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Kinetics of cellular permeability of phenoxazine and its dependence on P-glycoprotein expression

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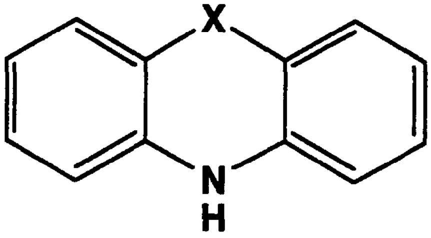
We present here the initial characterization of the mechanism of reversal of cellular resistance to *Vinca* alkaloids by phenoxazine (PZ). Changes in fluorescence upon cellular accumulation of PZ allowed measurement of the membrane transport kinetics in a sensitive KB-3-1 cell line and two multi-drug resistant (MDR) counterparts. The accumulation of PZ is characterized by two uptake routes, with pseudo-first order rate constants of 0.3 s^{-1} and 0.07 s^{-1}, while efflux of PZ from cells revealed rate constants of 0.2 s^{-1}. PZ rapidly reaches steady-state concentrations within cells, which may make it more clinically useful than modulators that accumulate more slowly (e.g. verapamil).

Multi drug; Resistance; *Vinca*; Modulator; Phenoxazine

1. INTRODUCTION

Cell lines expressing resistance to treatment via one agent often develop a resistance to many unrelated drugs [1]. This multi-drug resistance (MDR), has been connected to the over-expression of a 170kDa glycoprotein (pgp) typically located on the plasma membrane of the resistant cells. Although the function of this protein is not fully understood, it is associated with decreased accumulation of cytotoxic drugs within the cell, and has been postulated to act as an energy dependent multi-drug transporter [2].

Reversal of MDR has become a primary goal in the treatment of cancer. The most promising path toward this reversal seems to be co-treatment of cells with additional agents that prevent efflux of drugs from resistant cells. These agents, referred to as modulators, range from calcium channel antagonists to drug analogs [3,4]. PZ exhibits effective modulation (9- to 12-fold) of vincristine accumulation of KHCh^R-8-5 (KB85) and GC3/C1 (GC3) cell lines, while phenothiazine is almost completely ineffective [5].



X = O Phenoxazine
X = S Phenothiazine

In this report, we have begun a series of experiments designed to probe the mechanism of modulation of MDR by PZ and related derivatives. The fluorescence of PZ is environmentally sensitive, providing a wealth of information about the physical status and location of the drug. Accumulation of PZ into KB cells is accompanied by a significant fluorescence increase due to entry of the drug into hydrophobic regions in the cells (see below), making measurement of transport processes straightforward.

We have determined rate constants for the membrane flux of PZ in sensitive and MDR cells. The results of these experiments have provided key information in the elucidation of the mechanism of MDR reversal by PZ and the role of p-glycoprotein and cell morphology in this process.

2. MATERIALS AND METHODS

2.1. *Cell lines, cell culture and general chemicals*

Human epidermoid carcinoma KB-3-1 cells, a colchicine selected MDR variant KB-Ch^R-8-5, and a vinblastine selected MDR variant

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Abbreviations: PZ, phenoxazine; MDR, multi-drug resistance; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline (150 mM NaCl, 20 mM sodium phosphate, pH 7.4).

KB-V-1 (denoted KB31, KB85 and KBV1, respectively) were obtained from Dr. M. Gottesman [9] and maintained as previously described [5,9]. PZ was purchased from Aldrich Chemical Co. (Milwaukee, WI). Phenothiazine and non-enzymatic cell dissociation solution were purchased from Sigma.

2.2. *Drug uptake and efflux studies*

For uptake studies, cells were grown from 48 to 72 h and harvested by washing with PBS for 30 s, followed by a 4 min incubation with non-enzymatic cell dissociation solution. The dissociation solution was aspirated off and the cells were loosened from the flask by spanking. These were resuspended in 10 ml of PBS and centrifuged at 400 × *g* for 5 min. The resulting pellet was suspended in varying amounts of PBS to achieve the desired cell concentration. Cell concentrations were determined by nuclei counting using a Coulter Counter.

To measure uptake, a solution of 50 μM PZ or phenothiazine in PBS was mixed with the cell suspension in a hand-initiated stopped-flow apparatus (Hi-Tech Scientific) mounted in the cuvette holder of a Perkin-Elmer LS-5 Fluorescence Spectrometer. Fluorescence intensity was recorded with respect to time using an Apple IIGS microcomputer equipped with a data acquisition card connected to the chart recorder output of the fluorimeter. The excitation wavelength for both PZ and phenothiazine was 330 nm while the emission wavelengths were 397 nm and 445 nm for PZ and phenothiazine, respectively. Data were collected in 0.5 s intervals for 2 min. To correct for the contribution of cell settling to the fluorescence signal, the scattering peak occurring at 660 nm was monitored under identical conditions. This baseline was subtracted from the signal arising at the emission maximum of the drug.

For efflux studies, an aliquot from a concentrated solution of PZ or phenothiazine in dimethyl sulfoxide was added to the growth media in a 175 cm^2 T-flak to a final concentration of 64 μM. The cells were allowed to incubate at room temperature and atmosphere for 20 min, and were subsequently washed with PBS and then with dissociation solution, both of which contained 64 μM drug. The cells were suspended in PBS containing 64 μM PZ, centrifuged at 400 × *g* for 5 min, and then resuspended in PBS without PZ. The suspension was mixed with PBS using the stopped-flow apparatus and efflux was measured as the decrease in fluorescence with time using the same instrumentation as for the uptake studies.

For pseudo-first order kinetics, the relationship between fluorescence signal and integrated rate equation is:

$$F = C_1[1 - \exp(-\tau_1 t)] + C_2[1 - \exp(-\tau_2 t)] + C_3 \qquad \text{(Eq. 1)}$$

for uptake of PZ, and

$$F = C_1\exp(-\tau_{-1} t) + C_2 \qquad \text{(Eq. 2)}$$

for efflux, C_n are constants, τ_n are effective rate constants, and t is time. These equations describe a model for accumulation in which external drug is accumulated into cells by two routes:

$$A \xrightarrow{\tau 1} B \text{ and } A \xrightarrow{\tau 2} B$$

or effluxed as:

$$B \xrightarrow{\tau 1} A$$

The data wer fit to equations 1 and 2 using the non-linear least-squares curve-fitting routines of the commercial data analysis software Kaleidagraph (Synergy Software, Reading, PA).

2.4. *Fluorscence microscopy*

Plastic chamber well slides (Nunc, Inc., Naperville, IL) were plated with aliquots of the three KB cell lines, which were allowed to attach overnight in growth media. The cells were then incubated for 20 min with 64 μM PZ or phenothiazine, washed once with PBS (30 s), and fitted with a cover slip. The slides were examined using a Zeiss microscope with an epi-fluorescence condenser and 63 × oil immersion objectives. Excitation was performed with a broadband 450 nm filter, and emission was that > 520 nm. This produced faint but detectible fluorescence (Fig. 2). The images were photographed with a 35 mm camera mounted on the microscope.

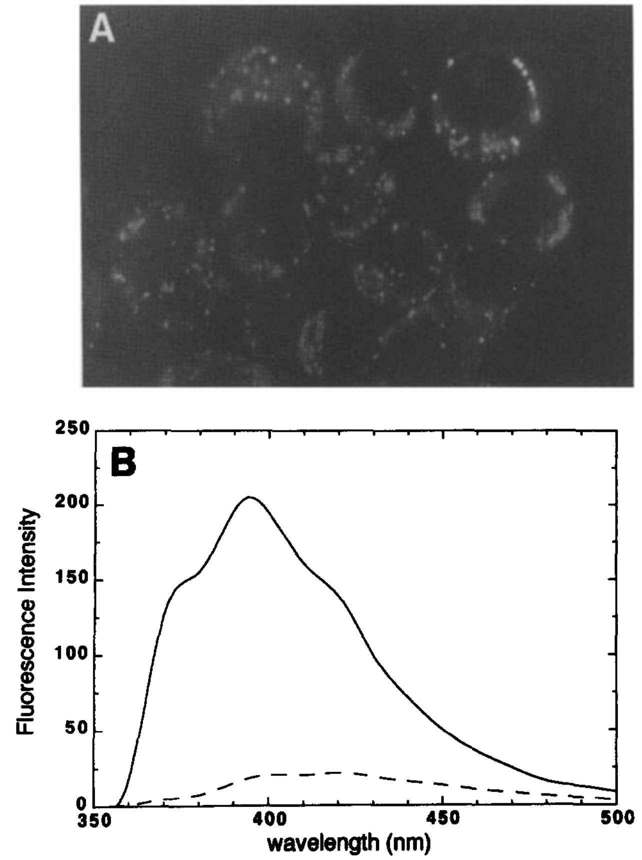

Fig. 1. (A) KB31 cells following incubation for 20 min with 64 μM phenoxazine. PZ is rapidly accumulated into lipid droplets distributed throughout the cytoplasm of the KB cell line. Similar results were observed with both the KB85 and KBV1 cell lines. These droplets also accumulate a wide variety of cytostatic agents such as doxorubicin (not shown), and may act to reduce the total intracellular drug concentrations available for biological activity. (B) Enhancement of phenoxazine fluorescence by organic solvents. The fluorescence of 60 μM PZ in PBX (broken line) and in 1-butanol (solid line). Phenothiazine showed a similar, but less pronounced, enhancement.

3. RESULTS

3.1. *Cellular uptake of phenoxazine and phenothiazine*

The three KB cell lines contains a heavy concentration of hydrophobic spheroids distributed throughout the cytoplasm. Upon entry into the cells, PZ is rapidly accumulated in these regions (Fig. 1A). The environmental change results in a pronounced enhancement in the fluorescence quantum yield for both PZ and phenothiazine similar to that which occurs when the drugs are dissolved in organic solvents (Fig. 1B). This enhancement provides an excellent marker for entry of the

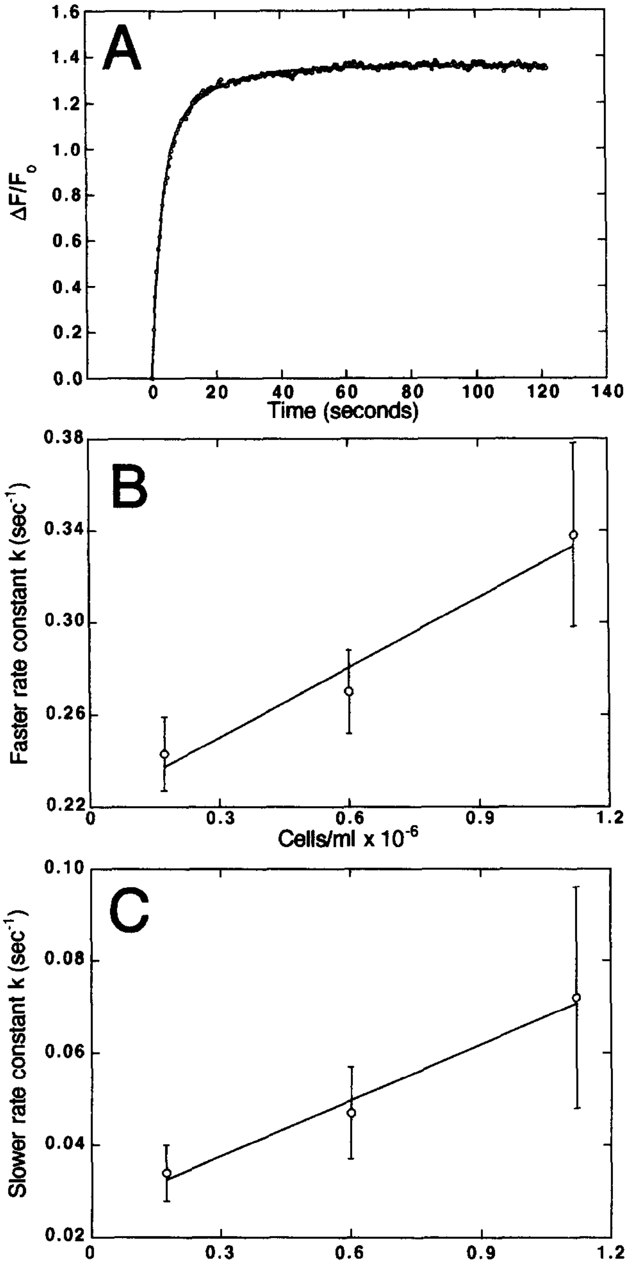

Fig. 2. Uptake of phenoxazine into KBV1 cells as monitored by fluorscence change at 397 nm (excitation at 330 nm). (A) A typical data set is shown (open circles) for the mixing of 50 μM PZ with 1×10^6 cells/ml in a stopped-flow apparatus. Similar data were obtained with KB31 and KB85 cell lines. The solid line represents the curve fit to a double exponential process as described by Equation 1 (see section 2). (B,C) The resulting rate constants τ_1 (fast) and τ_2 (slow) were linearly dependent on the total cell concentration for the three KB cell lines.

drugs into cells, and allows the use of fluorescence to monitor drug uptake.

Fig. 2A reproduces a typical data set for uptake of PZ into KBV1 cells. Similar data sets were also obtained for the KB31 and KB85 lines. The data could not be accurately fit to a single exponential process, and were therefore fit to the double exponential process described by Equation 1. The amplitude of the slower component was typically 30–40% of that of the faster. The resulting pseudo-first order rate constants were linearly dependent on the concentration of cells (Fig. 2B,C), as were the amplitudes of the signal. Data were collected for each cell line at a variety of cell concentrations, and the values of the rate constant at 1×10^6 cells/ml are reported in Table I. As can be seen, within experimental error, the rates of entry into the cells is independent of the drug sensitive or residant nature of the line.

The uptake of phenothiazine is very similar to that for PZ, and could generally be fit to a single exponential process. However, phenothiazine is much less environmentally sensitive and does not produce the fluorescence enhancement to the extent of that observed with PZ. Although no second component could be detected in the uptake, the decreased signal to noise obtained with phenothiazine makes resolution of a slower component difficult, and one may exist albeit with a magnitude much less than that for the fast component recorded in Table I. Interestingly, with all three cell lines the uptake of phenothiazine was significantly slower (ca. one half) than for PZ, suggesting different transport barriers for the two compounds.

3.2. *Cellular efflux of phenoxazine*

Cells loaded with PZ exhibited efflux that could be described by a single exponential process (Fig. 3A), and the rate constants were nearly independent upon cell count (Fig. 3B). Efflux of phenothiazine from the cells could not be detected, presumably due to the low signal to noise associated with this drug.

The determination of both uptake and efflux rates allows an estimate of the equilibrium partition coefficient of PZ with cells (at 10^6 cells/ml). This partition coefficient (P = [in]/[out]) has a value of 1.5 to 2.0 (Table I), calculated from the relationship: $P = (\tau_1 + \tau_2)/\tau_{-1}$. Therefore, the internal and external

Table I

Rate constants for MDR modulator-cell interactions*

Modulator	Cell line	τ_1 (fast) (s^{-1})	τ_2 (slow) (s^{-1})	τ_{-1} (s^{-1})	P**
Phenoxazine	KB31	0.38 ± 0.02	0.09 ± 0.01	0.29 ± 0.06	1.6
	KB85	0.31 ± 0.03	0.02 ± 0.01	0.22 ± 0.02	1.5
	KBV1	0.34 ± 0.04	0.07 ± 0.02	0.21 ± 0.05	2.0
Phenothiazine	KB31	0.16 ± 0.05			
	KB85	0.14 ± 0.02			
	KBV1	0.16 ± 0.01			

*At 25°C using a cell concentration of 1×10^6 cells/ml.

**Defined as [phenoxazine concentration inside cell]/[phenoxazine concentration outside cell].

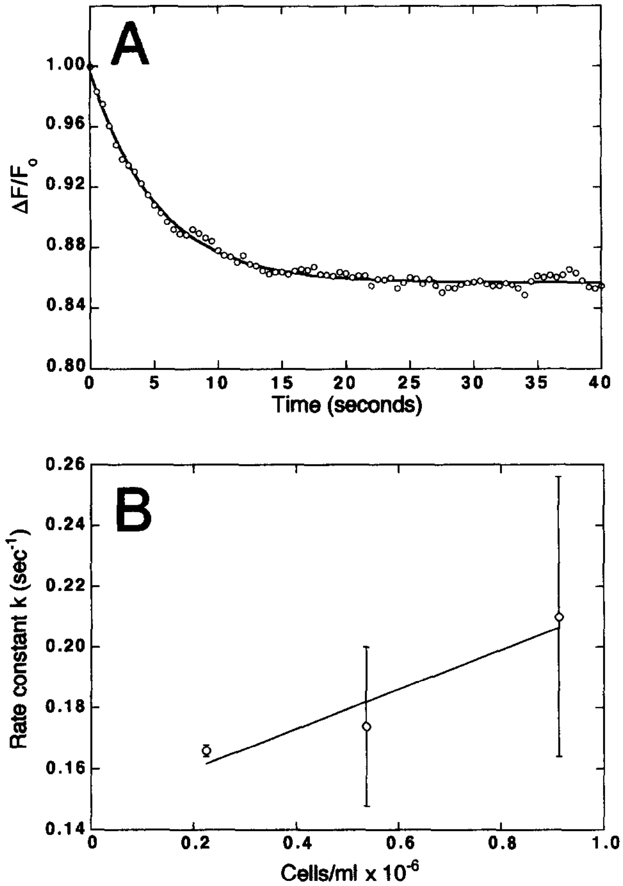

Fig. 3. Efflux of phenoxazine from KBV1 cells as monitored by fluorescence at 397 nm (330 nm excitation). (A) Data shown (open circles) is for the mixing of 1 × 10^6 cells/ml, which had been previously incubated with 64 μM PZ, with PBS in a stopped-flow apparatus. The data could be fit to a single exponential decay (solid line) according to Eqation 2 (see section 2). (B) The resulting rate constant τ was slightly dependent on cell concentration for the three KB cell lines.

concentrations of PZ are almost equal at physiological concentrations and temperatures.

4. DISCUSSION

As shown in Table I, both uptake and efflux of PZ is rapid, reaching steady-state levels within one minute. This is in sharp contrast to the rates of influx of the cytostatic drug doxorubicin, which is accumulated into cells with rate constants on the order of 10^{-5} to 10^{-4} s^{-1} for a 10^6 cells/ml suspension [6,7]. Likewise, this also differs from the uptake of verapamil, another MDR modulator, which is shown to accumulate within both sensitive and resistant cells over a period of two hours before reaching steady-state levels [8]. These results are in agreement with the previous study [5] in which significant levels of modulation of vinblastine accumulation were observed within the first 5 min of incubation. The consequence of this rapid entry is that PZ is able to reach its target (as yet unidentified) before significant levels of cytostatic drugs have entered the cell, and therefore sensitization, is on this time scale, virtually instantaneous. Interestingly, the uptake of phenothiazine is much slower than that for PZ: about one half as rapid under similar conditions.

Due to the low value of the partition coefficient, the intracellular concentration of PZ will be only slightly higher than that found extracellularly, and this steady-state concentration will be rapidly approached. This is in direct contrast to verapamil, in which only a small percentage of the modulator in the external incubation media is accumulated very slowly into cells [8]. Thus, modulation by PZ can be rapidly induced or decreased solely through changes in the extracellular concentration of modulator. Ramu et al. [7] have suggested that for effective modulation of MDR at the clinical level a period of pre-treatment with a modulator such as verapamil should accompany treatment with doxorubicin. This would not seem to be necessary with PZ, due to its rapid cellular equilibration.

The lack of correlation of cellular transport of PZ to p-glycoprotein content (Table I) suggests that direct interaction of drug with the protein (i.e. active PZ efflux by pgp170) is unlikely, or at least incidental. Similar conclusions have been reached in the method of verapamil and azidopine modulation [8].

An interesting result of these studies is the intracellular localization of PZ, as determined by fluorescence microscopy (Fig. 1A). The three KB cell lines contains a large concentration of lipid droplets within the cytoplasm. These droplets act as hydrophobic sponges for any number of lipophilic drugs, including PZ and phenothiazine, as well as other pgp170 substrates such as doxorubicin and rhodamine derivatives (data not shown). The droplets are absent from other cell lines such as GC3. The presence of these droplets in one cell line versus another can partially explain the cell line dependence of modulation by PZ previously reported [5], as well as the varying levels of PZ toxicity observed. Under identical extracellular concentrations, intracellular concentrations free to develop biological activity will be substantially lower in the KB lines than in others (approximately 5-fold less, based on PZ IC_{10} values in KB85 and GC3 lines [5]). Extrapolating this to general pharmacology suggests that it may, in fact, be possible to develop modulating agents that are cell line dependent in their function.

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