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Interaction of CJZ3, a lomerizine derivative, with ATPase activity of human P-glycoprotein in doxorubicin-resistant human myelogenous leukemia (K562/DOX) cells

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P-Glycoprotein, a 170–180 kDa membrane glycoprotein that mediates multidrug resistance, hydrolyses ATP to efflux a broad spectrum of hydrophobic agents. To observe the interaction of a P-gp reversal agent with P-gp ATPase activity should provide further insights into the mechanisms of P-gp modulator. In this study, we analysed the effect of CJZ3, a lomerizine derivative, on the adenosine triphosphatase (ATPase) activity of human P-glycoprotein. The results showed that the basal P-gp ATPase activity was increased by CJZ3 with half-maximal activity concentration (K_m) of $6.8 \pm 1.5 \mu\text{M}$, CJZ3 may interact with P-gp with a higher affinity and exhibit a more potent effect than verapamil (Ver). Kinetic analysis indicated a non-competitive inhibition of Ver-stimulated P-gp ATPase activity and a competitive inhibition of CJX2-stimulated P-gp ATPase activity by CJZ3, moreover, the effect of CsA on CJZ3-stimulated and Ver-stimulated P-gp ATPase activity showed a non-competitive and a competitive inhibition respectively. CJZ3 and CJX2 can bind P-gp either on overlapping sites or distinct but interacting sites, while CJZ3 and Ver as well as CsA can bind P-gp on separated sites in K562/DOX cells.

1. Introduction

P-Glycoprotein (P-gp), a 170-KDa plasma membrane glycoprotein found in several mammalian tissues, mediates one type of multidrug-resistance (MDR) in tumor cells. P-gp has been demonstrated to transport a broad spectrum of agents out of MDR cells by using ATP (via hydrolysis) as the source of energy (Horio et al. 1991; Shapiro and Ling 1995; Sharom et al. 1996), leading to a decreased intracellular drug concentration (Gottesman and Pastan 1993). Like many other enzymes that require energy, P-gp couples the transport process with the enzymatic hydrolysis of ATP to ADP and free orthophosphate. Experimental quantitation of the coupling of ATP hydrolysis to efflux events suggests a ratio of approximately 1 ATP per substrate molecule transported (Eytan et al. 1996; Ambudkar et al. 1997; Stein 1997; Shapiro and Ling 1998). A large number of compounds that interact with P-gp have been identified (Ford and Hait 1990). Agents can stimulate or inhibit P-gp adenosine triphosphatase (ATPase) activity (Watanabe et al. 1997). Our previous study showed that CJZ3, a lomerizine derivative exhibited potent effects *in vitro* in the inhibition of P-gp function and the reversal of P-gp-mediated MDR (Ji et al. 2006). In the present report, we observed the interaction of CJZ3 with ATPase activity of human P-gp to gain further insight into the mechanism of action of the compound in the modulation of P-gp function.

2. Investigations and results

2.1. Effect of CJZ3, Ver, CJX2 and CsA on the basal P-gp ATPase activity

A plasma membrane preparation from K562/DOX cells with high P-gp expression was used to determine the stimulation of CJZ3, Ver and CJX2, an amlodipine derivative (Ji et al. 2006), on the basal P-gp ATPase activity. In the presence of 1 mM EGTA (to inhibit Ca^{2+} -ATPase), 0.5 mM ouabain (to inhibit the Na^+/K^+ -ATPase), and 10 mM azide (to inhibit mitochondrial ATPase), the ATPase activity measured for the P-gp containing membranes can be attributed to P-gp which represents 20% of the total membrane protein, the basal ATPase activity determined in the absence of any added agent was about $78 \pm 8.1 \mu\text{mol}^{-1} \cdot \text{min} \cdot \text{g}^{-1}$ protein. The dose-response curves revealed biphasic characteristics. At lower concentrations, CJZ3, Ver and CJX2 stimulated P-gp ATPase activity in a concentration-dependent manner, when exceeding a critical concentration, a gradually lower stimulation of ATPase activity was observed (shown in Fig. 1). In contrast, as shown in Fig. 2, CsA showed an inhibitory characteristic with about half-maximal inhibitory concentration of $0.36 \mu\text{M}$, which was similar to previous results (Watanabe et al. 1997). Fitting the data via nonlinear least regression analysis and assuming simple Michaelis-Menten kinetics, the values of K_m (μM) for CJZ3, Ver and CJX2 were about 6.8 ± 1.5 , 16.2 ± 2.2 and 10.7 ± 1.8

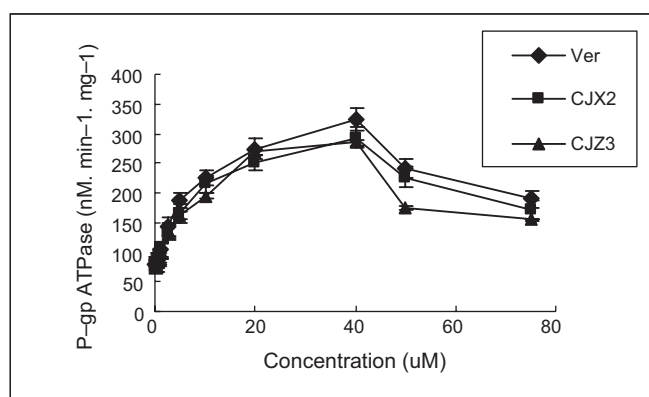


Fig. 1: Effect of CJZ3, Ver and Tet on the basal P-gp ATPase activity. The P-gp ATPase activity was measured as described in "Materials and methods". Each point represents the mean \pm SD from four experiments

respectively, and that of V_{max} for above agents were about 244.4 ± 43 , 322 ± 37 and 307 ± 42 $\text{nM} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, respectively.

2.2. Effect of CJZ3 on Ver-stimulated P-gp ATPase activity

As shown in Fig. 3 (A, B), in the presence of 0.5 and 0.75 μM CJZ3, the K_m (Ver) was essentially unchanged, while the value of V_{max} (Ver) was reduced from 322 ± 37 to 98 ± 12 and 52 ± 6 $\text{nM} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, indicating that CJZ3 inhibited Ver-stimulated P-gp ATPase activity in a non-competitive manner with the inhibition constant K_i of 0.42 ± 0.06 (μM).

2.3. Effect of CJZ3 on CJX2-stimulated P-gp ATPase activity

As shown in Fig. 4 (A, B), the CJZ3 inhibited CJX2-stimulated ATPase activity in a competitive manner accompanying unchanged V_{max} (CJX2) and increased K_m (from 10.7 ± 1.8 to 13.6 ± 2.6 and 15.1 ± 3.5), the inhibition constant K_i was about 0.76 ± 0.21 μM .

2.4. Effect of CsA on CJZ3-stimulated P-gp ATPase activity

As shown in Fig. 5 (A, B), in the presence of 0.1 and 0.25 μM CsA, the K_m (CJZ3) was essentially unchanged, however, the value of V_{max} (CJZ3) was reduced from 244.4 ± 43 to 96.5 ± 16

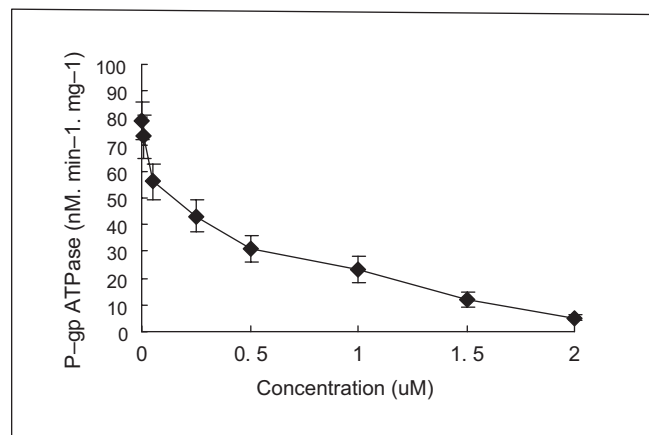


Fig. 2: Effect of CsA on the basal P-gp ATPase activity. The P-gp ATPase activity was measured as described in "Experimental". Each point represents the mean \pm SD from four experiments

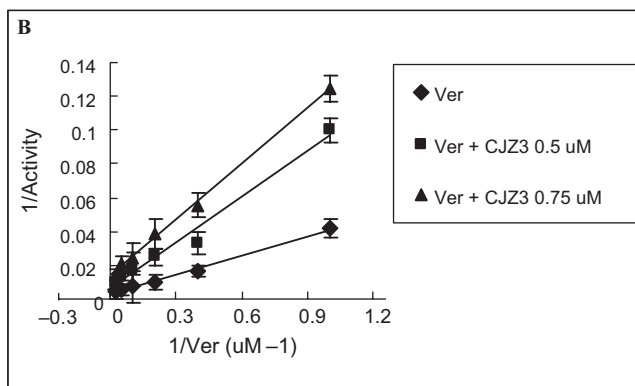
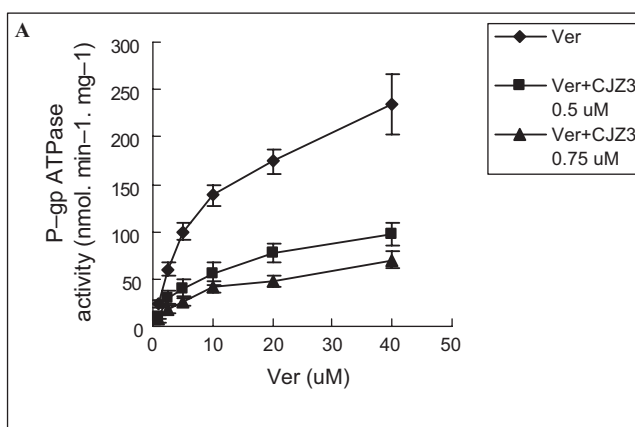


Fig. 3: Effect of CJZ3 on Ver-stimulated P-gp ATPase activity. K_m , K_i and V_{max} were determined by fitting data to Michaelis-Menten equation. Each point represents the mean \pm SD from four experiments

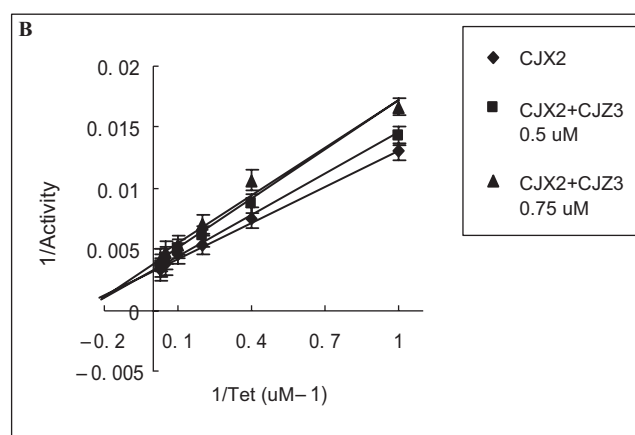
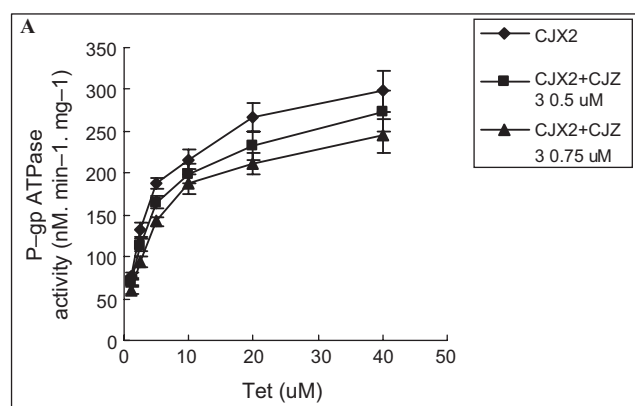


Fig. 4: Effect of CJZ3 on CJX2-stimulated P-gp ATPase activity. K_m , K_i and V_{max} were determined by fitting data to Michaelis-Menten equation. Each point represents the mean \pm SD from four experiments

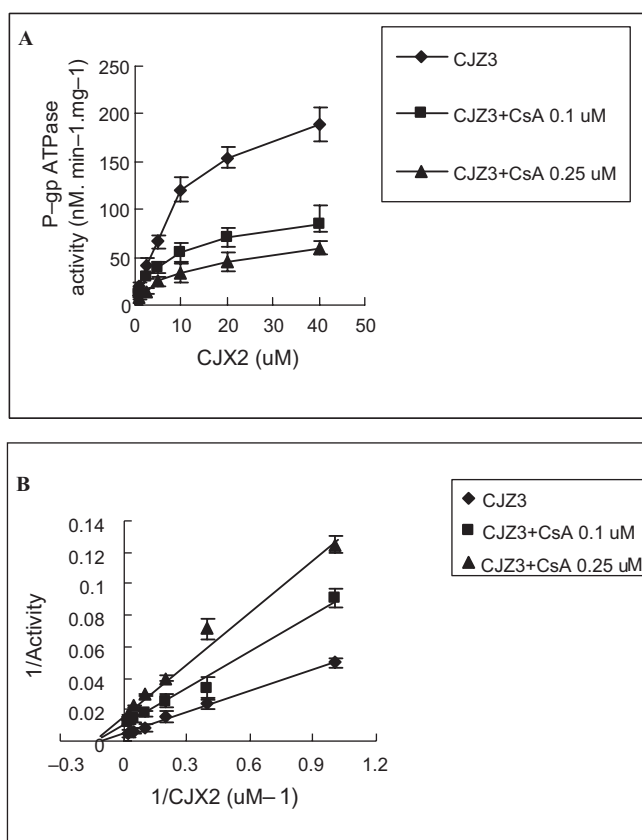


Fig. 5: Effect of CsA on CJZ3-stimulated P-gp ATPase activity. K_m , K_i and V_{max} were determined by fitting data to Michaelis-Menten equation. Each point represents the mean \pm SD from four experiments

and 52 ± 5 nM·min⁻¹·mg⁻¹, implying that CsA inhibited CJZ3-stimulated P-gp ATPase activity in a non-competitive manner with the inhibition constant K_i of 0.113 ± 0.009 μM.

2.5. Effect of CsA on Ver-stimulated P-gp ATPase activity

As shown in Fig. 6 (A, B), in the presence of 0.1 and 0.25 μM CsA, the V_{max} (Ver) was unchanged, the value of K_m (Ver) was increased from 16.2 ± 2.2 to 23.5 ± 3.6 and 28.3 ± 4.2 μM, suggesting that CsA inhibited Ver-stimulated P-gp ATPase activity in a competitive manner with the inhibition constant K_i of 0.080 ± 0.005 μM.

3. Discussion

The ATP-dependent transport enzyme known as P-gp confers multidrug resistance (MDR) against unrelated drug and xenobiotics, many experiments have focused on strategy to inhibit the efflux action of this protein. Clearly, an understanding of the mechanism of P-gp reversal agents will provide much useful information regarding drug delivery to the tumor cells. P-gp has 12 transmembrane domains contained in two homologous halves and there are two ATP-binding cassette domains in each of the halves that catalyse ATP hydrolysis (Hamada and Tsuruo 1988a,b; Sharom et al. 1993; Ambudkar et al. 1992). The hydrophobicity of the substrates interacting with P-gp makes difficult to measure precisely transmembrane transport of different cytotoxic agents as well as their inhibition by the MDR-reversal agents because of huge non-specific binding to lipids and passive diffusion through the membranes. The measurement of MgATP should allow a complementary and more thorough analysis of P-gp function and provide further views about the mechanisms of P-gp modulator. Thus, we used

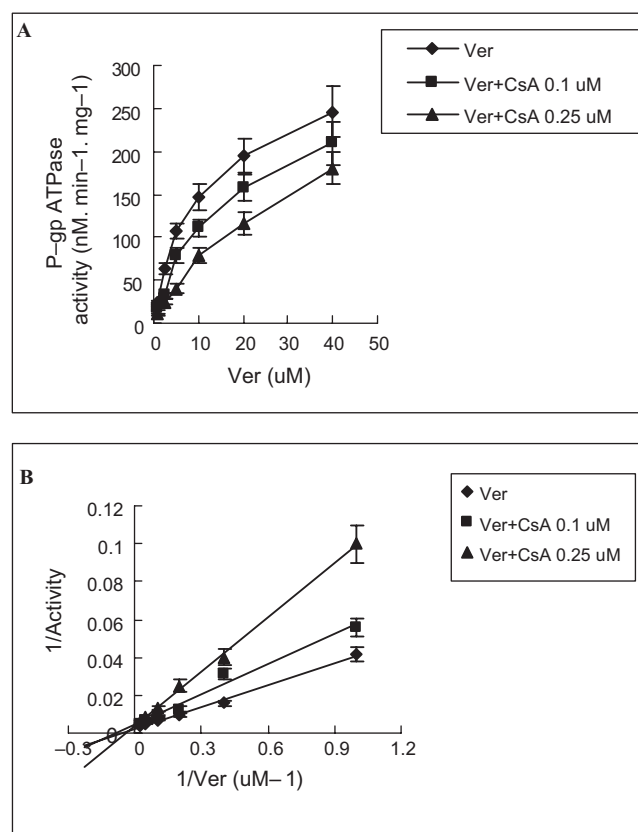


Fig. 6: Effect of CsA on Ver-stimulated P-gp ATPase activity. K_m , K_i and V_{max} were determined by fitting data to Michaelis-Menten equation. Each point represents the mean \pm SD from four experiments

the CJZ3, a lomerizine derivative, to delineate its modulating site on P-gp by measuring its effect on P-gp ATPase activity. P-gp commonly exhibits a basal activity that is purported to be caused by endogenous substrates (Shapiro and Ling 1998), such as membrane lipids. To our knowledge, there are two classes of MDR reversal agents interacting with P-gp: one activates ATPase activity, suggesting that these drug were transported by P-gp and also appeared as substrates of P-gp with a higher rate of transport than endogenous substrate, such as Ver and progesterone (Garrigos et al. 1997), the another, such as PSC833, causes a decrease in the baseline ATPase activity with a hyperbolic relationship to concentration (Borgnia et al. 1996), this response is consistent with a rate of transport that is even slower than putative endogenous substrate. In the present report (shown in Fig. 1), the ascended dose-response curves indicated that CJZ3 and Ver as well as CJX2 can be considered as substrate of P-gp with higher transporting rate. CsA showed an inhibitory effect on the basal P-gp ATPase activity (shown in Fig. 2), this result was similar to that of a previous study (Watanabe et al. 1997). The value of K_m for CJZ3 was less than that of Ver, suggesting that CJZ3 may interact with P-gp with a higher affinity and exhibit more potent effect than Ver, which has also been verified in our previous report (Ji et al. 2006).

P-gp presents two types of binding sites, one for transport and another for modulation, it is quite possible that more than three sites may exist for drug interaction on P-gp (Martin et al. 2000). The P-gp reversal agents may block the efflux of therapeutic drugs in a competitive or non-competitive manner via binding to transport sites or to modulation sites. The binding of reversal agents to modulation sites will elicit conformation changes in transport sites, resulting in the inhibition of therapeutic agent binding or transport, this phenomenon was defined as allosteric effect (Martin et al. 2000). In the present report, the effect of CJZ3 on Ver-stimulated P-gp ATPase activity and effect of

CsA on CJZ3-stimulated P-gp ATPase activity showed a non-competitive manner of inhibition (shown in Figs. 3–5), while the inhibitory effect of CJZ3 on CJX2-stimulated P-gp ATPase activity and effect of CsA on Ver-stimulated P-gp ATPase activity revealed a typical competitive inhibition mechanism (shown in Figs. 4–6), these results implied that CJZ3 and Ver (or CsA) were non-exclusive for their modulating effects on P-gp ATPase, CJZ3 and CJX2 were mutually exclusive for their modulating effects on P-gp ATPase, moreover, Ver and CsA were mutually exclusive for their P-gp ATPase. The present results also indicated that the binding sites of CJZ3 and CJX2 on P-gp in K562/DOX cells may be either identical or partially overlapping and subject to negative allosteric interaction, while CJZ3 and Ver (or CsA) can bind P-gp on separated sites in K562/DOX cells. It has been proposed that if the two modulators bind at different or separated sites, they will act synergistically on the inhibition of P-gp function via allosteric interaction, each can contribute to the overall interaction with P-gp leading to a combined effect which is greater than that given either drug alone (Ayeshe et al. 1996), this will be of great importance in the clinical practice. According to the present results, the experiments to observe the effect of pair CJZ3 and Ver or CsA on P-gp efflux function will be carried out in the near future in our laboratory.

4. Experimental

4.1. Materials

K562/DOX cells were purchased from the Shanghai Institutes for Biological Science, Chinese Academy of Sciences; CJZ3 and CJX2 were obtained from School of Traditional Chinese Pharmacy, China Pharmaceutical University; verapamil, cyclosporin A (CsA), EGTA, ouabain, and azide were purchased from Sigma Co; All other chemicals were of analytical grade and commercially available.

4.2. Cell culture

Doxorubicin-resistant human myelogenous leukemia (K562/DOX) cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO₂. K562/DOX cells were cultured in the presence of 0.5 μM doxorubicin and were grown in drug-free medium 2 weeks before the experiments.

4.3. Preparation of membrane vesicles

Membrane vesicles were prepared as described previously (Garrigos et al. 1997). Isolated cells were suspended in hypotonic lysis buffer (mM: Tris-HCl 10, pH 7.8, KCl 10, MgCl₂ 2, dithiothreitol 1, egtazi acid 1) and allowed to swell for 20 min at 4 °C. Swollen cells were disrupted by sonication for 10 s at 20% maximum power (Sonicator W-225R Heat system Ultrasonics) and the resulting homogenate was centrifuged (1400 × g, 10 min, 4 °C). The supernatant was layered on a 46% sucrose cushion in lysis buffer and centrifuged (7000 × g, 20 min, 4 °C). The layer at the sucrose interface was removed, diluted twice with lysis buffer, and sedimented (13500 × g, 15 min, 4 °C). The pellet of total membrane was washed twice in lysis buffer and finally resuspended in lysis buffer supplemented with 100 mM NaCl at a total membrane protein concentration of 2.0 g/L. The protein concentration was determined by the Lowry method, with bovine serum albumin as standard.

4.4. P-gp ATPase activity measurement

The ATPase activity of the isolated K562/DOX cells membrane was estimated by measuring inorganic phosphate liberation (Sarkadi et al. 1992). Membrane suspensions (about 20 μg of membrane protein, as determined by a modified Lowry method) were incubated at 37 °C in 0.1 ml of a medium containing (mM): Tris-HCl 50 (pH 6.8), dithiothreitol 2, MgCl₂ 5, ouabain 2 (to eliminate Na⁺, K⁺-ATPase activity), egtazi acid 2 (to eliminate Ca²⁺-ATPase activity), sodium azide 5 (to eliminate F₁-F₀-ATPase activity), and the ATPase reaction was started by the addition of MgATP 5 mM. Inorganic phosphate (Pi) was measured by a modification of the sensitive colorimetric reaction described previously. The samples were supplemented with 0.4 ml of reagent containing H₂SO₄ 2.5 M, 1% ammonium molybdate, 0.014% antimony potassium tartrate, and 1 ml of distilled water. For the reduction of the complex, 0.2 ml of 1% ascorbic acid (freshly prepared) was added and the optical density read at 660 nm. Activities were calculated from the

initial linear rate of Pi production and ATPase activity was estimated by difference obtained in Pi levels between 0 min (reaction stopped immediately) and 30 min incubation period.

4.5. Kinetic analysis

Data from experiments measuring ATPase activity were fitted to the Michaelis-Menten equation by nonlinear least square regression analysis (Watanabe et al. 1997) (Eq. (1)). V_{max} and K_m values with standard errors were derived from these curves (Fig. 1) and K_i values were calculated using the equation for the competitive (Eq. (2)) or noncompetitive (Eq. (3)) inhibition.

$$V = V_{\max} \cdot C / (K_m + C) \quad (1)$$

$$V = V_{\max} \cdot C / [K_m(1 + I/K_i) + C] \quad (2)$$

$$V = V_{\max} \cdot C / [K_m(1 + I/K_i) + C(1 + I/K_i)] \quad (3)$$

Double-reciprocal treatment:

$$1/V = 1/V_{\max} + (K_m/V_{\max}) \cdot 1/C \quad (4)$$

$$1/V = 1/V_{\max} + [K_m(1 + I/K_i)/V_{\max}] \cdot 1/C \quad (5)$$

$$1/V = (1 + I/K_i)/V_{\max} + [K_m(1 + I/K_i)/V_{\max}] \cdot 1/C \quad (6)$$

where V and V_{max} are the drug-stimulated ATPase activity and the maximum velocity, respectively; K_m and K_i are the half-maximal activity concentration (Michaelis constant) and inhibition constant, respectively; C and I are the concentrations of stimulator and inhibitor, respectively.

References

- Ambudkar SV, Lelong IH, Zhang J, Cardarelli CO, Gottesman MM, Pastan I (1992) Partial purification and reconstitution of the human multidrug-resistance pump: characterization of the drug-stimulatable ATP hydrolysis. *Proc Natl Acad Sci U S A* 89: 8472–8476.
- Ambudkar SV, Cardarelli CO, Pashinsky I, Stein WD (1997) Relation between the turnover number for vinblastine transport and for vinblastine-stimulated ATP hydrolysis by human P-glycoprotein. *J Biol Chem* 272: 21160–21166.
- Ayeshe S, Shao YM, Stein WD (1996) Co-operative, competitive and non-competitive interactions between modulators of P-glycoprotein. *Biochim Biophys Acta* 1316: 8–18.
- Bian-Sheng Ji, Ling He, Guo-Qing Liu (2006) CJZ3, a Lomerizine derivative, reverses P-glycoprotein-mediated multidrug-resistance in doxorubicin-resistant human myelogenous leukemia (K562/DOX) Cells. *Drug Devel Res* 67: 862–869.
- Borgnia MJ, Eytan GD, Assaraf YG (1996) Competition of hydrophobic peptides, cytotoxic drugs, and chemosensitizers on a common P-glycoprotein pharmacophore as revealed by its ATPase activity. *J Biol Chem* 271: 3163–3171.
- Eytan GD, Regev R, Assaraf YG (1996) Functional reconstitution of P-glycoprotein reveals an apparent near stoichiometric drug transport to ATP hydrolysis. *J Biol Chem* 271: 3172–3178.
- Ford JM, Hait WN (1990) Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* 42: 155–199.
- Garrigos M, Mir LM, Orlowski S (1997) Competitive and non-competitive inhibition of the multidrug-resistance-associated P-glycoprotein ATPase—further experimental evidence for a multisite model. *Eur J Biochem* 244: 664–673.
- Gottesman MM, Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. *Ann Rev Biochem* 62: 385–427.
- Hamada H, Tsuruo T (1988 a) Characterization of the ATPase activity of the Mr 170,000 to 180,000 membrane glycoprotein (P-glycoprotein) associated with multidrug resistance in K562/ADM cells. *Cancer Res* 48: 4926–4932.
- Hamada H, Tsuruo T (1988 b) Purification of the 170- to 180-kilodalton membrane glycoprotein associated with multidrug resistance. 170- to 180-kilodalton membrane glycoprotein is an ATPase. *J Biol Chem* 263: 1454–1458.
- Horio M, Lovelace E, Pastan I, Gottesman MM (1991) Agents which reverse multidrug-resistance are inhibitors of (3H) vinblastine transport by isolated vesicles. *Biochim Biophys Acta* 1061: 106–110.
- Ji BS, Ling H (2006) CJX2, an amlodipine derivative, reverses p-glycoprotein-mediated multidrug-resistance in doxorubicin-resistant human myelogenous leukemia (K562/DOX) cells. *Drug Devel Res* 66: 278–285.

ORIGINAL ARTICLES

- Martin C, Berridge G, Higgins CF, Mistry P, Charlton P, Callaghan R (2000) Communication between multiple drug binding sites on P-glycoprotein. *Mol Pharmacol* 58: 624–632.
- Sarkadi B, Price EM, Boucher RC, Germann UA, Scarborough GA (1992) Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. *J Biol Chem* 267: 4854–4858.
- Shapiro AB, Ling V (1995) Reconstitution of drug transport by purified P-glycoprotein. *J Biol Chem* 270: 16167–16175.
- Shapiro AB, Ling V (1998) Stoichiometry of coupling of rhodamine 123 transport to ATP hydrolysis by P-glycoprotein. *Eur J Biochem* 254: 189–193.
- Sharom FJ, Yu X, Doige CA (1993) Functional reconstitution of drug transport and ATPase activity in proteoliposomes containing partially purified P-glycoprotein. *J Biol Chem* 268: 24197–24202.
- Sharom FJ, Yu X, DiDiodato G, Chu JW (1996) Synthetic hydrophobic peptides are substrates for P-glycoprotein and stimulate drug transport. *Biochem J* 320: 421–428.
- Stein WD (1997) Kinetics of the multidrug transporter (P-glycoprotein) and its reversal. *Physiol Rev* 77: 545–590.
- Watanabe T, Kokubu N, Charnick SB, Naito M, Tsuruo T, Cohen D (1997) Interaction of cyclosporin derivatives with the ATPase activity of human P-glycoprotein. *Br J Pharmacol* 122: 241–248.