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Research Article

Interaction of CJX2, an Amlodipine Derivative With Human P-Glycoprotein ATPase Activity

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Strategy, Management and Health Policy				
Enabling Technology, Genomics, Proteomics	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT P-glycoprotein, a plasma membrane protein overexpressed in multidrug-resistant (MDR) cells, is responsible for the energy-dependent efflux of structurally unrelated cytotoxic agents and MDR-reversing drugs from the cells. Understanding the interaction of P-gp reversing agents with P-gp ATPase activity should provide useful information for understanding the mechanisms of P-gp modulator. The aim of present report was to elucidate the possible mechanism of CJX2, an amlodipine derivative, in modulating P-gp function by determining its effect on P-gp ATPase activity. Basal P-gp ATPase activity in K562/DOX cells was increased by CJX2 with a half-maximal activity concentration (K_m) of 10.7 ± 1.8 μM. Kinetic analysis indicated a noncompetitive inhibition of verapamil (Ver)-stimulated P-gp ATPase activity by CJX2 and a competitive inhibition of CJX2-stimulated P-gp ATPase activity by tetrandrine; moreover, the effect of Cyclosporin A (CsA) on CJX2-stimulated and Ver-stimulated P-gp ATPase activity showed noncompetitive and a competitive inhibition, respectively. CJX2 and Tet can bind P-gp either on overlapping sites or distinct but interacting sites, while CJX2 and Ver as well as CsA can bind P-gp on separate sites in K562/DOX cells. Drug Dev Res 69:42–47, 2008 ©2008 Wiley-Liss, Inc.

Key words: CJX2; P-glycoprotein; multidrug resistance; ATPase

INTRODUCTION

P-glycoprotein (P-gp), a 170-kDa plasma membrane glycoprotein found in mammalian tissues, mediates one type of multidrug-resistance (MDR) in tumor cells. P-gp can transport a broad spectrum of agents out of MDR cells using ATP hydrolysis as an energy source [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 ~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 CJX2, an amlodipine derivative, exhibited potent effects in vitro in the inhibition of P-gp function and the reversal

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of P-gp-mediated MDR [Ji and He, 2006]. In the present report, we studied the interaction of CJX2 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.

MATERIALS AND METHODS

Materials

K562/DOX cells were purchased from Shanghai Institutes for Biological Science, Chinese Academy of Sciences; CJX2 was obtained from the School of Traditional Chinese Pharmacy, China Pharmaceutical University; verapamil, Cyclosporin A (CsA), EGTA, ouabain, and azide were purchased from Sigma; and tetrandrine (Tet) was kindly donated by Professor Guo-Qing Liu (China Pharmaceutical University, China). All other chemicals were of analytical grade and commercially available.

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% CO_2. 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.

Preparation of Membrane Vesicles

Membrane vesicles were prepared as described previous [Garrigos et al., 1997] Isolated cells were suspended in hypotonic lysis buffer (mM: Tris-HCl 10, pH 7.8, KCl 10, $MgCl_2$ 2, dithiothreitol 1, egtazic 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 centrifuged (1,400 *g*, 10 min, 4°C). The supernatant was layered on a 46% sucrose cushion in lysis buffer and centrifuged (7,000 *g*, 20 min, 4°C). The layer at the sucrose interface was removed, diluted twice with lysis buffer, and sedimented (13,500 *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 (BSA) as the standard.

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 (~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, $MgCl_2$ 5, ouabain 2 (to eliminate Na^+, K^+-ATPase activity), egtazic acid 2 (to eliminate Ca^{2+}-ATPase activity), and sodium azide 5 (to eliminate F_1-F_0-ATPase activity). The ATPase reaction was initiated by addition of MgATP 5 mM. Inorganic phosphate (P_i) was measured by a modification of the sensitive colorimetric reaction described previously [Ji and He, 2006]. The samples were supplemented with 0.4 ml of reagent containing H_2SO_4 2.5 M, 1% ammonium molybdate, 0.014% antimony potassium tartrate, and 1 ml of distilled water. For reduction of the complex, 0.2 ml of 1% ascorbic acid (freshly prepared) was added, and the optical density read at 660 nm. Activity were calculated from the initial linear rate of P_i production and ATPase activity was estimated by difference obtained in P_i levels between 0 min (reaction stopped immediately) and the 30-min incubation period.

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:

$$V = Vmax \cdot C/(Km + C) \quad (1)$$

$$V = Vmax \cdot C/[Km(1 + I/Ki) + C] \quad (2)$$

$$V = V\mathit{max} \cdot C/[Km(1 + I/Ki) + C(1 + I/Ki)] \quad (3)$$

Double-reciprocal treatment:

$$1/V = 1/V\,max + (Km/V\,max) \cdot 1/C \quad (1)$$

$$1/V = 1/V\,max + [Km(1 + I/Ki)/V\,max] \cdot 1/C \quad (2)$$

$$1/V = (1 + I/Ki)/V\,max + [Km(1 + I/Ki)/V\,max] \cdot 1/C \quad (3)$$

where V and $_{max}$ are the compound-stimulated AT Pase 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 concentration of stimulator and inhibitor, respectively.

RESULTS

Effect of CJX2, Ver, Tet, and CsA on the Basal P-gp ATPase Activity

The plasma membrane preparation from K562/DOX cells with high P-gp expression was used to

determine the stimulation of CJX2, Ver, and Tet on 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 $\sim 78 \pm 8.1\ \mu mol^{-1} \cdot min \cdot g^{-1}$ protein. Dose-response curves revealed biphasic characteristics. At lower concentrations, CJX2, Ver, and Tet stimulated P-gp ATPase activity in a concentration-dependent manner. When exceeding a critical concentration, a gradually lower stimulation of ATPase activity was observed (Fig. 1). In contrast (Fig. 2), CsA showed an inhibitory characteristic with a half-maximal inhibitory concentration of ~0.36 μM, which was similar to that previously reported [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 CJX2, Ver, and Tet were $\sim 10.7 \pm 1.8$, 16.2 ± 2.2 and 3.1 ± 0.4, respectively, and that of $_{max}$ (nM · min^{-1} · mg^{-1})for the above agents were $\sim 307 \pm 42$, 322 ± 37, and 312 ± 33, respectively.

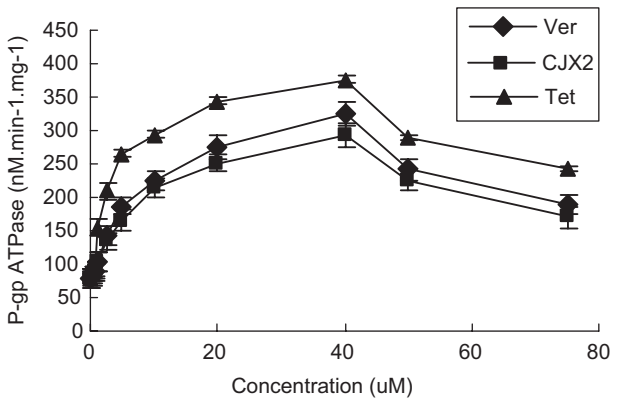

Fig. 1. Effect of CJX2, Ver, and Tet on basal P-gp ATPase activity. P-gp ATPase activity was measured as described in Materials and Methods. Each point represents the mean ± SD from four experiments.

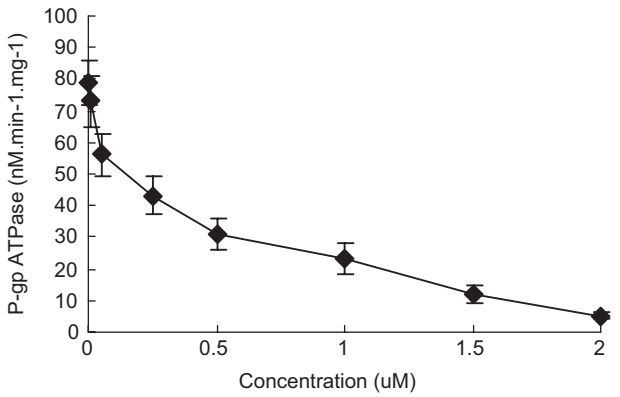

Fig. 2. Effect of CsA on basal P-gp ATPase activity. P-gp ATPase activity was measured as described in Materials and Methods. Each point represents the mean ± SD from four experiments.

Effect of CJX2 on Ver-Stimulated P-gp ATPase Activity

As shown in Figure 3A,B, in the presence of 0.5 and 0.75 μM CJX2, the K_m (Ver) was essentially unchanged, while the value of $_{max}$ (Ver) was reduced from 322 ± 37 to 114 ± 19 and 66.8 (nM · min^{-1} · mg^{-1}), indicating that CJX2 inhibited Ver-stimulated P-gp ATPase activity in a noncompetitive manner. The K_i was 0.62 ± 0.07 μM.

Effect of Tet on CJX2-Stimulated P-gp ATPase Activity

As shown in Figure 4A,B, Ter at 0.5 μM and 0.5 μM inhibited CJX2-stimulated ATPase activity in a competitive manner, as reflected in an unchanged $_{max}$ (CJX2) and increased K_m (10.7 ± 1.8 to12.6 ± 2.2 and 14.8 ± 2.5). The K_i was 0.72 ± 0.13 μM.

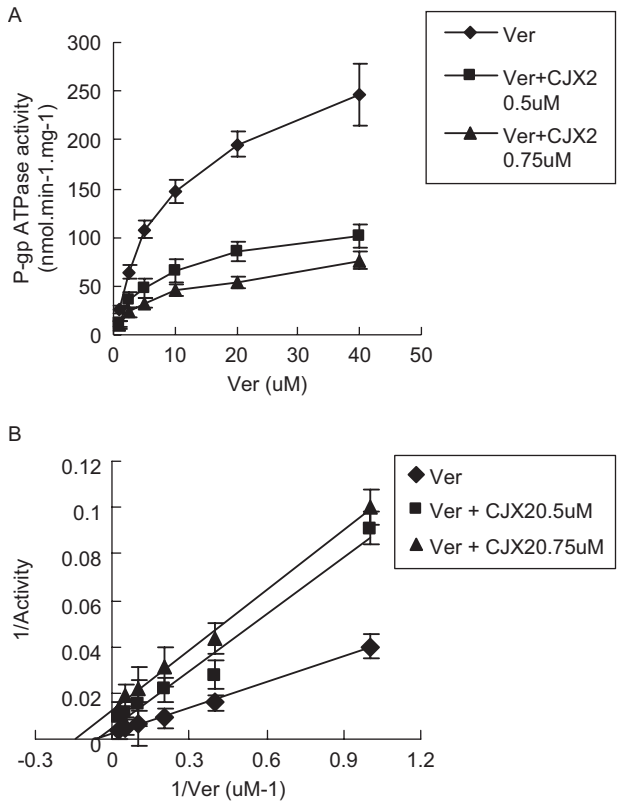

Fig. 3. Effect of CJX2 on Ver-stimulated P-gp ATPase activity. K_m, K_i, and $_{max}$ were determined by fitting data to Michaelis–Menten equation. Each point represents the mean ± SD from four experiments.

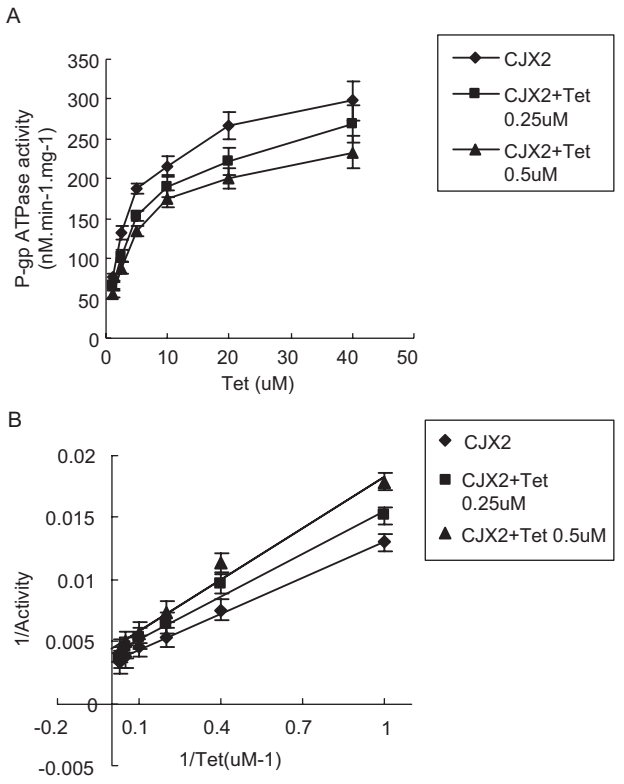

Fig. 4. Effect of Tet on CJX2-stimulated P-gp ATPase activity. K_m, K_i, and $_{max}$ were determined by fitting data to Michaelis–Menten equation. Each point represents the mean ± SD from four experiments.

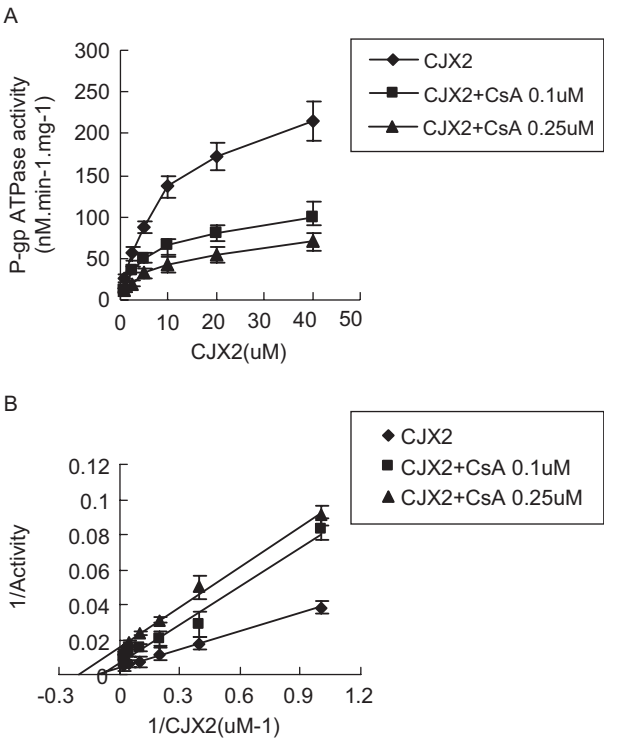

Fig. 5. Effect of CsA on CJX2-stimulated P-gp ATPase activity. K_m, K_i, and $_{max}$ were determined by fitting data to Michaelis–Menten equation. Each point represents the mean ± SD from four experiments.

Effect of CsA on CJX2-Stimulated P-gp ATPase Activity

As shown in Figure 5A,B, in the presence of 0.1 and 0.25 μM CsA, the K_m (CJX2) was e unchanged. However, the value of $_{max}$ (CJX2) was reduced from 307 ± 42 to 112 ± 18 and 63 ± 7 (nM · min^{-1} · mg^{-1}), implying that CsA inhibited CJX2-stimulated P-gp ATPase activity in a noncompetitive manner. The K_i value was 0.096 ± 0.006 (μM).

Effect of CsA on Ver-Stimulated P-gp ATPase Activity

As shown in Figure 6A,B, in the presence of 0.1 and 0.25 μM CsA, the $_{max}$ (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 an inhibition constant, K_i of 0.080 ± 0.005 (μM).

DISCUSSION

The ATP-dependent transport enzyme, 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 catalyze 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 nonspecific 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 the CJX2, an amlodipine 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 activated ATPase activity, suggesting that these drug were transported by P-gp and also appeared as substrates of P-gp with

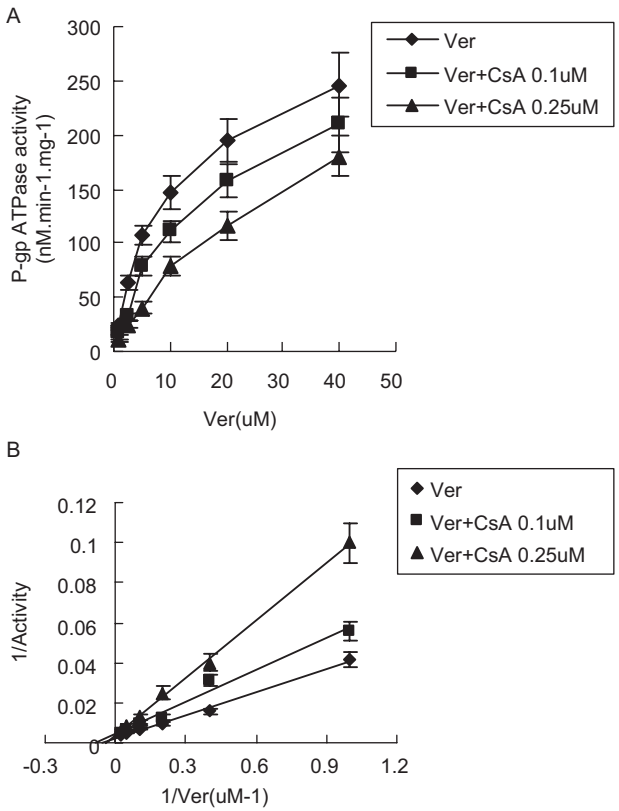

Fig. 6. Effect of CsA on Ver-stimulated P-gp ATPase activity. K_m, K_i, and $_{max}$ were determined by fitting data to Michaelis–Menten equation. Each point represents the mean ± SD from four experiments.

higher rate of transport than endogenous substrate, such as Ver and progesterone [Garrigos et al., 1997]. The other, 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 CJX2 and Ver as well as Tet can be considered as a substrate of P-gp with higher transporting rate. CsA showed an inhibitory effect on the basal P-gp ATPase activity (see Fig. 2), this result was similar with the previous study [Watanabe et al., 1997]. The value of K_m for CJX2 was less than that of Ver, suggesting that CJX2 may interact with P-gp with a higher affinity and exhibit more potent effect than Ver, which has also been verified by our previous report [Ji and He, 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 noncompetitive manner via binding to transport sites or to modulating 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 an allosteric effect [Martin et al., 2000]. In the present report, the effect of CJX2 on Ver-stimulated P-gp ATPase activity and effect of CsA on CJX2-stimulated P-gp ATPase activity showed noncompetitive manner of inhibition (shown in Figs. 3 and 5), while the inhibitory effect of Tet 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 and 6), these results implied that CJX2 and Ver (or CsA) were nonexclusive for their modulating effects on P-gp ATPase, CJX2 and Tet 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 CJX2 and Tet on P-gp in K562/DOX cells may be either identical or partially overlapping and subject to negative allosteric interaction, while CJX2 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 that is greater than that given either drug alone [Ayesh 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 CJX2 and Ver or CsA on P-gp efflux function will be carried out in the near future in our laboratory.

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