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# *In vitro* evaluation of P-glycoprotein functions in human neuroblastoma cell lines

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**ABSTRACT**

**Background:** Neuroblastoma (NB) remains one of the most puzzling of paediatric cancers in which most patients develop progressive disease that is refractory to chemotherapy. Effective treatment is hampered by drug resistance related to the expression of multidrug-resistant proteins belonging to the ATP-binding cassette transporters family, especially P-glycoprotein (P-gp). Most previous studies focused on molecular evidence of P-gp expression, however, functional studies of P-gp efflux have not clearly demonstrated.

**Objectives:** The aim of this study was to determine whether human neuroblastoma cell lines (SH-SY5Y and SK-N-SH) express the functionally active P-gp efflux pump.

**Materials and methods:** Functional studies on P-gp–mediated pumping were performed using pirarubicin, a P-gp substrate, with verapamil, a multidrug resistance inhibitor, and analyzed by a spectrofluorometer. To confirm the gene expression, reverse transcription polymerase chain reaction (RT-PCR) was performed with specific primers for human multidrug resistance 1 (MDR1).

**Results:** MDR1 expression was observed in neuroblastoma cell line (SH-SY5Y) in the same degree of expression as in the sensitive K562 cell line, a negative P-gp model. Kinetic analysis showed that there was no difference in drug accumulation in the presence or absence of verapamil, indicating that no function of P-gp influenced the accumulation of pirarubicin (PIRA) in both human neuroblastoma cell lines. Combination treatment of verapamil and PIRA was also not found to increase the sensitivity of PIRA.

**Conclusion:** This study suggests that the existence of P-gp in neuroblastoma cell lines is not significant function.

## Introduction

Neuroblastoma is a type of cancer in the sympathetic nervous system of infants and is characterized by highly heterogeneous clinical behaviour, ranging from spontaneous regression to rapid progression and, finally, patient death.[1-3] Strategy for treatment of NB remains a challenge because disease relapse and progression despite receiving intensive multimodal therapy are common in patients with high risk tumors.[2] Most patients achieve some response to aggressive chemotherapy. However, those in the poor-prognosis group fail the treatment and have early relapse. Subsequently, the tumors become refractory to a variety of chemotherapeutic agents.

One of the most significant impediments that hamper treatment of the cancer is resistance to the standard types of chemotherapy. This is a phenomenon in which tumor cells that have been exposed to a cytotoxic agent develop cross resistance to a whole range of drugs with unrelated function and structure.[4] This phenomenon is known as multidrug resistance (MDR). The development

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of drug resistance involves alteration of the signaling pathway,[5,6] induction of anti-apoptotic proteins,[7,8] alteration of tumor microenvironment,[9,10] and induction of drug metabolizing systems.[11] Clearly, the clinical obstacle associated with drug resistance is responsible for the reduced effect of chemotherapeutic drugs, mainly due to uptake from the targeted cells via the ABC transporter cassette and P-gp, which is a subfamily B member.[11,12] P-gp is a member of the ATP-binding cassette superfamily of active transporters and functions as an energy-dependent uptake pump that reduces intracellular concentration of cytotoxic compounds and their toxicity.[13,14] P-gp has a broad substrate specificity and can confer resistance to a wide range of different cytotoxic compounds.[15]

Most pre-clinical and clinical aim to overcome MDR in NB which is modulated by P-gp activity, however, success has been limited as regards efficiency in the outcome. To date, the focus has been primarily on molecular evidence of P-gp expression. However, evidence for functional expression has not been clearly elucidated. Therefore, it may be more appropriate to clearly understand the functionality of P-gp. Numerous methods have been adopted in order to elucidate the P-gp function. Up until now, feasibility of a method that deals with living cells without disturbing their drug equilibrium has not been investigated. Many proposed methods have been used in studies, such as calcein assay,[16,17] rhodamine 123 efflux assay,[16,18,19] and trans-epithelial transporter assay.[20] It was found that one step of these methods is based on incubation of the cells with drugs, centrifugation and washing, suspension in a buffer, and detection of the concentration of the drug within the cell or in the supernatant. However, these steps could not reflect exactly what happens with living cells; also, these steps might render the equilibrium among the drug concentration outside and inside the cell disturbed.[21] For this reason, to analyze the function of the multidrug transporter that mimics living cells, a method that uses the change in the fluorescent characteristics of the transported compound when it moves between intracellular and extracellular medium was employed.[21,22] This method allows measuring accurately the overall concentration of anthracycline accumulated inside the cell and the concentration that is intercalated between the base pairs in the nucleus in the steady state.[23,24]

In the present work, P-gp function in neuroblastoma cell lines, SK-N-SH and SH-SY5Y, was evaluated by using the kinetic uptake of anthracycline monitored by a spectrofluorometer. Pirarubicin (PIRA; a fluorescent P-gp substrate) was selected to examine the function of P-gp *in vitro* because its pKa is relatively low and it can penetrate the cells very rapidly.[21] Fluorescence behaviour of PIRA was made use of and the accumulation of PIRA was directly monitored in the presence of verapamil. By this technique, the sensitive and the resistant K562 cells were selected as the model and defined as the negative and the positive control, respectively, for the P-gp function study. Data obtained showed that the presence of endogenously expressed P-glycoprotein in neuroblastoma cells is not functional.

## Materials and methods

### *Cell lines and cultures*

Experiments were performed with human neuroblastoma SH-SY5Y and SK-N-SH. SH-SY5Y cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). SK-N-SH cells were a gift from Assistant Professor Dr. Chainarong Tocharus (Department of Anatomy, Faculty of Medicine, Chiang Mai University, Thailand). SH-SY5Y cells were maintained in minimum essential medium (Eagle's)/Ham's F12 medium with L-glutamine (Caisson, USA) and SK-N-SH cells in DMEM high glucose medium (Sigma-Aldrich, USA), all supplemented with 10% fetal bovine serum (Gibco®, Invitrogen, USA) and 1% penicillin/streptomycin (Caisson, USA), and kept in a humidified atmosphere of 5% $CO_2$ at 37 °C. For the experiment, both cell lines were initiated at a density of 20% confluence (T75 flask) in culture medium for 3 days to reach about 80-90% confluence. The K562 cell line is a human myelogenous leukemia derived from a 53-year-old female patient with chronic myeloid leukemia and the resistant phenotype line (K562/adr) were used as the control for studying the P-gp function.[25,26] These two lines were provided by Dr. Ruoping Tang (Hôpital de l'Hôtel Dieu, Pairs, France). Both cell lines were routinely cultured in RPMI-1640 medium (Caisson, USA) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin in a humidified atmosphere of 95%air and 5%$CO_2$. K562 and K562/adr cell initiated at a density of $10^5$ cells/mL grew exponentially to about $10^6$ cells/mL in 72 hrs. For the assays, the cells were initiated at $5\times10^5$ cells/mL to have the cells in the exponential growth phase and allowed to grow for 24 h until the experiments. Cell viability was assessed by trypan blue exclusion. The number of cells was determined by using a hemocytometer.

### *Drugs and chemicals*

Pirarubicin (4-Q-tetrahydropyrayldoxorubicin) was purchased from APExBIO Technology (Houston, USA). Stock solutions were prepared in DMSO just before use. Concentrations were determined by diluting stock solutions in distilled water to approximately 1 mM and using $\varepsilon_{480}$=11500 $M^{-1}cm^{-1}$. Verapamil hydrochloride (Sigma, Singapore) was dissolved in absolute ethanol. Deionized double distilled water was used throughout the experiments for solutions and buffers. Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt) was purchased from Sigma and dissolved in deionized double distilled water. The experiments were performed in Luckoff-$Na^+$ buffer solutions at pH 7.25 containing 20 mM HEPES, 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5 mM D-glucose.

### *Real-time PCR*

To evaluate the expression of MDR1 levels in the cell lines, total RNA was isolated from each of the cell lines using the PureLink® RNA Mini Kit (Thermo scientific, Invitrogen, USA) according to the manufacturer's instructions. UV absorbance of the spectrophotometrically isolated RNA was measured at 260 nm to determine its concentration and at 280 nm to check its purity from the 260/280 nm

absorbance ratio. cDNA was prepared from 5 µg of the extracted total RNA with MMLV reverse transcriptase using oligo(dT)18VN nucleotides and RiboSafe RNase inhibitor (Tetro cDNA Synthesis Kit, Bioline, London, UK). RT-PCR was performed using the primer sequences listed in Table 1. The amplification reaction components consisted of 5 µL of 10X PCR buffer, minus Mg (ThermoFisher Scientific, Invitrogen, USA), 1 µL of 10 mM dNTP mixture (ThermoFisher Scientific, Invitrogen, USA), 2.5 µL of specific-primer pair (10 µM), 0.1 µL of Taq DNA polymerase (ThermoFisher Scientific, Invitrogen, USA), and 1 µL of cDNA template (250 ng), and it was adjusted to a final volume of 50 µL with RNase-free water. The amplification reaction was performed under the following thermal cycling condition: 94 °C for 3 min, 36 cycles of 94 °C for 45 sec, 50 °C for 30 sec, 72 °C for 45 sec, and the final extension step at 72 °C for 10 min in a thermal cycler machine (TProfessional, Biometra, Göttingen, Germany). The PCR products were subjected to electrophoresis on 3% agarose gels and imaged using a ChemiDoc™Touch imaging system (Bio-Rad, Hercules, CA, USA); the band intensities were determined using the relative mRNA expression levels of the genes.

**Table 1** Primer Sequence in RT-PCR

| Gene | Primer sequence | Amplicon (bp) |
|---|---|---|
| MDR1 | 5'-GAA ACC AAC TGT CAG TGT-3' (forward)<br>5'-AGC ATC ATG AGA GGA AGT-3' (reverse) | 120 |
| β-actin | 5'-AAG GCC AAC CGC GAG AAG ATG A-3' (forward)<br>5'-TGG ATA GCA ACG TAC ATG GCT G-3' (reverse) | 74 |

***Cytotoxicity assay***

Cells were seeded in 96-well plates ($5\times10^3$ cells for K562 and K562/adr, $5\times10^4$ cells for SH-SY5Y and SK-N-SH,100 µL/well) and incubated with increasing concentrations of PIRA (0–5 µM) in the presence and absence of verapamil (at final concentrations of 0.25 µM, 2.5 µM, and 5.0 µM) for 3 days. After incubation, 20 µL of resazurin stock solution (0.02 mg/mL) was added to each well. The plates were further incubated for 4 h in a 5% $CO_2$ incubator. Cell viability was assessed by the ability of the remaining viable cells to reduce resazurin to resorufin. The fluorescence intensity of resorufin (570 nm excitation/590 nm emission) was measured with a spectrofluoroscopic microplate reader (PerkinElmer LS55 spectrofluorometer). The 50% inhibitory concentration ($IC_{50}$), defined as the drug concentration causing 50% reduction in cell viability, was determined by plotting the concentration of the drug in the x-axis and the percentage of the cell inhibition in the y-axis. The experiments were run in triplicate.

***Determination of P-gp–mediated uptake of pirarubicin in presence or absence of verapamil***

Kinetic uptake of PIRA by cells was followed by monitoring of the decrease in the fluorescence signal at 590 nm ($\lambda_{ex}$ = 480 nm) by following the method previously described.[23,24,27] Using this method, it is possible to accurately quantify the kinetic parameters of the drug uptake by the cells without compromising cell viability. All experiments were conducted in 1 cm quartz cuvettes containing 2 mL of buffer at 37 °C using a circulating thermostat water bath. Briefly, cells ($10^6$ cells/mL, 2 mL per cuvette) were incubated with or without various concentrations of verapamil (0–10 µM) for 10 min in glucose containing Luckoff-$Na^+$ at pH 7.25, under incessant stirring. After the addition of 20 µL of $10^{-4}$ M pirarubicin, yielding a concentration $C_T$ equal to 1 µM, the decrease in the fluorescence intensity $F_0$ at 590 nm was monitored as a function of time. In the steady state, the curve F=f(t) reached a plateau and the fluorescence intensity was equal to $F_n$. The drug-cells system was thus in a steady state and the overall concentration $C_n$ of the drug intercalated between the base pairs in the nucleus was $C_n=C_T.(F_0-F_n)/F_0$. Once the steady state was reached, the cell membranes were permeabilized by the addition of 0.02% Triton X-100, yielding the equilibrium state which was characterized by a new value $F_N$ of the fluorescence intensity. The overall concentration $C_N$ of the drug intercalated between the base pairs in the nucleus was then $C_n=C_T.(F_0-F_N)/F_0$. P-gp function was assessed by the ratio value of $C_n/C_N$ as a function of the verapamil concentration. In the presence of verapamil, the uptake of PIRA was found to increase, resulting in an increase in the overall concentration $C_n$. In case of complete inhibition of the P-gp function or no effect on the P-gp function, the overall concentration of the drug intercalated between the base pairs in the nucleus in the steady state is expected to be the same as that in the equilibrium state: thus, the ratio of $C_n/C_N$ equals to 1.

***Statistical analysis***

All data are expressed as mean±standard deviation (SD). Statistical significance was determined using Student's t-test between the groups treated and the control. A probability (*p*) value less than 0.05 was considered statistically significant.

## Results

***Expression of MDR1/P-gp mRNA in cell lines***

The objective was to investigate whether P-gp, encoded by the *mdr1* gene, is expressed in neuroblastoma cell lines. The determination of MDR1 mRNA was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). The PCR products of P-gp, 120 bp, and β-actin, 74 bp, are shown in Figure 2a. Expression of the MDR1 mRNA was determined relative to the β-actin used as the internal control. Data on the expression of MDR1 mRNA relative to β-actin are shown in Figure 2b. The MDR1 mRNA expression was identified in K562 cells as equal to 0.45±0.19. In contrast, their resistance cell line, K562/adr, showed higher expression with value equal to 0.74±0.17.

The expression of MDR1 mRNA in the neuroblastoma cells demonstrated the low-level expression in the SH-SY5Y cells, with value equal to 0.33±0.17, while no expression was observed in the SK-N-SH cells. Unexpectedly, there was found the presence of non-targeted PCR in the SK-N-SH cells in the PCR product range of 200-300 base pairs.

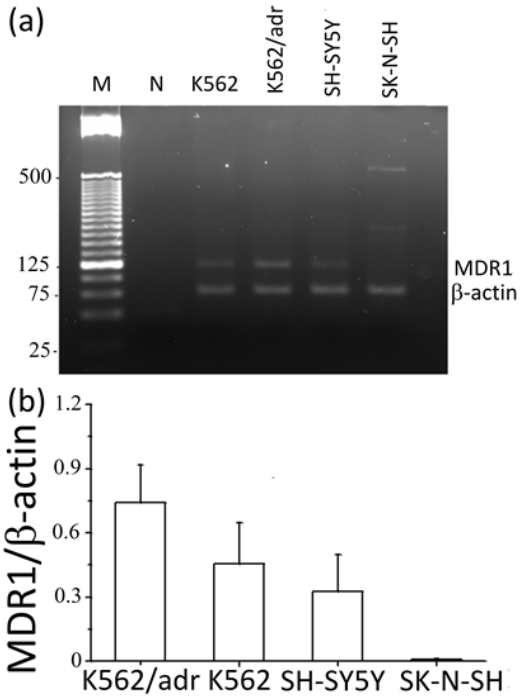


***Figure 3*** *RT-PCR analysis of MDR1 expression in K562, K562/adr, SH-SY5Y, and SK-N-SH cells. Total RNA from each cell line was subjected to RT-PCR analysis with primers specific for human MDR1. RT-PCR products were run on a 3% agarose gel to show the expression of the MDR1 genes. (a) The expected 120-bp product for MDR1 was present in the cell lines. The housekeeping gene human β-actin, 74-bp, was used as the internal control. (b) Relative mRNA expression of MDR1/P-gp normalized to human β-actin. Each value represents mean ± SD (n = 3).*

### *Uptake of pirarubicin by cells*

Typical pattern of PIRA uptake by a P-gp overexpression model, K562/adr, is shown in Figure 1. After the addition of PIRA, the decrease in the fluorescence intensity at 590 nm was monitored as a function of time of incubation. Quenching of fluorescence intensity refers to the accumulation of PIRA within the nuclei of the cells. Once the steady state was reached, 0.02% Triton X-100 (v/v) was added, and the fluorescence intensity decreased to $F_N$. The overall nuclear concentration in the steady state ($C_n$) and the overall nuclear concentration in the equilibrium state ($C_N$) were calculated. As shown in Figure 1, $C_n$ was lower than $C_N$, rendering the ratio of $C_n/C_N$ less than 1. This observation provided evidence of a gradient in the concentration of PIRA, with a low free intracellular concentration against a high extracellular concentration. The gradient was generated by the plasma membrane protein transporter, P-gp. Therefore, the accumulation of PIRA in K562/adr was very low as a result of the P-gp function. In the negative control for the P-gp function, the sensitive K562 cell, the same experiment was performed (Figure 3a). It was observed that upon adding PIRA, the decrease in the fluorescence intensity at 590 nm was higher than that of the K562/adr cells. The fluorescence intensity did not change after the addition of 0.02% (v/v) Triton X-100; $C_n$ was similar to $C_N$, rendering the ratio of $C_n/C_N$ close to 1. These events indicated that no gradient in the concentration of PIRA was observed in the sensitive K562 cells. As for the drug uptake in both the neuroblastoma cell lines, SH-SY5Y and SK-N-SH, the behaviour of kinetic drug uptake was similar to that observed in the sensitive K562 cells. The ratios of $C_n/C_N$ in all the cell lines tested are shown in Figure 3b.

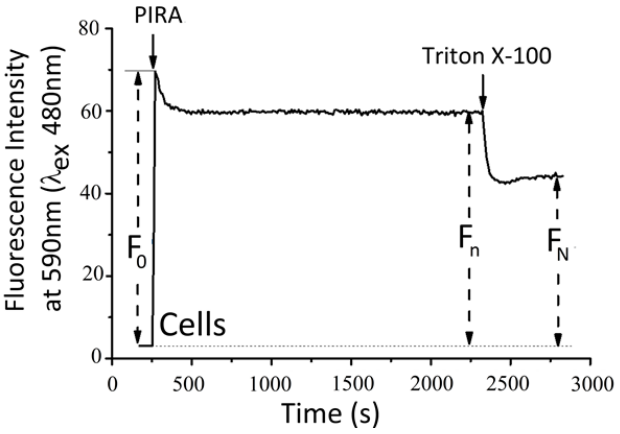


***Figure 1*** *Uptake of pirarubicin by K562/adr, the P-gp overexpression model, with fluorescence intensity at 590 nm ($\lambda_{ex}$ =480 nm) was recorded as a function of time until the steady state. Cells, 2×10$^6$ cells, were suspended in a cuvette filled with 2 mL buffer of pH 7.25. At t=0, 20 µL of 100 µM PIRA was added to the cells, which yielded a $C_T$=1 µM PIRA solution. The fluorescence intensity was then $F_0$. Once the steady state was reached, the fluorescence was $F_n$, and the concentration of the drug intercalated between the base pairs in the nucleus was $C_n=C_T$. $(F_0 - F_n)/F_0$. The addition of 0.02% Triton X-100 yielded the equilibrium state. The overall concentration $C_N$ of the drug intercalated between the base pairs in the nucleus was then $C_N=C_T$. $(F_0 - F_N)/F_0$.*

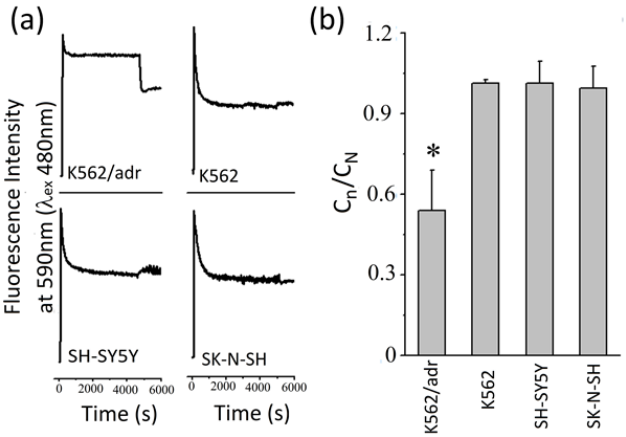


***Figure 3*** *The representative data of the uptake of pirarubicin (PIRA) by K562/adr, K562, SH-SY5Y, and SK-N-SH (a). The cells were analyzed in the experiment, as previously described. The ratio of $C_n/C_N$ was determined to investigate the effect of the P-gp function on drug accumulation (b). The data are presented as mean±SD (n=3). *P<0.05 versus K562, SH-SY5Y, and SK-N-SH.*

***Kinetic P-gp–mediated uptake of pirarubicin in the presence of verapamil***

Verapamil, a calcium channel blocker, is a known P-gp inhibitor.[28,29] It was found that verapamil has the capability to reverse multidrug resistance.[30,31] Therefore, to evaluate the function of P-gp, the uptake of PIRA by cells was determined in the presence of verapamil. The typical experiment in the K562/adr cells, the positive control of the P-gp function, is shown in Figure 4a. In the experiment, when the cells were incubated in the presence of verapamil, the fluorescence signal decreased as a function of time of incubation and then plateaued after reaching the steady state. The addition of Triton X-100 did not yield any modification in the fluorescence signal, indicating that the P-gp function was completely blocked and there was no effect of the P-gp–mediated PIRA pump outside the cell. However, when the cells were incubated without verapamil, the accumulation of PIRA in the nucleus in the steady state was very low. The addition of Triton X-100 yielded a decrease in the fluorescence signal, indicating a gradient in the drug concentration which was generated by the presence of the pump. From the experiment, the overall concentration of the drug bound to the nucleus in the steady state, $C_n$, and the overall concentration of the drug bound to the nucleus in the equilibrium state, $C_N$, were determined. The ability of verapamil to inhibit P-gp-mediated uptake of PIRA, with the ratio $C_n/C_N$ as the function of the verapamil concentration added, is demonstrated in Figure 4b. In K562/adr cells, the ratio of $C_n/C_N$ increased continuously as the concentration of verapamil increased. When the same experiment was performed in K562, SH-SY5Y, and SK-N-SH cells, no modification in the fluorescence signal was observed either in the condition of the presence of verapamil or in the condition of the absence of verapamil. Thus, the concentration of the drug bound to the nucleus in the steady state, $C_n$, was not significantly altered by verapamil, rendering the ratio of $C_n/C_N$ close to 1.0 (Figure 4b), which indicates that no function of P-gp was observed on the accumulation of PIRA in these cell lines.

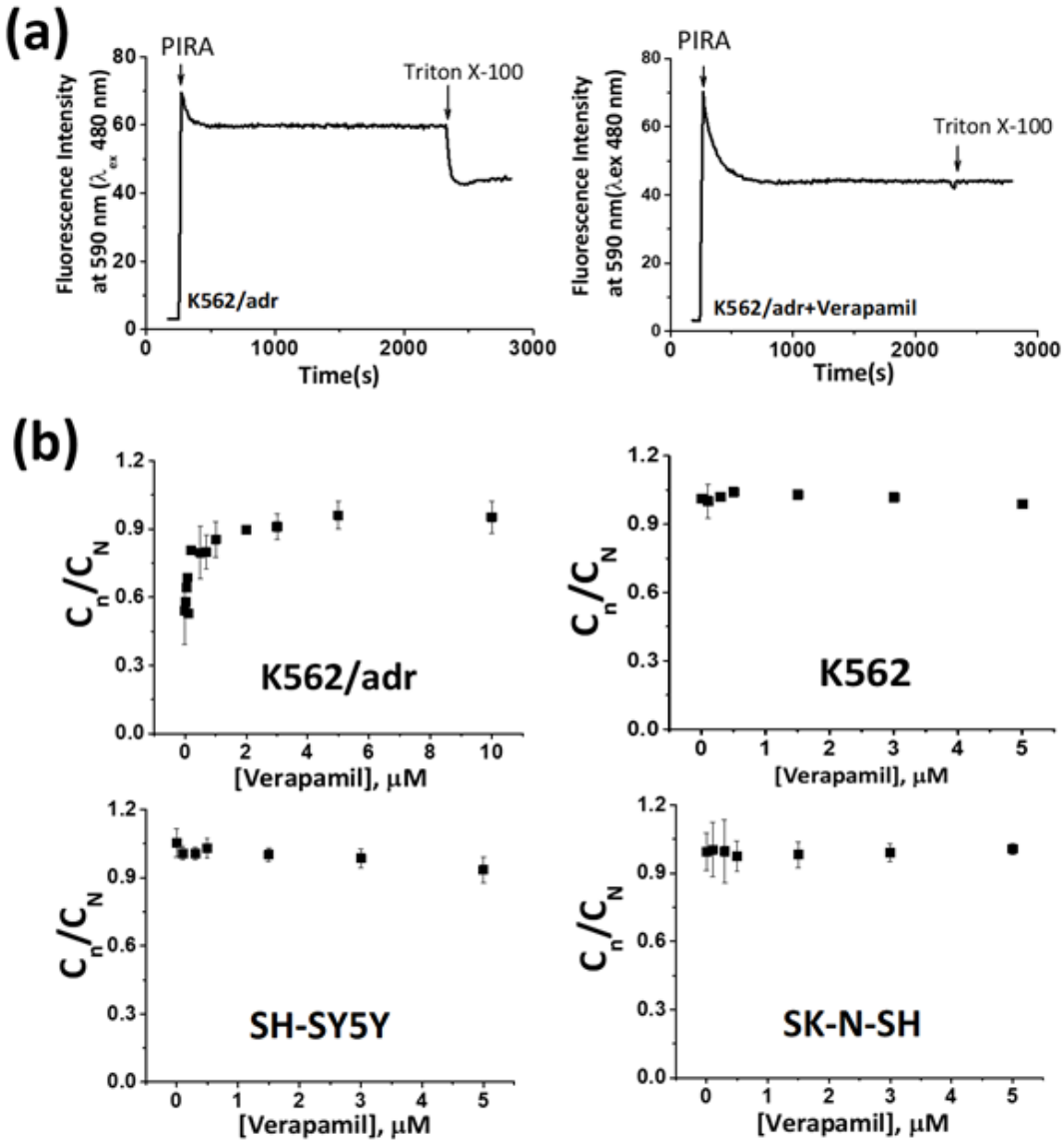


***Figure 4*** *The effect of verapamil on the drug accumulation in K562/adr, K562, SH-SY5Y, and SK-N-SH. In the model of the P-gp overexpressing cell line, K562/adr, the typical characteristics of P-gp–mediated uptake of PIRA were studied in controlled conditions and exposed to verapamil (a). The values of $C_n/C_N$ are plotted as a function of the concentration of verapamil (b). Each value represents mean±SD (n=3).*

***Effect of verapamil on cellular sensitivity to pirarubicin***

To confirm the function of P-gp in long-term treatments, cotreatment assay of PIRA with various concentrations of verapamil was performed. The results of the cotreatment assays were expressed as $IC_{50}$ values. As shown in Figure 5, addition of verapamil significantly increased the sensitivity of PIRA to K562/adr cells in a dose-dependent manner; however, no effect of verapamil was observed in K562, SH-SY5Y, or SK-N-SH. Interestingly, it was observed that 5 μM verapamil significantly decreased the $IC_{50}$ value when compared with untreated control in SK-N-SH.

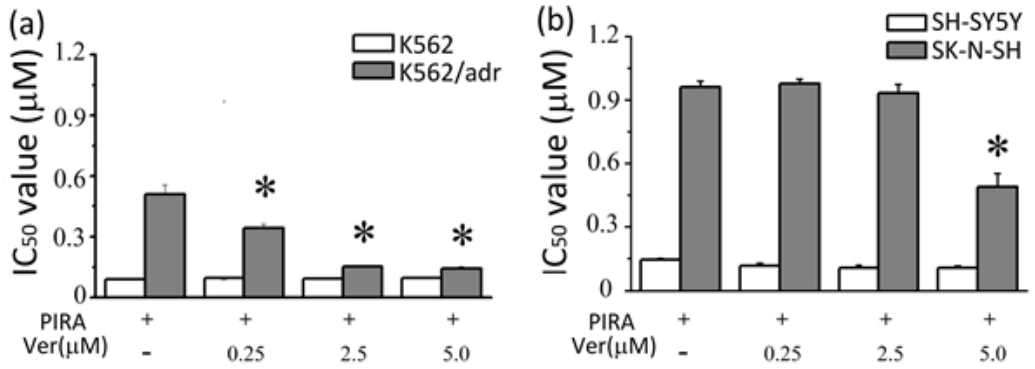


***Figure 5*** *The $IC_{50}$ value after cotreatment of PIRA with verapamil in K562 and K562/adr cells (a), and SH-SY5Y and SK-N-SH (b). The cells were treated with different concentrations of PIRA in the presence of various concentrations of verapamil (0–5.0 μM) for 72 h and analyzed using the AlamarBlue® assay. The data are presented as mean±SD (n=3). *p<0.05 versus the control of each cell line.*

## Discussion

Multidrug resistance phenomenon is a major hindrance to successful treatment of neuroblastoma patients. In tumor cells, the drug penetration across cell membranes can be hindered by increased presence of the ATP-binding cassette (ABC) which contains membrane proteins that translocate a wide variety of substrates across extra- and intracellular membranes.[32] As a result, intracellular drug concentration within the targets is reduced to a level below its therapeutic threshold. It should be noted that certain ABC transporters confer multidrug resistance to numerous drugs differing in chemical structure and mechanisms of action.[4] An important member of this family that has been extensively studied is P-gp.[33,34] Regarding the type of drug to be transported, conventional chemotherapeutics have been the obvious first consideration because those are the antitumor drugs in most widespread use. In this study, the sensitive and the resistant K562 cells were selected as the negative control and the positive control, respectively, for P-gp expression. The confirmation was by observing the increase in the $IC_{50}$ value of anthracycline, PIRA, in the drug-resistant K562/adr cells compared with their corresponding sensitive cells, as shown in Table 2. Thus, the *in vitro* setup that was used can be considered to be a suitable model to investigate the function of P-gp.

**Table 2** $IC_{50}$ Values and ratio of $C_n/C_N$ of Human Cancer Cell Lines after Exposure to pirarubicin (PIRA)

| Cell line | $IC_{50}$ (μM) | $C_n/C_N$ |
|---|---|---|
| Erythroleukemic cell line | | |
| K562/adr | 0.51±0.05 | 0.54±0.15 |
| K562 | 0.09±0.01 | 1.01±0.01 |
| Neuroblastoma cell line | | |
| SH-SY5Y | 0.15±0.01 | 1.01±0.08 |
| SK-N-SH | 0.96±0.03 | 1.00±0.08 |

***Note:*** *Each value is expressed as mean±SD (n=3). Means with different letters are significantly different at p<0.05.*

***Abbreviations:*** *$IC_{50}$, 50% inhibitory concentration; $C_n$, overall concentration of PIRA bound to the nucleus in the steady state; and $C_N$, overall concentration of the drug bound to the nucleus in the equilibrium state.*

It is known that acquisition of drug resistance and/or changes in cellular drug accumulation may be related to changes in the molecular expression of the drug transporter.[35] Initial experiments with mRNA from neuroblastoma cell lines demonstrated that MDR1 mRNA expression was common as demonstrated by reverse transcription PCR. In this study's experiments, included as controls for MDR1 mRNA expression were the drug-sensitive parental K562 cell line and the drug-resistant sublines, K562/adr, which are 6-fold resistant to PIRA. Detectable levels of MDR1 mRNA were seen in SH-SY5Y, and not in SK-N-SH. The level of MDR1 mRNA expression in SH-SY5Y was close to the same level of expression in the sensitive K562 cells, indicating the presence of endogenously expressed P-glycoprotein. It was found that SH-SY5Y expressed both *mdr1* gene and *mrp1* gene.[36-38] A study by Bates et al. found that expression of *mdr1* gene in human neuroblastoma is related to the degree of differentiation modulated by retinoic acid.[36] SH-SY5Y cells are derived from a subclone of the parental neuroblastoma cell line SK-N-SH and can be differentiated into mature human neurons through a variety of different mechanisms including the use of retinoic acid.[39] Interestingly, apart from MDR1 mRNA expression, one study characterized the ABC transporter protein in SH-SY5Y and found that MRP1 mRNA in human SH-SY5Y cells had the highest expression levels of ABC transporters, while MDR1 mRNA was expressed at levels approximately 30% of those for MRP1 mRNA.[38] Michelle et al. showed that high levels of MRP1 mRNA expression are related to poor clinical outcome in a large prospective study of primary neuroblastoma;

MDR1 mRNA expression, nevertheless, was demonstrated to have no prognostic significance.[37] However, no evidence has been found for the expression of human BCRP mRNA in SH-SY5Y.[38] It seems that MRP1 protein plays a more prominent role in drug resistance than P-gp. However, no evidence has been found proving the prominent role of MDR protein in neuroblastoma cells. Further studies should be carried out to clearly demonstrate this phenomenon.

As is known, mRNA levels cannot be used as surrogates for corresponding protein levels without verifying their function. All mRNAs are not equal with regard to function of proteins. Based on this notion, it was investigated whether the P-gp function takes place in the neuroblastoma cells where they are expressed. In this study, drug kinetic uptake using the spectrofluorometric method has been used to get a better understanding of the function of multidrug transporters that mimic living cells. This principle is used to investigate whether human neuroblastoma cells express the functionally active P-gp efflux pump. To demonstrate the functionality of P-gp, the impact of inhibiting P-gp–mediated PIRA efflux was investigated in the presence of verapamil. Verapamil is a specific first-generation MDR1 inhibitor. Its efficiency has been confirmed in certain types of cancer, both in preclinical studies and in clinical use.[28] In contrast to the findings of mRNA expression, kinetic data demonstrated that no gradient, generated by P-gp, of PIRA concentration was present in both the neuroblastoma cells. Thus, it can be suggested that accumulation of PIRA is not associated with the function of P-gp. Not only the inhibitory effect of verapamil on P-gp–mediated PIRA transport but also the effect of reversal of P-gp activity when combined with conventional chemotherapy, pirarubicin, for long-term treatment, was observed. Importantly, the apparent $IC_{50}$ values for various concentrations of verapamil were not significantly different between SH-SY5Y and SK-N-SH, except at 5 µm verapamil, which is similar to what was observed in the sensitive K562 cells. At the same time, in the positive control of the P-gp model, verapamil enhanced the chemosensitivity of PIRA in the resistant K562 cell line in which the expression of MDR1 was highly detectable. In SK-N-SH, it was found that 5 µM verapamil enhanced the efficacy of PIRA which was not consistent with the kinetic study. However, it was found that the percentage of viable cells of SK-N-SH was reduced to 5% after treated with 5 µM verapamil (data not shown). Therefore, it was possible that this discrepancy results affect from the toxicity of verapamil on SK-N-SH. From both the kinetic and the cotreatment data, it can safely conclude that endogenous expression of P-gp in neuroblastoma cells is not functional and is associated with increased cytotoxic drug accumulation. The reason for this discrepancy was unclear. However, possible explanations for this effect have been proposed. It might be possible that the MDR phenotype is not fully developed at these lower levels of P-gp expression, as can be observed in the sensitive K562 cell. Other *in vitro* studies have also supported this notion that MDR proteins effect drug resistance more actively in higher levels of resistance.[40-42] Notion that different isoforms of P-gp may possess different specificities was proposed. This hypothesis is supported by Sieczkowski et al. who found that core-glycosylated P-gp, in contrast to the fully glycosylated 180-kDa species of P-gp, is inactive and accumulates in the endoplasmic reticulum.[43] Bates et al. showed that drug accumulation failed to show decrease in 3H-vinblastine accumulation in the SK-N-SH and the SH-SY5Y cell lines after treatment with retinoic acid over a time period in which a clear increase in P-gp was observed.[36] It can be inferred that increased levels of MDR1 mRNA expression are not necessarily associated with increased cytotoxic drug accumulation. This is in accordance with the finding of this study that verapamil showed no effect on the toxicity of PIRA in SH-SY5Y, where there is endogenous expression of P-gp.

## Conclusion

This study has demonstrated no significant P-gp function. This result correlates with the mild expression of MDR1 mRNA in both human neuroblastoma cell lines.

## Conflict of interest

The authors declare that they have no conflict of interest.

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