CHAPTER II

Rhodamine B as a Mitochondrial Probe for Measurement and Monitoring of Mitochondrial Membrane Potential in Drug-Sensitive and -Resistant Cells

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Rhodamine B as a Mitochondrial Probe for Measurement and Monitoring of Mitochondrial Membrane Potential in Drug-Sensitive and -Resistant Cells

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Abstract

In order to get more insight into the energetic state of multidrug-resistance (MDR) cell compared with its corresponding sensitive cell, a noninvasive fluorescence method for determining and monitoring the mitochondrial membrane potential ($\Delta\Psi_m$), using rhodamine B and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was established. Rhodamine B distributes across biological membranes in response to the electrical transmembrane potential. P–glycoprotein and MRP1–protein–mediated efflux do not create a concentration gradient, leading the cell–rhodamine B system to reach a steady state, where the ratio of cytosolic to extracellular rhodamine B was equal to 1. The mitochondrial matrix rhodamine B concentration was precisely determined as a decrease of rhodamine B fluorescence in the presence of formazan, a rhodamine B fluorescence quencher, which locally accumulates in the matrix of mitochondria. The kinetics of decrease in rhodamine B fluorescence (*Vi*) can be used to estimate $\Delta\Psi_m$ using the Nernst equation: $\Delta\Psi_m = -61.54 \log Vi - 258.46$. The $\Delta\Psi_m$ values determined were -160 ± 4 mV for K562 cell, -146 ± 6 mV for K562/adr cell, -161 ± 10 mV for GLC4 cell and -168 ± 2 mV for GLC4/adr cell. An increase or a decrease in $\Delta\Psi_m$ consequently followed an increase or a decrease in the cellular ATP contents. An increase ATP content in the two MDR cell lines can protect cells from cytotoxicity induced by pirarubicin.

Keywords: Multidrug resistance associated protein (MRP1), P–glycoprotein (P–gp), Mitochondrial membrane potential ($\Delta\Psi_m$), Rhodamine B, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), spectrofluorometry, Cyclosporin A (CsA)

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1. INTRODUCTION

Multidrug resistance (MDR) is a major cause of failure in cancer chemotherapy. MDR is associated with a decrease of cellular drug accumulation, thus reducing its efficacy, resulting from enhanced drug efflux by the membrane proteins such as P–glycoprotein and MRP1 protein (Bradley *et al.*,1988; Ambudkar *et al.*, 1999; Almquist *et al.*, 1995). It was demonstrated that MDR cells need more cellular ATP contents than that of their corresponding sensitive cells due to the addition of the ATPase activity of P–glycoprotein and MRP1 protein (Miccadei *et al.*, 1996; Dorward *et al.*, 1997; Mieminen *et al.*, 1994; Jia *et al.*, 1996). There is strong evidence that when MDR cells were deprived of ATP, even partially, it would block the P–glycoprotein and MRP1 pump activity, leading to an increase in cellular drug accumulation (Broxterman & Pinedo, 1991; Mankhetkorn *et al.*, 1996). Indeed, understanding the source of cellular ATP production and cellular energetic state of MDR cells is crucial to overcome the MDR phenomena.

To get more insight into the energetic state of cells, numerous types of lipophilic cation probes such as $DiOC_6$, MIBI, JC–1, TPP^+ and rhodamine dyes have been used to estimate the electrical potential across the inner mitochondrial membrane ($\Delta\Psi_m$) (Zinkewich–Peotti & Andrews, 1992; Rottenberg & Wu, 1998; Shtil *et al.*, 2000; Vergote *et al.*, 1998; Eytan *et al.*, 1997; Hagen *et al.*, 1997). Such compounds are not only sufficiently lipophilic to partition into biological membranes, but also contain a delocalized charge distributed throughout the molecule, thereby allowing passive distribution of the compounds across a bilayer in proportion to an imposed transmembrane electrical potential. Among these compounds, rhodamine 123, tetramethylrhodamine methyl ester (TMRM) and tetremethylrhodamine ethyl ester (TMRE) have been used to measure the $\Delta\Psi_m$ by fluorescence imaging (Loew *et al.*, 1993). Unfortunately, these compounds were demonstrated to be effluxed by MDR transporters.

Rhodamine B, a rhodamine derivative, may distribute across biological membranes in response to the transmembrane potential; this action is well characterized in lipophilic cations such as $DiOC_6$, MIBI, JC–1, TPP^+ and rhodamine dyes. Russell *et al.* (Russell *et al.*, 1999) found that rhodamine B is a very poor substrate of P–glycoprotein. We report herein that the gradient concentration, being high in the extracellular compartment and low in the cytosol, was not created by P–glycoprotein and MRP1 protein due to the higher rate of uptake coefficient than the efflux coefficient mediated by these two membrane proteins.

This work shows an advantage of using rhodamine B as a probe to estimate the $\Delta\Psi_m$ in drug–sensitive and, particularly, in drug–resistant cells. This is a noninvasive functional study that can be used to determine and to monitor a spontaneous change in mitochondrial function in drug–sensitive as well as MDR cell. The method is simple and direct and can be easily employed using a standard spectrofluorometer. It also alleviates the difficulty in using either electrodes or radioactive tracers to estimate the distribution of lipophilic cations in the measurement of $\Delta\Psi_m$.

2. MATERIALS AND METHODS

2.1 *Cell culture*

The erythromyelogenous leukemic cell K562 and its P–glycoprotein–overexpression cell K562/*adr* (Zilstra, *et al.*, 1987; Lozio & Lozio, 1975), and the human small cell of lung cancer GLC4 and its MRP1–overexpression GLC4/*adr* (Tsuro *et al.*, 1986; Muller *et al.*, 1994; Versantvoort *et al.*, 1995), were routinely cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco Biocult). The resistant GLC4/*adr* and K562/*adr* cells were cultured with 100 nMdoxorubicin, 2 weeks before experiments. For the assays, a culture was initiated at 5×10^5 cells/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/ml. Cell viability was assessed by Trypan blue exclusion. The number of cells was determined by a hemocytometer.

The cytotoxicity assay was performed as follows: 5×10^4 cells/ml were incubated in the presence of various pirarubicin concentrations. The viability of cells was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl -2*H*-tetrazolium bromide (MTT) reduction. The IC_{50} was determined by plotting the percentage of cell growth inhibition versus the pirarubicin concentration: IC_{50} is the pirarubicin concentration that inhibits cell growth by 50% when measured at 72 h. The resistant factor (RF) was defined as the IC_{50} of resistant cells divided by the IC_{50} of the sensitive cells. The resistant factors were 40 and 7 for K562/*adr* and GLC4/*adr*, respectively.

2.2 Drugs and chemicals

Rhodamine B and tetrazolium salt (MTT) were from Amresco. Cyclosporin A (CsA) was from Sandoz. ATP (K^+ salt), oligomycin, *p*-trifluoromethoxyphenyl hydrazone cabonyl cyanide (FCCP) and luciferin-luciferase were from Sigma. Purified pirarubicin (4-Q-tetrahydropyrayl-doxo rubicin) was kindly provided by Laboratoire Roger Bellon (France). Deionized double–distilled water was used throughout the experiments, which were performed in HEPES–Na^+– or HEPES–K^+–rich media.

HEPES–Na^+–rich medium consisted of 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose and 20 mM HEPES (pH 7.25, 37 °C).

HEPES–K^+–rich medium consisted of 132 mM KCl, 3.5 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose and 20 mM HEPES (pH 7.25, 37 °C).

To adjust the $\Delta\Psi_m$ to the indicated values using valinomycin, Na^+–rich and K^+–rich media were mixed in the appropriated proportions.

A stock solution of 12 mM MTT was prepared in HEPES–Na^+ buffered solution, filtered through a 0.22–μm filter and stored in 4 °C. The pirarubicin solution was freshly prepared before use and its concentration was spectrophotometrically determined by using ε_λ = 480 nm equal to 11,500 $M^{-1}cm^{-1}$ (Shimadzu UV2501–PC).

2.3 Flow cytometric assay

Cells (10^6 /mL) were incubated with rhodamine B, in the absence or in the presence of 7 μM FCCP or 5 μM cyclosporin A or 100 AM MTT for 20 min. Rhodamine B was excited at 488 nm and fluorescence was analyzed at 580 nm (FL2) after logarithmic amplification (F_{cyto}). The analysis was performed on Becton Dickinson and values were given as mean fluorescence

of the popularity calculated on a linear scale (± S.D.). Forward scatters (FSC) and side scatters (SSC) were analyzed after linear amplification. The ratio FSC/SSC was calculated for each cell individual during the analysis and plotted as a distribution histogram on a linear scale.

2.4 Spectrofluorometric assay

The uptake of rhodamine B into the cells was monitored by following the decrease of the fluorescence intensity at 582 nm (excited at 553 nm) due to the local quencher of rhodamine B fluorescence, formazan crystals, which took place in the mitochondria (Perkin–Elmer model LS 50B spectro-fluorometer). The kinetics of decrease of rhodamine B fluorescence was sensitive to the $\Delta\Psi_m$. The initial rate of rhodamine B fluorescence extinction could be determined by the slope of the tangent of the curve of $F = f(t)$, where F is the rhodamine B fluorescence intensity. By using this technique, it was possible to monitor the alteration of mitochondrial function in intact cells because the incubation of the cells with the drug proceeded without compromising cell viability. All experiments were conducted in 1–cm quartz cuvettes containing 2 ml of buffer vigorously stirred at 37 °C.

2.5 Measurement of ATP in cell extracts

To measure intracellular ATP, Cells (5×10^5) were incubated in the presence or the absence of MTT or oligomycin at 37 °C for 10 minute to 1 hour. The cells were pelleted and were permeabilized by 500 µl lysis solution containing 0.1% Triton X–100, 20 mM Tris, 0.1 mM EDTA, and 5 mM $MgSO_4$. ATP in the extracts was quantitated using a luciferase assay (Kimmich *et al.*, 1975). The intensity of light was recorded by Perkin–Elmer LS 50B spectrofluorometer after addition of 50 µl luciferin-luciferase (20 mg/ml).

2.6 Reduction of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)

For determination of the physiological state of the mitochondrial, MTT reduction by the cells was used as an indicator of mitochondrial function. MTT was reduced by succinate dehydrogenase, a component of complex II of the respiratory chain (Berridge & Tan, 1993). 5×10^5 cells/ml were incubated in RPMI 1640 medium at 37 °C in the presence of various MTT concentrations. The blue formazan crystals were recovered, dissolved in DMSO at various times and the absorbance at 560 nm was measured. The amount of formazan produced was calculated using molar extinction coefficient of 51,000 $M^{-1}\cdot cm^{-1}$.

3. RESULTS AND DISCUSSION

3.1 Intracellular accumulation of rhodamine B by flow cytometry:

F_{cyto} is proportional to the total concentration of rhodamine B added to cell ranging from 0.01 to 2 µM for the four cell lines. This signifies that the association of rhodamine B to cell at least up to 2 µM does not yield any quenching of rhodamine B fluorescence intensity. In this study, we had used a 40 nM concentration of rhodamine B. This is a minimal concentration wherein the intracellular accumulation would not disturb the function of the mitochondria. It also provides a significant change in fluorescence intensity, which can be detected by flow cytometry and conventional spectrofluorometry.

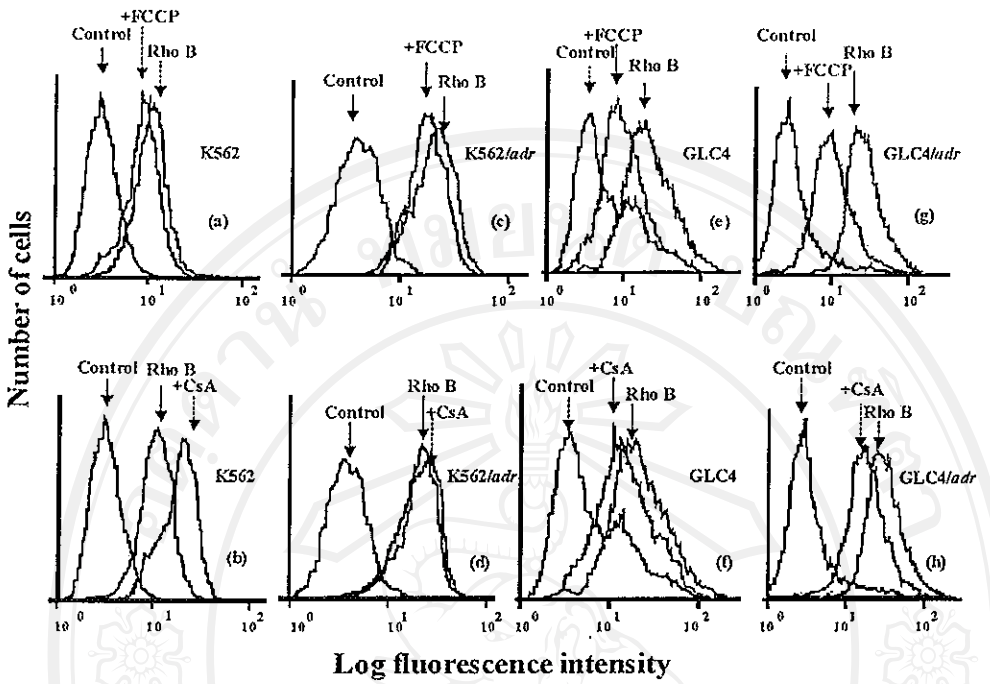

Figure 1. Typical histograms of cell–rhodamine B bound fluorescence; effect of 7 μM FCCP for (a) K562, (c) K562/*adr*, (e) GLC4 and (g) GLC4/*adr* and of 5 μM cyclosporin A for (b) K562, (d) K562/*adr*, (f) GLC4 and (h) GLC4/*adr*. Cells (2×10^6) were incubated with 40 nM rhodamine B in 2 ml of buffer HEPES–Na^+ for 20 minutes at 37 °C. FCCP (7 μM) or cyclosporin A (5 μM) was added, and the incubation was continued for an additional 20 minutes. The cell fluorescence was measured by flow cytometry. Each histogram was obtained from 5000 cells.

The role of the plasma membrane potential ($\Delta\Psi_p$) that contributed to F_{cyto} was investigated in the four cell lines. Cells (2×10^6) were incubated in 2 ml of buffer solution containing 40 nM rhodamine B and different K^+ concentrations ranging from 3.5 mM to 132 mM without valinomycin for 30 minutes; then, F_{cyto} were measured. In these conditions, $\Delta\Psi_p$ was decreased when the extracellular K^+ concentration is increased, and dissipated when the extracellular K^+ concentration was equal to 132 mM. However, any modification of F_{cyto} was not observed. The $\Delta\Psi_p$ does not contribute to the cellular rhodamine B uptake.

In order to localize the intracellular targets of rhodamine B, cells were incubated with 40 nM rhodamine B in the presence of the $\Delta\Psi_m$ modulators such as FCCP (Max *et al.*, 1989) and Cyclosporin A (Bernardi, 1992) for 30 minutes; F_{cyto} was then measured. FCCP is a protonophore, an uncoupling agent that induce depolarization of mitochondria by dissipating the H^+ electrochemical gradient across the inner membrane, while cyclosporin A is a specific inhibitor of the permeability transition pores, which induce repolarization of mitochondria.

Figure 1 shows that 7 μM FCCP collapsed only the $\Delta\Psi_m$, resulting in a reduction of F_{cyto}, particularly in GLC4 and GLC4/*adr* cells. The K562 and K562/*adr* cells are less sensitive to FCCP than both GLC4 and GCL4/*adr* cells. The addition of 5 μM cyclosporin A clearly increased F_{cyto} in K562, but not in K562/*adr*, and slightly decreased F_{cyto} in GLC4 and GLC4/*adr* cells. These results indicated that the accumulations of rhodamine B in the four cell lines were sensitive to the $\Delta\Psi_m$.

The determination of the $\Delta\Psi_m$ in this study is based on the observation that rhodamine B is accumulated within the mitochondrial matrix in accordance to the Nernst equation. The quantity of mitochondrial dye accumulation can be determined by measuring the quenching of F_{cyto} after addition of MTT to the rhodamine–B–loaded cells (Figure 2). The addition of MTT yields a stimulation of the activity of SDHase that reduces MTT to produce formazan. As formazan, a rhodamine B fluorescence quencher (inset of Figure 3), is located exclusively in the mitochondrial matrix, only the rhodamine B located in this compartment will be quenched. However, the flow fluorocytometric measurement does not provide any information concerning the concentration of dye.

3.2 Estimation of the mitochondrial membrane potential by spectrofluorometry

Figure 3 shows that, in contrast to flow cytometric results, none of the mitochondrial modulators used, FCCP (7 μM), cyclosporin A (5 μM) and valinomycin (3 μM), affect the fluorescence intensity of rhodamine–B–loaded cells as measured by conventional spectrofluorometer. This demonstrates that they are not quenchers of rhodamine B fluorescence. It also shows that spectrofluorometry per se cannot give information about the rhodamine B distribution between the extracellular and intracellular compartments. Interestingly, when MTT was added to rhodamine–B–loaded cells, one can observe a decrease of rhodamine B fluorescence intensity due to the formation of mitochondrial matrix formazan as mentioned above. The difference of the fluorescence intensity before and after addition of MTT, $(\Delta F)_{MTT}$, corresponds to the mitochondrial matrix concentration of rhodamine B. To use this method in estimation of the $\Delta\Psi_m$, several factors must be taken into account. The kinetics of MTT uptake and SDHase activity as well as the kinetics of rhodamine B uptake in the four cell lines were analysed as indicated in Table 1. It is clear that the reduction of MTT occurred in the same manner for the MDR cells compared with their corresponding sensitive cells, but varied from one cell type to another. The kinetics of MTT uptake and MTT reduction are about 2500 (K562, K562/*adr* and GLC4) to 5000 (GLC4/*adr*) and about 2900 times faster than that of the kinetics of rhodamine B uptake of the four cell lines, respectively. Since the formazan immediately quenched the fluorescence intensity of rhodamine B (inset of Figure 3), the MTT concentrations under these experimental conditions are largely in excess compared with rhodamine B concen-tration, the reasonable hypothesis is that the quantities of formazan is not limited in this reaction.

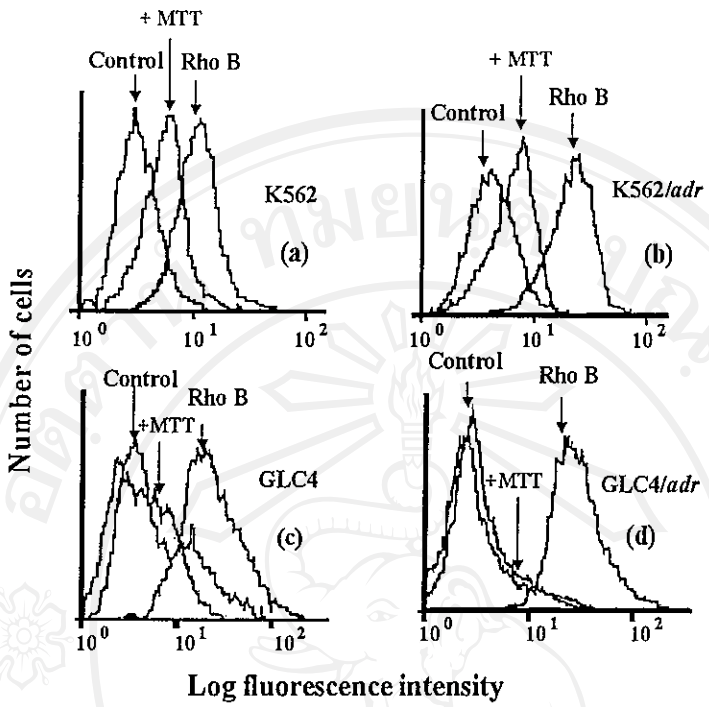

Figure 2. Typical histograms of cell–rhodamine B bound fluorescence: effect of 100 μM MTT on (a) K562, (b) K562/*adr*, (c) GLC4, and (d) GLC4/*adr*. Cells (2×10^6) were incubated with 40 nM rhodamine B in 2 ml of buffer HEPES–$\dot{N}a^+$ for 20 minutes at 37 °C. MTT was then added and the incubation was continued for an additional 20 minutes. The cell fluorescence was measured by flow cytometry. Each histogram was obtained from 5000 cells.

3.2.1 Drug-sensitive cells

To simplify the system, cells were divided into three: extracellular, cytoplasmic and mitochondrial compartments, respectively. At steady state, rhodamine B was equilibrated between the extracellular (C_e^o), cytosolic (C_i^o) and mitochondrial compartments (C_m^o), where the ratio of C_i^o / C_e^o is equal to 1, and $C_m^o > C_i^o$ and C_e^o. The progressive decrease of rhodamine B fluorescence intensity is due to the translocation of rhodamine B from the outside to the inside of mitochondria. Indeed, the reduction of MTT induces repolarisation of the mitochondria, yielding an increase in the $\Delta\Psi_m$, which can be illustrated by increasing the cellular ATP contents (Figure 6). 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 rate of rhodamine B uptake (V_{rhoB}) into the mitochondrial matrix can be written as:

$$V_{rhoB} = PC_i^o \qquad (1)$$

where P is a permeability coefficient that depends on rhodamine B and on

the mitochondria membrane. When 1 mole leaves the extracellular medium to go to cytosol and then to the mitochondria at $\Delta\Psi_m$, and the variation of the fluorescence per mole is:

$$\delta F = F_e - F_m \qquad (2)$$

where F_e and F_m are the rhodamine B fluorescence intensity in the extracellular compartment and mitochondrial compartments, respectively. When MTT is added to the cells, 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 fluorescence signal:

$$n = V_{rhoB}\,\Delta t \qquad (3) \text{ or}$$

$$n = PC_i^{\circ}\Delta t \qquad (4)$$

$$\Delta F = n\delta F \qquad (5)$$

According to Eq.(4), Eq. (5) can be written as:

$$\Delta F = PC_i^{\circ}\Delta t\delta F \qquad (6)$$

According to Eq. (2), Eq. (6) becomes:

$$\Delta F/\Delta t = PC_i^{o}(F_e - F_{mtt}) \qquad (7)$$

P does not depend on the $\Delta\Psi_m$ and the sign of $\Delta F/\Delta t$ does not depend on C_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 s) 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 4, corresponding to V_i, the initial rate of the decrease of rhodamine B fluorescence intensity.

$$V_i = PC_i^{o}(F_e - F_{mtt}) \qquad (8)$$

In 50 s, the change in extracellular rhodamine B concentration is negligible compared with that before the addition of MTT; however, $\Delta F/\Delta t$ is easily determined. Indeed, only 8% of rhodamine B were found to partition into cells. It is reasonable to assume that at steady state, C_e^{o} was equal to C_i^{o}. The distribution of free dye across the mitochondrial membrane can be precisely determined by using an advantage of specific accumulation of MTT reduction product and rhodamine B in the mitochondria. This method has its foundation in the quantitation 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 an estimated measurement of $\Delta\Psi_m$:

$$C_m^{o}/C_i^{o} = 10^{-(\Delta\Psi_m F/2.303RT)} \qquad (9)$$

$$\Delta\Psi_m = -61.51 \log V_i - 258.46 \qquad (10)$$

where $\Delta\Psi_m$ is the mitochondrial membrane potential in mV, RT/F is 26 mV at 37 °C and C_i^{o} is 40 nM and V_i is in nM·s^{-1}. The V_i should reflect the $(\Delta\Psi_m)_{mtt}$ value which is nearest to the *in situ*, $\Delta\Psi_m$. The expression of $\Delta\Psi_m = -61.54 \log V_i - 258.46$ can then be used to estimate $\Delta\Psi_m$ under other conditions, provided that the concentration of cell, rhodamine B and MTT used in the standardization procedure is maintained. The $\Delta\Psi_m$ value determined by this method is indicated in Table 1. The $\Delta\Psi_m$ values obtained in our experiments are in total

agreement with those obtained by other methods as mentioned in Table 2. V_i is sensitively changed such that when cells were incubated in HEPES buffer with incremental K^+ concentration that induced a decrease in the $\Delta\Psi_m$, even a narrow window change as indicated in Figure 4 (a). As shown in Figure 4(b) and (c), log V_i was linearly decreased when the absolute value of $\Delta\Psi_m$ increased.

Table 1. The mean coefficient of kinetics of rhodamine B uptake ($K^+_{rhoB,}$), the mean coefficient of kinetics of MTT uptake ($K^+_{MTT,}$), the mean coefficient of MTT–reduction ($k^+_{MTT-reduction}$) by cells, and the absolute mitochondrial membrane potential values determined by using rhodamine B as molecular probe.

Cell lines	$K^+_{rhoB,}$ (pl.cell^{-1}.s^{-1})	$K^+_{MTT,}$ (pl.cell^{-1}.s^{-1})	$K^+_{MTT-reduction,}$ (pl.cell^{-1}.s^{-1})	$\Delta\Psi_m$ (mV)
K562	0.021 ± 0.004	50 ± 4	21.96 ± 3.3	160 ± 4
K562/*adr*	0.018 ± 0.002	48 ± 6	21.96 ± 3.3	146 ± 6
GLC4	0.0075 ± 0.001	50 ± 4	21.96 ± 3.3	161 ± 10
GLC4/*adr*	0.019 ± 0.001	50 ± 4	21.96 ± 3.3	168 ±2

Table 2. Lipophilic cations used to estimate the mitochondrial membrane potential ($\Delta\Psi_m$).

Mitochondrial Dye	Types of study	$\Delta\Psi_m$ (mV)	Ref.
$DiOC_6$	intact hepatocytes of mice	168 ± 8	Rotenberg, 1998
(^{3}H)TPP$^+$	intact hepatocytes of young rat	154 ± 20	Hagen, 1997
(^{3}H)TPP$^+$	intact hepatocytes of old rat	101 ± 18	Hagen,1997
tetramethylrhodamine methyl ester (TMRE)	Neuroblastoma cells	150	Shtil, 2000

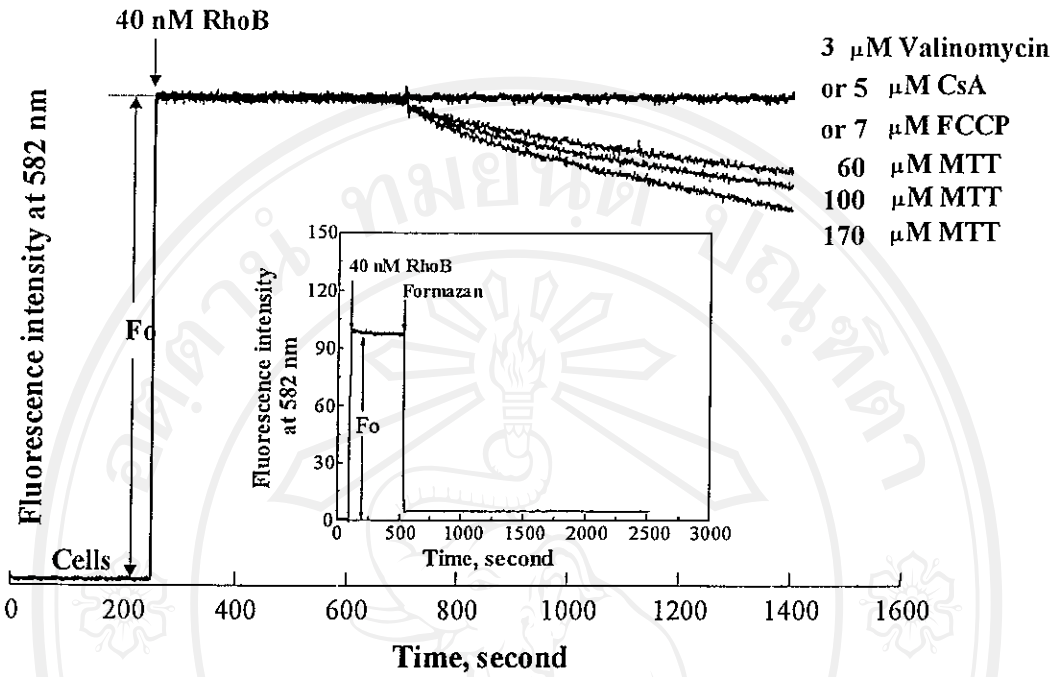

Figure 3. Typical kinetics of the uptake of rhodamine B by cells. Fluorescence intensity, F, at 582 nm (λ_{ex} = 553 nm) was recorded as a function of time. Cells (2×10^6) were suspended in 2 ml of HEPES–Na^+ at 37 °C and vigorously stirred. A small volume of rhodamine B stock was added to the solution, yielding a final concentration of 40 nM rhodamine B. The corresponding fluorescence intensity F_0 was recorded, for 20 minutes after the indicated concentration of valinomycin, FCCP or MTT was added. Inset: The quenching effect of the MTT–reduction on production rhodamine B fluorescence. The MTT–reduction product was added to the solution of 40 nM rhodamine B.

3.2.2 Drug-resistant cells

As mentioned above, this method of the $\Delta\Psi_m$ estimation is based on the mitochondrial, cytosolic and extracellular rhodamine B concentration. The value of $\Delta\Psi_m$ is exact in the case of K562 and GLC4 cells. However, in case of drug-resistant K562/*adr* and GLC4/*adr* cells, the mitochondrial and cytosolic rhodamine B concentration may be affected by the P–glycoprotein– or MRP1–mediated efflux activity. The investigation of the capability of both membrane transport proteins that pumps the rhodamine B out of cell, leading to lowering intracellular concentration, thus decreased the mitochondrial concentration was performed.

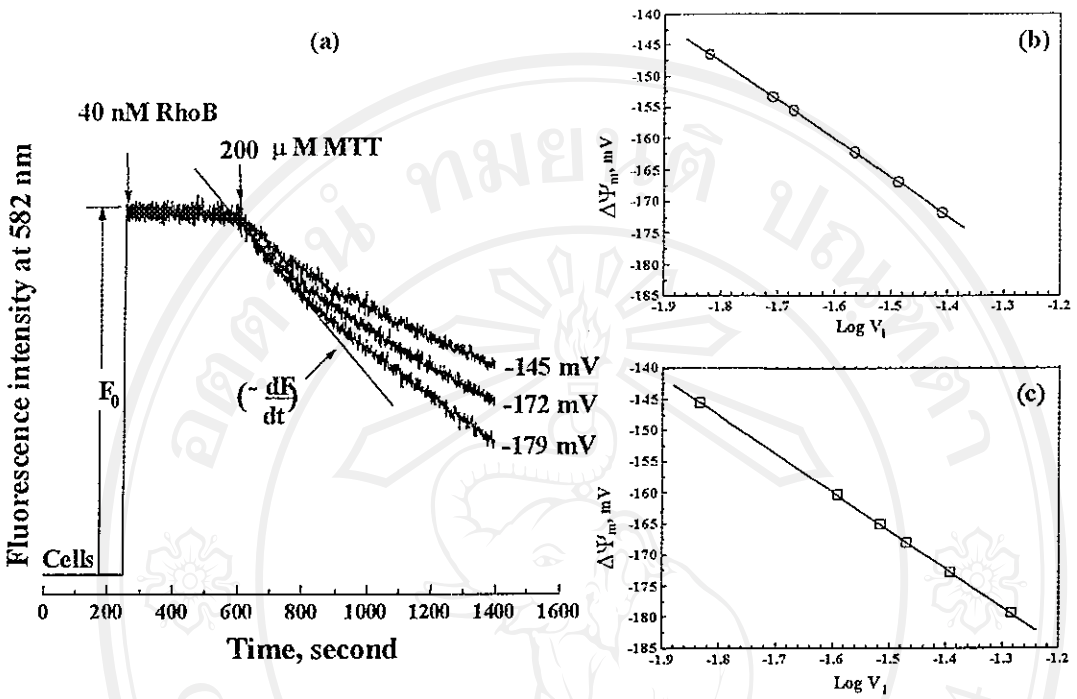

Figure 4. (a) Typical kinetics of the uptake of rhodamine B by cells. Fluorescence intensity, F, at 582 nm (λ_{ex} = 553 nm) was recorded as a function of time. 2×10^6 cells were suspended in 2 ml of HEPES–Na^+ buffer at 37 °C and vigorously stirred. A small volume of rhodamine B stock was added to the solution yielding a final concentration of 40 nM. The corresponding fluorescence intensity F_0 was recorded for 20 minutes after 200 μM MTT was 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 rhodamine B fluorescence extinction was equal to; $Vi = (dF/dt).(C_T/F_0)$. To set $\Delta\Psi_m$ at the indicated values, cells (2×10^6) were suspended in 2 ml of HEPES–Na^+/HEPES–K^+ buffer in varying ratios in the presence of 20 nM valinomycin. (b) and (c) Variation of log V_i as a function of known $\Delta\Psi_m$ for K562 cell and for GLC4 cell, respectively.

Cells (2×10^6) were incubated with 40 nM rhodamine B in the presence of 5 μM cyclosporin A or 10 μM PAK–104P for 30 minutes, then the F_{cyto} was measured. Cyclosporin A is a well–known P–glycoprotein inhibitor (Mankhetkorn & Garnier–Suillerot, 1998) and we have checked that at 5 μM, cyclosporin A completely inhibited the P–glycoprotein function, while PAK–104P is the most efficient inhibitor of MRP1 protein (Marbeuf–Gueye *et al.*, 2000). We have also checked that at 10 μM, PAK–104P inhibited about 80% of MRP1 function.

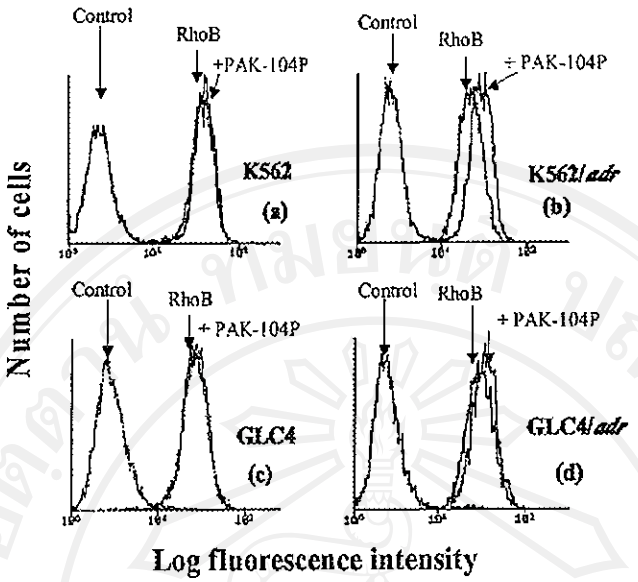

Figure 5. Typical histograms of cell–rhodamine B bound fluorescence: effect of 10 μM PAK–104P on (a) K562 cell, (b) K562/*adr* cell, (c) GLC4 cell and (d) GLC4/*adr* cell. Cells (2 x 10^6) were incubated with 40 nM rhodamine B in 2 ml of buffer HEPES–Na^+ for 20 minutes at 37 °C. PAK–104P (10 μM) was then added and the incubation was continued for an additional 20 minutes. Cell fluorescence was measured by flow cytometry. Each histogram was obtained from 5000 cells.

Figure1(d) and (h) and Figure 5 shows that neither cyclosporin A nor PAK–104P affects F_{cyto} in K562/*adr* and GLC4/*adr* cells, which indicates that gradient rhodamine B concentration is not created by the two membrane protein. For drug–resistant cells, rhodamine B behaves in the same manner as in drug–sensitive cell; at steady state, it was equilibrated among extracellular (C_e^o), cytosolic (C_i^o) and mitochondrial compartment (C_m^o), where the ratio of C_i^o/C_e^o is equal to 1 and $C_m^o > C_i^o$ and C_e^o. Rhodamine B is then a suitable molecular probe for determining the $\Delta\Psi_m$ of both K562/*adr* and GLC4/*adr* cells.

Similar experimental conditions, as in drug–sensitive cells, for determining a V_i were performed for both MDR cells, and then the $\Delta\Psi_m$ was determined as indicated in Table 1. As can be expected, the $\Delta\Psi_m$ of GLC4/*adr* cell was slightly higher than that of the GLC4 cell, but on the contrary, the $\Delta\Psi_m$ of K562/*adr* cell was lower than that of the K562 cell. The same direction of results were reported by Shtil *et al.* (Shtil *et al.*, 2000); the $\Delta\Psi_m$ of K562/*adr* cells was also lower than in K562 cell, but it was found to be comparable for MCF7 cell and MCF7–VP cell with overexpression MRP1–protein. These results suggest that altered mitochondrial dynamics is non–random in MDR cells; thus, the mitochondria may be involved in the production of a pleiotropic MDR phenotype. It was proposed that rapid overexpression of MDR1/P–glycoprotein is associated with a decrease of $\Delta\Psi_m$ (Shtil *et al.*, 2000).

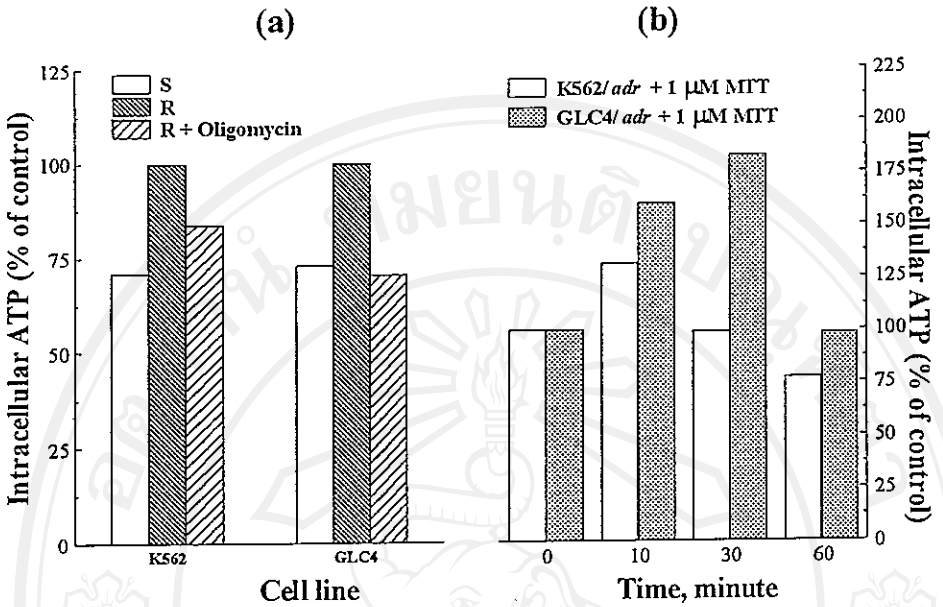

Figure 6. (a) Intracellular ATP levels of drug–sensitive and MDR cells and the effect of 5 μM oligomycin on intracellular ATP levels of MDR cells. (b) Time course of intracellular ATP concentration in MDR cells treated with 1 μM MTT (see material and methods). The results are representative of three similar experiments

3.3 Determination of cellular ATP contents

In order to evaluate the cellular response, according to a decrease or an increase of the $\Delta\Psi_m$, the cellular ATP contents were determined. The cellular ATP content was found in significantly greater degree in both drug–resistant K562/*adr* and GLC4/*adr* cell than in their corresponding drug–sensitive cells. It was decreased by about 25% for the four cell lines, when cells were incubated with 5 μM oligomycin, a specific inhibitor of oxidative phosphorylation (Penefsky, 1985) , for 30 minutes.

Figure 6 shows typical results of evolution of the cellular ATP contents as a function of time, when both MDR cells were incubated with 1 μM MTT. The cellular ATP contents increased to reach a maximal value about 150% for K562/*adr* and 175% for GLC4/*adr* and then returned to the initial value at about 30 minutes for K562/*adr* cells and 60 minutes for GLC4/*adr* cells. Similar results were obtained when the cells were incubated with 100 μM MTT (data not shown).

The cellular response pattern under the supplemented intracellular ATP concentration occurring during MTT reduction was demonstrated in Figure 7. It should be noted that an increase of intracellular ATP contents does not affect the toxicity induced by pirarubicin in both drug–sensitive cell lines, but clearly does in MDR cell lines.

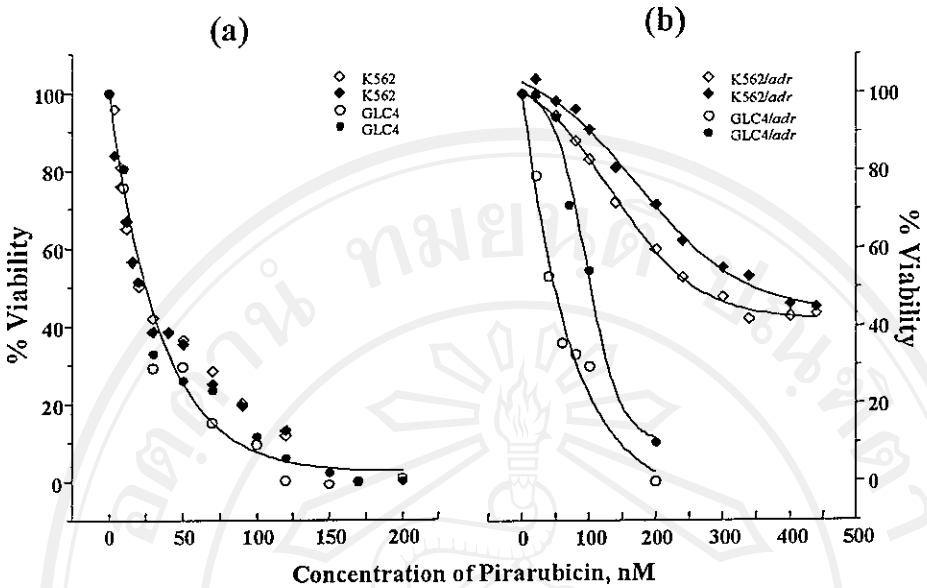

Figure 7. Comparative cytotoxicity of pirarubicin (a) to drug–sensitive cells; (◊, ◆) K562 cell and (O, ●) GLC4 cell and (b) to drug–resistant cells; (◊, ◆) K562/*adr* cell and (O, ●) GLC4/*adr* cell. Percent Viability of cell resulting from 72 hours exposure to pirarubicin was evaluated by comparing the cell numbers to that of untreated cells. Closed symbols refer to experiments performed in the presence of 1 μM MTT. Other conditions were as described under materials and methods.

In both MDR cell lines, the concentration of pirarubicin required to decrease 50% viability (IC_{50}), in the series of co–treatment using pirarubicin and 1 μM MTT, is two times higher than that of the series of treatment with pirarubicin alone. This indicates that an increase of intracellular ATP concentration assures the P–glycoprotein and MRP1 protein function, leading to long–term cell protection from pirarubicin–induced toxicity.

4. CONCLUSION

We have established a noninvasive fluorescence technique for determining the $\Delta\Psi_m$ of intact drug–sensitive and drug–resistant cells by using rhodamine B as a mitochondrial probe. Rhodamine B is a lipophilic cation whose cellular accumulation depends on the $\Delta\Psi_m$. We propose that it is a suitable probe for monitoring the function of mitochondria. The C_m° was precisely determined by using formazan, a rhodamine B fluorescence quencher, which locally accumulates in the matrix of mitochondria. The kinetics of decrease in rhodamine B fluorescence (V_i) can be used to estimate the $\Delta\Psi_m$ applying the Nernst equation:

$$\Delta\Psi_m = -61.54 \log V_i - 258.46.$$

The $\Delta\Psi_m$ values determined were indicated in Table 1. The $\Delta\Psi_m$ is a sensitive indicator for the energetic state of mitochondria and cell. (Sharkey, 1993; Lyon, 1998).

An increase or a decrease in $\Delta\Psi_m$ consequently followed an increase or a decrease in cellular ATP content. We have found that an increase in cellular ATP content in MDR cells via oxidative phosphorylation can protect cells from toxicity induced by pirarubicin. This probably assures the function of the P–glycoprotein and MRP1 protein. The origin of ATP synthesis, such as the mitochondria, can be used as a specific target for MDR modulator research.

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