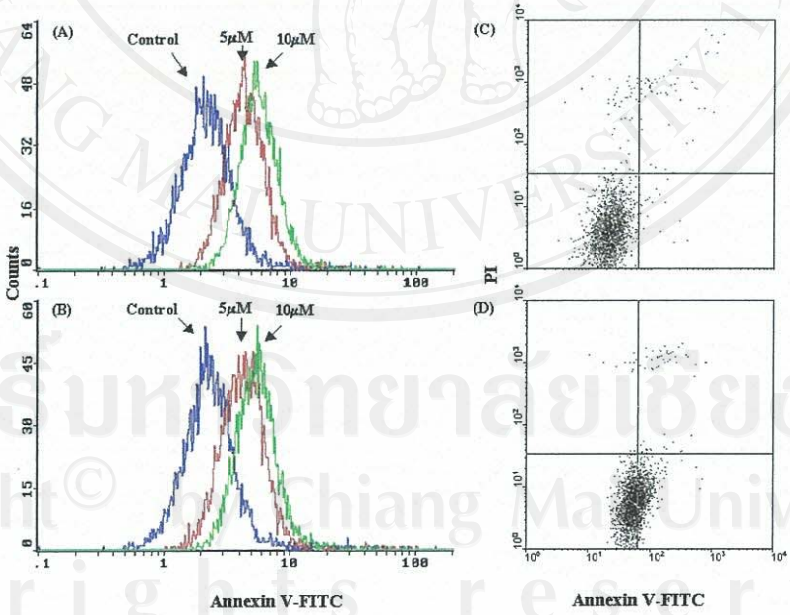

Figure 2. Monoparametric histogram of cell bound radiolabeled compound Annexin V-FITC: effects of 5 μM, 10 μM and without quercetin (control) in (A) K562 and (B) K562/*adr*. Representative biparametric histrogram of an Annexin V-FITC versus PI; (C) without and (D) 3 h incubation with 10 μM quercetin in K562 cell.

Quercetin induced apoptosis

Previous studies have shown that quercetin exert its cytotoxicity via induction of apoptosis in various cancer cell lines (Wang *et al.*, 1999; Yin *et al.*, 1999; Choi *et al.*, 2001). To get further insight the mode of action of quercetin, its ability induction of apoptotic cell death was monitored. Without treatment, basal apoptotic level of the 2 cell lines was 1.5 ± 0.1%. The typical histogram of the time course of Annexin V-FITC associated with the phosphatidylserine (PS) on apoptotic cells was shown in Fig. 2. An increase in number of apoptotic cells was observed in both K562 and K562/*adr* cells when incremental quercetin concentration was used (Fig. 2A and 2B). Early apoptotic cells were the number of cells found in quadrant 4, by which the cells were PI negative and Annexin V-FITC positive and late apoptotic cells were PI and Annexin V-FITC positive (Fig. 2C and 2D).

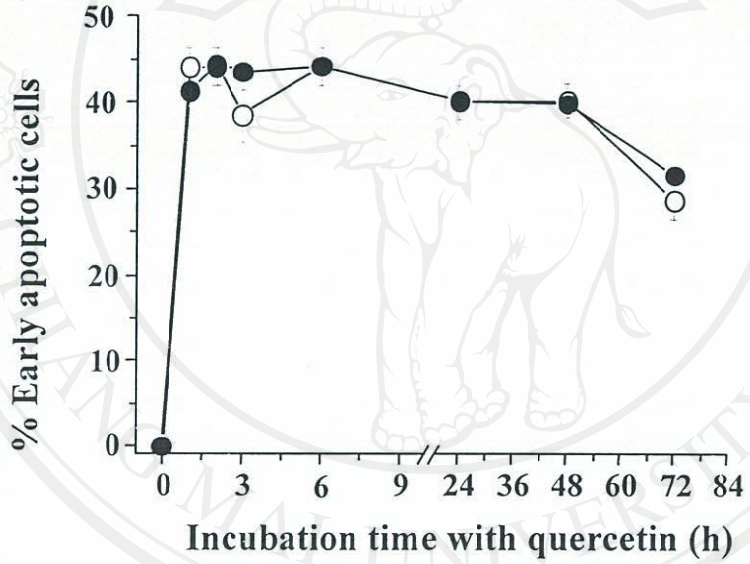

Figure 3. Variation of percentage of early apoptotic cells as a function of time in (○) K562 and (●) K562/*adr*. Cell bound Annexin V-FITC was detected after incubated with 10 μM of quercetin. Data are the mean ± SD of 3 independent experiments.

Quercetin induced early apoptotic cell death is demonstrated in Fig. 3. After using 10 μM quercetin, the percentage of early apoptotic cells increased in was the first hour (42 ± 2%) and this percentage appeared unmodified until 24 h. After 24 h, the percentage of late apoptotic and necrotic cells increased slightly while the percentage of early apoptotic cells decreased slowly to 32 ± 2% at 72h. A similar series of experiments were performed using various quercetin concentrations ranging from 0 to 10 μM. The percentage of early apoptotic cells increased with an increase in quercetin concentrations. At the maximal quercetin concentration used (10 μM), about 45% of early apoptotic cell was found in both K562 and K562/*adr*.

The ability of quercetin to induce apoptosis compared with etoposide was determined using ^{99m}Tc-AnnexinV as an apoptotic probe. Fig. 4 demonstrates that there is an increase in binding of ^{99m}Tc-Annexin V of both K562- and K562/*adr*- treated cells using quercetin and etoposide. After treatmenting K562- and K562/*adr* cells with 10 μM quercetin for 3 h and 100 μM etoposide for 24 h, a similar percentage of early apoptotic cells of both drugs of K562 and K562/*adr* were found, about 41 ± 7% for quercetin and about 54 ± 8% for etoposide. Under these experimental conditions, a 10-fold lower concentration of quercetin induced a similar level of apoptosis as etoposide in both cell lines. These results indicate that the existence of Pgp dose not contribute to the etoposide gradient of concentration, high on the extracellular and low on the intracellular side. Moreover, etoposide rapidly diffuses into cells but emanates out of cells to a lesser degree because it is a very poor substrate of Pgp (Guo *et al.*, 2002).

Modulation of mitochondrial membrane potential by quercetin

A typical result of the series of experiments with cellular ^{99m}Tc-MIBI uptake in the presence of quercetin and GG918 is illustrated in Fig. 5. Without treatment, the absolute accumulation of ^{99m}Tc-MIBI was higher in K562 than in K562/*adr* cells. A significant increase in ^{99m}Tc-MIBI accumulation in the presence of 10 μM quercetin was observed but not with 5μM GG918 in K562. In the presence of GG918, a cellular tracer in K562/*adr* increased almost equally to the cell tracer in K562, but an increased in cellular tracer was not significantly observed with quercetin. This indicates that quercetin does not inhibit the Pgp-mediated efflux of ^{99m}Tc-MIBI, but any increase in cellular ^{99m}Tc-MIBI accumulation in these experiments might be due to increase in the $\Delta\Psi_m$.

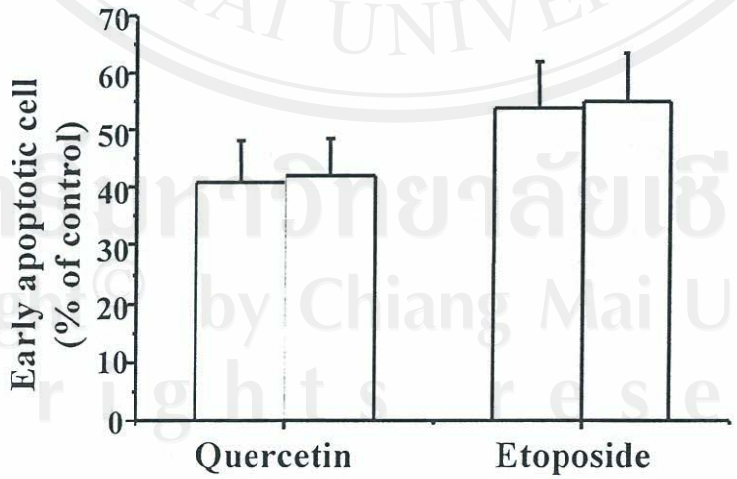

Figure 4. Detection of early apoptotic cells using ^{99m}Tc-Annexin V. Cells were treated with 10 μM quercetin for 3 h and with 100 μM etoposide for 24 h in K562 (white bar) and K562/*adr* cell (Grey bar). Each bar represents the means ± SD of 3 independent experiments.

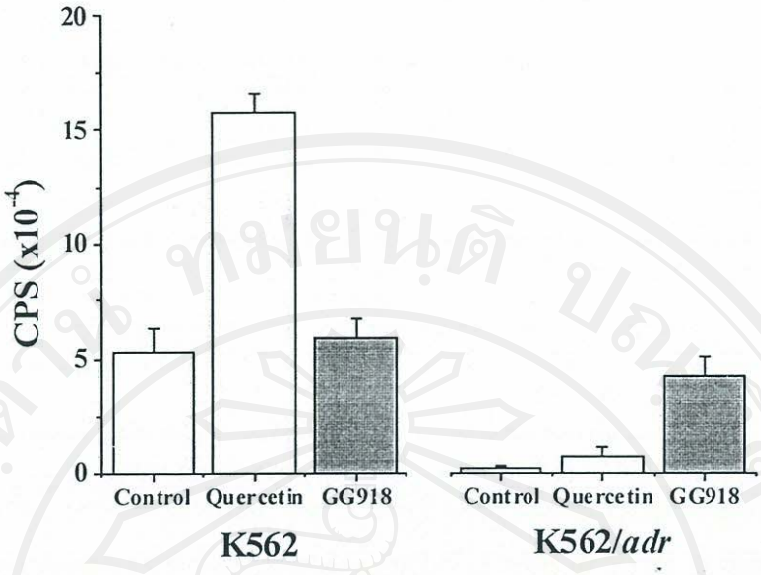

Figure 5. Accumulation of ^{99m}Tc-MIBI in sensitive (K562) and resistant (K562/*adr*) cell lines; (white bar) without inhibitor, (grey bar) 10 μM quercetin and (black bar) 5 μM GG918. Each bar represents the means ± SD of 3 independent experiments.

Modulation of absolute value of $\Delta\Psi_m$ ($|\Delta\Psi_m|$) in K562 and K562/*adr* cells in the presence of quercetin was investigated as a function of times and quercetin concentrations. At basal level (without quercetin), $|\Delta\Psi_m|$ was 160 ± 1.0 mV and 145 ± 1.2 mV in K562 and K562/*adr* cells, respectively. Typical results of quercetin- induced $\Delta\Psi_m$ change as a function of time was indicated in Fig. 6. After adding quercetin (10 μM), $|\Delta\Psi_m|$ increased slightly to reach a maximal value within 1 h, then progressively decreased by 5.5% of the initial value at 3 h for K562 cells. For K562/*adr* cells, the maximal value of $|\Delta\Psi_m|$ was reached at 30 min followed by decrease of 3.8%. An increase or a decrease in $|\Delta\Psi_m|$ after addition of quercetin, due to an increase or a decrease in mitochondrial martix rhodamine B concentration, which depends on the mitochondrial energetic state, is not a result of direct inhibition of P-glycoprotein function by quercetin. This signifies that quercetin mediates action at mitochondrial level. $|\Delta\Psi_m|$ determined at 3 h in the 2 cell lines with the increasing quercetin concentration was presented in Fig. 7.

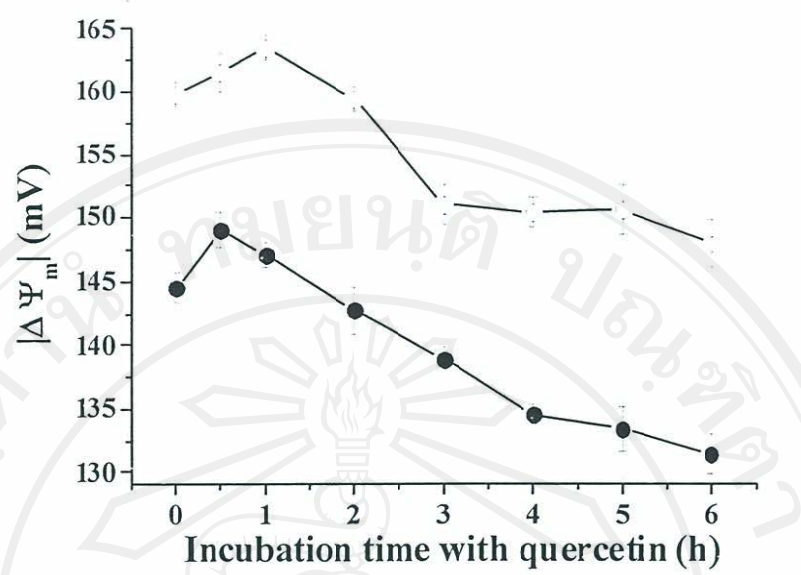

Figure 6. Absolute value of mitochondrial membrane potential ($\Delta\Psi_m$) of (○) K562 and (●) K562/*adr* in the presence of 10 μM quercetin as a function of time. Data represent mean ± SD of 3 independent experiments.

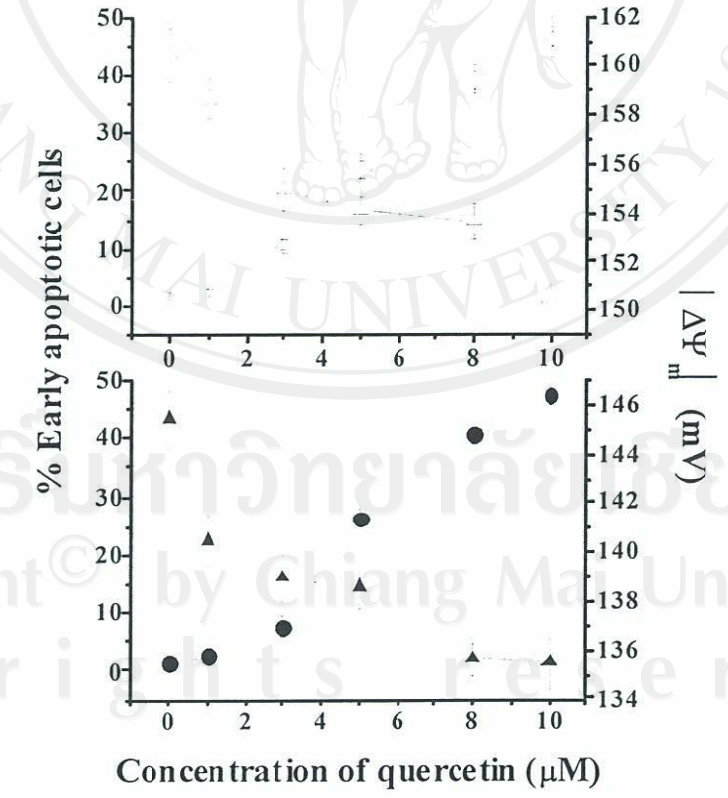

Figure 7. Variation of Absolute value of $\Delta\Psi_m$ (Δ, ▲) and percentage of early apoptotic cell (○, ●) as a function of quercetin concentration in (A) K562 and (B) K562/*adr*. Cells were treated with quercetin for 3 h. Data are the mean ± SD of 3 independent experiments.

DISCUSSION

The potential beneficial use of quercetin, the most widely distributes flavonoids in vascular plants, for preventing ischemia-reperfusion-induced myocardial damage by reactive oxygen species has been reported. By using a normal cell such as H9c2 cardiomyoblast, quercetin could protect hydrogen peroxide from inducing H9c2 cardiomyoblast cells (Park *et al.*, 2003). It was also reported that quercetin showed higher value of antioxidant activity than of Vitamin C, Vitamin E and β-carotene on molar basis (Rice-Evans *et al.*, 1995). Owing to the high antioxidant activity, the quercetin prevented the generation of reactive oxygen species by cyclosporine, which suppressed the cyclosporine-induced nephrotoxicity (Satyanarayana *et al.*, 2001). These authors proposed that quercetin might play an expression modulator of Mn-SOD, an enzyme which is located on the mitochondrial matrix. The results obtained from various normal cell types reveal that quercetin protected various cell types effectively from oxidative injury and from induction of apoptosis via inhibition of the mitochondrial dysfunction.

However, bioflavonoids have been considered as drugs for various pathologies such as cancer, viral infection, inflammation, allergy, hypertension as well as atherosclerosis. Among bioflavonoids, quercetin is most tested for pharmacological properties such as induction of apoptosis resulting in an inhibition of the growth of various cancer cell types (Wang *et al.*, 1999; Yin *et al.*, 1999; Choi *et al.*, 2001). The mechanisms quercetin's anticancer activity is likely complicated, starting on metabolic changes, decreases in IP3 concentration, and down-regulation of oncogenes (*c-myc* and *Ki-ras*). The products of *c-myc* and *Ki-ras* oncogenes are required for induction of proliferation and of apoptosis (Ellis *et al.*, 1991; Evan *et al.*, 1992).

Our results show that K562/*adr* cells are approximately 2-fold more sensitive than K562 cells to cytotoxic effects of quercetin. Quercetin induces apoptosis in a concentration- and time-manner, and K562 and K562/*adr* cells displayed similar levels of apoptosis 1h after exposure to 10 μM quercetin. This indicates that a mechanism other than apoptosis was inducing cell cycle arrests (Yoshida *et al.*, 2002) and was probably contributing to the cytotoxic effects of quercetin. Quercetin does not inhibit Pgp function. This observation is consistent with the results of Ikegawa *et al.* (Ikegawa *et al.*, 2002), which show that quercetin derivatives were potent MDR-reversing agents, although quercetin was not.

The effect of quercetin on mitochondrial energetic state is determined in this work. At such a low quercetin concentration as 10 μM, the spontaneous change in $|\Delta\Psi_m|$ indicated the energetic state of mitochondria and cells could be induced. An increase followed by a decrease in $|\Delta\Psi_m|$ value was associated with an induction of apoptosis that could be detected at 1h. Our results are in an agreement with the other studies, which reported that quercetin (60 μM) and other flavonoids induced apoptosis in HL-60 cells by releasing Cytochrome c and inducing caspase-9 processing (Wang *et al.*, 1999; Murphy, 1999). The $|\Delta\Psi_m|$ of K562 and K562/*adr* cells is equal to 160 ± 1.0 mV and 145 ± 1.2 mV, respectively. Our previous work found that artemisinin and its derivatives

reversed MDR phenomenon at mitochondrial level (Reungpatthanaphong *et al.*, 2003). The correlation between the impairment of mitochondrial energetic state (decreasing in $|\Delta\Psi_m|$ value) and an induction of apoptosis (the percentage of early apoptotic cells) by quercetin is shown in Fig. 7. It can be noted that at increasing higher concentrations of quercetin, the $|\Delta\Psi_m|$ decreases in very narrow range (from 160 ± 1.0 mV to 150 ± 0.6 mV for K562 and from 145 ± 1.2 mV to 135 ± 1.1 mV in K562/*adr*), whereas the percentage of early apoptotic cells increased in greater degree of range (from 1.5 ± 0.4% to 45 ± 3.2%).

The results of this study reveal that quercetin provokes its cytotoxicity at mitochondria level, impairing mitochondrial energetic state, and then inducing apoptosis and inhibition of caner cell growth. Quercetin, therefore, might be a new generation of antitumour drugs, particularly for overcoming MDR phenomena.

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