JPET Fast Forward. Published on September 15, 2003 as DOI:10.1124/jpet.103.055574

Original articles

Interaction of Imatinib Mesilate with Human P-glycoprotein

Running title: Interaction of Imatinib with P-gp

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Key words: Imatinib mesilate, P-glycoprotein, Multidrug resistance, Transport, calcein-AM, interactions

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JPET Fast Forward. Published on September 15, 2003 as DOI: 10.1124/jpet.103.055574
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Abstract

The interaction of imatinib mesilate with P-glycoprotein (P-gp) was examined using pig kidney epithelial LLC-PK1 cells versus L-MDR1 cells which overexpress human P-gp on the apical membrane. The basal-to-apical transport of imatinib mesilate in L-MDR1 cells significantly exceeded that in the parental LLC-PK1 cells. The intracellular accumulation of imatinib mesilate after its basal application to LLC-PK1 and L-MDR1 cells was 35% and 15%, respectively. A P-gp modulator, cyclosporin A, inhibited the basal-to-apical transport in L-MDR1 cells. The intracellular accumulation of imatinib mesilate in L-MDR1 cells was also increased by cyclosporin A. The rhodamine 123 efflux assay showed that the efflux of rhodamine 123 in K562/DXR cells, which overexpress human P-gp, could be blocked markedly by imatinib mesilate in a dose-dependent fashion. The Ki values for the inhibition of P-gp function by cyclosporin A and imatinib mesilate were estimated to be 6.1μM and 18.3μM, respectively, using a calcein-AM efflux assay. These observations demonstrate that imatinib mesilate-caused is a substrate as well as a modulator of human P-gp, suggesting that imatinib mesilate drug interactions may occur via P-gp. It is necessary to consider the pharmacokinetic and pharmacodynamic interactions of imatinib mesilate with other drugs via P-gp.

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Introduction

Imatinib mesilate (Glivec) has been approved in various countries as a drug for the treatment of interferon α (IFN)-resistant chronic myeloid leukemia (CML) (Artaga et al., 2002). Some other drugs targeting particular cell components, including gefitinib, a non-small cell lung cancer agent, and trastuzumab, a breast cancer agent, have received a great deal of attention (Artaga et al., 2002; Fortunato et al., 2000; Goel et al., 2002; Hoelzer et al., 2002; Moloney et al., 2001). Imatinib mesilate was developed as a Bcr-Abl tyrosine kinase inhibitor (Maruo et al., 2001). Imatinib mesilate, which competes for the ATP binding site of Bcr-Abl tyrosine kinase, selectively inhibits the kinase activity (Goldman et al., 2001). Imatinib mesilate has high efficacy in comparison with IFN, a conventional CML drug (Thiesing et al., 2000). On the other hand, acquired drug resistance in patients treated by continuous administration of imatinib mesilate is a problem (Mahon et al., 2000; le Coutre et al., 2000; Gorre et al., 2001). These studies have shown that amplification of Bcr-Abl gene, increased expression of Bcr-Abl protein, and upregulation of P-glycoprotein occurred in some imatinib-resistant cell lines, and indicated that the mechanism of imatinib resistance could be due to overexpression not only the Bcr-Abl protein but also of P-glycoprotein. Multidrug resistance is one of the most serious problems responsible for the failure of chemotherapy. ATP-binding cassette (ABC) transporters are known to be multiple drug

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resistance factors in cancer chemotherapy (van Helvoort et al., 1996). ABC transporters have an ATP binding domain, and are key proteins in the expulsion of xenobiotics via a mechanism requiring the hydrolysis of ATP. P-gp, multidrug resistance-associated protein (MRP), and lung resistance protein (LRP) are known to be ABC transporters (Gottesman et al., 1988; Liminga et al., 1994; Scheper et al., 1993). P-gp contains 12 transmenbrane domains and ATP binding sites (van Helvoort et al., 1996). The molecular weights of P-gp substrates range from 300 to 2000. Substrates of P-gp need to have both moderate hydrophobicity and hydrophilicity in order to be recognized. The immunosuppressive agent cyclosporin A, calcium antagonist verapamil, and cancer chemotherapy agents etoposide, doxorubicin, and paclitaxcel are known to be substrates of P-gp. These drugs are important as substrates or inhibitors of cytochrome P-450, CYP3A4. It has been reported that many substrates of CYP3A4 appeared to be substrates of P-gp (Schuetz et al., 1996; Wacher et al., 2000). Imatinib mesilate is metabolized by CYP3A4, CYP2C9, and CYP2D6 (Goldman et al., 2001), however, little information is available on the pharmacokinetics of imatinib mesilate, i.e., whether or not it is transported through the cells (Hegedus et al., 2002). In this study, we evaluated the P-gp-mediated transport of imatinib mesilate using the L-MDR1 cell line, which expresses human P-gp on the apical membrane. This cell line is a useful tool for the identification of substrates for P-gp and moreover for quantitative

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characterization of the transport via P-gp (Shiraki et al., 2002).

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Materials and Methods

Drugs. Imatinib mesilate and cyclosporin A were gifts from Novartis Pharma (Basel, Switzerland). Doxorubicin was a gift from Kyowa Hakko Kogyo Co., Ltd.(Tokyo, Japan). Vincristine was a gift from Eli Lilly KK. (Hyogo, Japan). Rhodamine 123 (R 123), calcein acetoxymethyl ester (calcein-AM), and alamar blue were purchased from Sigma Chemical Co. (St. Louis, U.S.A.), Molecular Probe Co. (Oregon, U.S.A.) and Alamar Bioscience, Inc. (California, U.S.A.), respectively. All other chemicals were of the highest purity available.

Cell culture Porcine kidney epithelial LLC-PK1 and L-MDR1 cells transfected with human *MDR1* cDNA were generously gifts from Dr. Erin G. Schuetz, St. Jude Children's Research Hospital, Memphis, Tennessee, USA and cultured as described previously (Schinkel et al., 1995). In brief, LLC-PK1 and L-MDR1 cells were maintained in complete medium consisting of Medium 199 supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin, and L-MDR1 cells were maintained at 640 nM vincristine. K562 erythroleukemia cells, doxorubicin-resistant K562/DXR cells, and vincristine-resistant K562/VCR cells were provided by Dr. T. Tsuruo, Institute of Molecular and Cellular Biosciences, University of Tokyo. K562/DXR and K562/VCR cells were previously shown to express a high level of P-gp, as revealed by Northern blot analysis (Shibata et al., 1990).

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Other transporters may be localized in K562/DXR cells, but MRP and LRP could play a minor role in multidrug resistance of K562 (Grandjean et al., 2001). The cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. All cells were grown under an atmosphere of 5% CO_2-95% air at 37℃.

Cytotoxicity assay To quantify the cytotoxic effects of imatinib mesilate, alamar blue was used in a semiautomatic fluorometric method. (Back et al., 1998) Alamar blue is non-fluorescent, and is cleaved to a fluorescent product by living cells. This activity is dependent on the cell viability. The amount of fluorescenc thus correlates with the number of living cells. Cells were harvested and plated at 5.0 x 10^3 cells/well in a volume of 90 μl in 96-well plates. Ten microliters of culture medium containing the drug at the desired concentration were added and the cells were then cultured for 48 h under the conditions described above. Ten microliters of alamar blue were added to each well of the plates, which were then incubated under the culture conditions for an additional 4 h. Fluorescence was measured using a fluorescence plate reader (Fluoroscan Ascent; Labsystems, Finland) with excitation at 485 nm and emission at 528 nm.

Transcellular transport and intracellular accumulation of imatinib mesilate Transport and accumulation assays were performed as

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described previously (Miller et al., 1999; Regina et al., 1998; Schinkel et al., 1995; Ueda et al., 1996). For the transport studies, L-MDR1 and LLC-PK1 cells were seeded on polycarbonate membrane filters inside Transwell cell culture chambers (24 mm in diameter, 3.0 μm pore size, Costar) at a cell density of 2 x 10^6cells / filter. Cells in each chamber were cultured as described above for 3 days. The medium was replaced after 2 days by fresh medium and the cells were used in the transport studies 3 days after plating. Two hours before transport experiments, the culture medium was replaced with fresh medium. The cell monolayers were preincubated with 2 ml of Medium 199 on each side for 2 h at 37℃. Transcellular transport of imatinib mesilate was determined using the cell monolayers. Medium on either the basal or apical side of the monolayers was replaced with 2 ml of fresh medium containing imatinib mesilate, and that on the opposite side was replaced with 2 ml of fresh medium alone. In the inhibition study, an inhibitor was added to the medium of both sides. The monolayers were incubated in 5% CO_2-95% air at 37℃ for up to 8 h, and 50 μl aliquots on the other side were taken at the indicated time points. For accumulation studies, the medium was aspirated off at the end of the incubation period, and the monolayers were lysed with 1 ml of 1 N NaOH. Lysed cells were neutralized with 1 mL of 1 N HCl. The concentration of imatinib mesilate in each sample was measured by high pressure liquid chromatography (HPLC).

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HPLC Analysis The concentration of imatinib mesilate were determined using HPLC (model LC-6A, Shimadzu, Kyoto, Japan). One hundred microliters of sample and 100 µl of methanol were mixed in a 0.5-ml tube, and tubes were centrifuged at 10,000 rpm for 5 min. The supernatant (50 µl) was injected into the HPLC column. Separation was performed on a reversed-phase column (Lichrospher 100 RP-18, 5 µm particle size, 250 mm x 4 mm i.d.) at 40℃. The mobile phase was a 4:6:0.1 mixture of acetonitrile, water, phosphoric acid, and the flow rate was 1.0 ml/min. Imatinib mesilate was detected by UV absorption at 267 nm.

Calcein-AM efflux assay Efflux assays were performed as described previously (Liminga et al., 1994). A kinetic fluorometric assay was used to study the interaction of imatinib mesilate with P-gp. For the calcein-AM efflux assay, L-MDR1 and LLC-PK1 cells were seeded in 96-well tissue culture plates at a cell density of 1 x 10^5 cells / well. Cells were cultured in 200 µl of Medium 199 supplemented with 10% fetal bovine serum, 1% benzylpenicillin and streptomycin in each wells in an atmosphere of 5% CO_2-95% air at 37℃ for 1 day. Cells were plated in 96-well tissue culture plates in Medium 199 containing imatinib mesilate or cyclosporin A. After a 30-min incubation, calcein-AM was added to a final concentration of 2 µM and the plates were placed into a Fluoroscan Asent (Labsystems,

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Finland). Fluorescence was measured from 0 to 30 min with 485 nm excitation and 530 nm emission filters. The rate of calcein accumulation in the presence and absence of drugs was calculated by linear regression analysis using the Asent software (Labsystems, Finland). The Ki value for P-gp was calculated using a modified form of the Michaelis-Menten equation. (Yasuda et al., 2002; Shiraki et al., 2002)

Cellular efflux assay To study the transport function of P-gp in K562/DXR cells, rhodamine 123 was used in an efflux assay (Getie et al., 1999). Cells（1.0 x 10^6）were washed, resuspended in 1 ml of serum-free RPMI-1640 and incubated with 1 μM rhodamine 123 and/or imatinib mesilate, or Cyclosporin A for 1 h at 37℃. After 1 h, the cells were washed twice with serum-free medium to remove excess rhodamine 123 or imatinib mesilate, resuspended in 1 ml of serum-free medium, and recultured at 37℃ for 2 h. After 30 min of culture, the cells were washed and analyzed by flow cytometry using a FACScan by measuring events in the FL1（rhodamine 123）channel. To quantify the effect of drug treatment on the rhodamine 123 efflux from K562/DXR cells, we measured the shift of the histogram to the right as compared with that of the control（rhodamine 123 alone）.

Statistical analysis The Student's *t* test was used to evaluate the

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significance of differences between groups. A *p* value of 0.05 or less was considered to be significant.

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Results

Antitumor effect of imatinib mesilate on P-gp expressing cell line

The in vitro antitumor effect of imatinib mesilate on K562, K562/DXR, and K562/VCR cells was evaluated. The cytotoxic activity against K562/DXR and K562/VCR cells, which overexpress P-gp, was weaker than that against K562 cells (Fig. 1). The IC_{50} values were approximately 0.17, 1.3 and 1.6 μM for K562, K562/DXR and K562/VCR cells, respectively. These results of the antitumor activity studies suggest that P-gp may mediate the efflux of imatinib mesilate, leading to decreased accumulation of imatinib mesilate in the P-gp over-expressing cells.

Intracellular accumulation and transcellular transport of imatinib mesilate in LLC-PK1 and L-MDR1 cells The intracellular accumulation of imatinib mesilate after its basal application to LLC-PK1 and L-MDR1 cells was 35% and 15%, respectively (Fig. 2). This result also suggests that over-expression of P-gp in L-MDR1 cells excludes imatinib mesilate into the extracellular space. Cyclosporin A (1 μM) restored the intracellular accumulation of basally applied imatinib mesilate to 24.1 and 26.8% in LLC-PK1 and L-MDR1 cells, respectively (Fig. 3). Since LLC-PK1 cells expressed endogenous P-gp at markedly lower levels than the exogenous P-gp expressed in L-MDR1 cells, the intracellular accumulation of imatinib mesilate after its basal application to LLC-PK1

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cells treated with 1 μM cyclosporin A was moderately lower than that in LLC-PK1 cells without cyclosporin A (24.1 vs 15.4%). Fig. 4 shows the transcellular transport of 10 μM imatinib mesilate in LLC-PK1 and L-MDR1 cells. The basal-to-apical transport of imatinib mesilate in L-MDR1 cells significantly exceeded that in LLC-PK1 cells, where the apical-to-basal transport was decreased. Cyclosporin A (1 μM) inhibited the basal-to-apical transcellular transport of imatinib mesilate in L-MDR1 cells, but had little effect on the transcellular transport of an imatinib mesilate in LLC-PK1 cells (Fig. 5). These results suggest that imatinib mesilate is a substrate of P-gp.

Effect of imatinib mesilate on calcein-AM efflux in L-MDR1 cells

Fig. 6 shows the calcein-AM efflux in LLC-PK1 and L-MDR1 cells treated with 5 μM cyclosporin A or 5 μM imatinib mesilate. When calcein-AM is taken up into LLC-PK1 or L-MDR1 cells, it is converted into calcein by esterase and becomes fluorescent. So calcein-AM is a substrate for P-gp, it is expelled from L-MDR1 cells. The level of calcein fluorescence in L-MDR1 cells without drugs was significantly lower than that of LLC-PK1 cells. Both cyclosporin A and imatinib mesilate significantly increased the calcein fluorescence in L-MDR1 cells. The Ki values of imatinib mesilate and cyclosporin A were calculated based on the calcein fluorescence at 30 min, as described in detail in Material and

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Methods. The Ki values of imatinib mesilate and cyclosporin A for the inhibition of P-gp function were 18.3μM and 6.1μM, respectively, when the drugs were tested at the concentration of 5μM.

Effect of imatinib mesilate on the efflux of rhodamine 123 We next examined the rhodamine 123 efflux as a functional test to evaluate the effects of imatinib mesilate on P-gp activity in K562 and K562/DXR cells. As shown in Fig. 7, rhodamine 123 was accumulated in K562 cells but not in K562/DXR cells, although the efflux of rhodamine 123 from K562/DXR cells could be blocked by imatinib mesilate at the concentration of 50 μM, and the effect was a dose dependent. The inhibitory effect of 10μM cyclosporin A on the efflux of rhodamine 123 was comparable with that of 50μM imatinib mesilate. Thus, the potency of the inhibitory effect on P-gp using this method was in the order of cyclosporin A > imatinib mesilate.

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Discussion

Most drug interactions occur as a result of the unexpected changes in pharmacokinetics of drugs coadministered. Anticancer drugs are usually given in combination to ensure higher efficacy. The combination of IFN, cytarabine, etoposide, and doxorubicin with imatinib mesilate showed a strong effect against CML in vitro (Kano et al., 2001). Recently, it was reported that combined treatment with SCH 66336, a farnesyl protein transferase inhibitor that has P-gp inhibitory action, enhanced the therapeutic efficacy of imatinib mesilate (Hoover et al., 2002; Wang et al., 2001), but the mechanism of this effect remains unknown. Previously, it was thought that the mechanisms of resistance to imatinib mesilate might involve a point mutation in the Bcr-Abl kinase domain, amplification of the Bcr-Abl gene or protein, binding of imatinib mesilate to α-acid glycoprotein, efflux of the drug mediated by P-gp, or inactivation by glutathione (Gorre et al., 2001; le Coutre et al., 2000; Mahon et al., 2000). A notable report demonstrated that cells resistant to imatinib mesilate showed an over-expression of P-gp (Druker et al., 2001). On the other hand, Mahon et al. reported that imatinib mesilate did not upregulate the expression of MDR1 gene in various cell line (Mahon et al., 2000). In the present study, the interaction of imatinib mesilate with P-gp was examined from the viewpoint of pharmacokinetic drug interactions. P-gp over-expression has previously been demonstrated as one of the various mechanisms of drug

resistance. P-gp-mediated efflux of anticancer drugs from tumor cells and the intracellular concentration of the anticancer drugs are decreased in some drug-resistant cells. The present study was therefore performed to obtain information about the pharmacokinetics and pharmacodynamic drug interactions of imatinib mesilate.

The results of the antitumor effects of imatinib mesilate in P-gp expressing cell lines suggested that imatinib mesilate could be a substrate of P-gp (Fig. 1). The intracellular accumulation of imatinib mesilate was decreased in L-MDR1 cells in comparison with the parental LLC-PK1 cells, and the accumulation of imatinib mesilate was restored by treatment with cyclosporin A. As the P-gp expression in brain and intestine, and tumor might be higher than that in other normal tissue, the P-gp in these tissue is an important determinant of distribution of it. Recently, it was reported that imatinib mesilate does not cross the blood-brain-barrier (Senior, 2003), that imatinib mesilate is a substrate of P-gp, and that this efflux transporter is an important determinant of the distribution of imatinib mesilate to the central nervous system (Dai et al. 2003). The results in this study support the notion that imatinib mesilate is a substrate of P-gp.

Moreover, these data indicate that imatinib mesilate is not only a substrate of P-gp but also a modulator of P-gp. The P-gp-inhibitory effect of imatinib mesilate is slightly weaker than that of cyclosporin A; however, the effect of imatinib mesilate is stronger than those of verapamil, or calcium

antagonist (Fig.6). Previously we reported that the Ki value of verapamil was 540μM, as determined by the calcein-AM efflux test (Shiraki et al., 2002). These findings suggest that imatinib mesilate has an effect on the efflux transport of a substrate of P-gp. Imatinib mesilate, not only a substrate but also inhibitor of P-gp, may therefore change the pharmacokinetics and pharmacodynamics of a substrate of P-gp; for example, the anticancer drugs etoposide and doxorubicin, the diuretic drug, spironolactone, and the anti-hypertensive drugs verapamil and nifedipine might have such effects. Furthermore, it should be clarified whether drug interaction of imatinib mesilate with P-gp enhanced antitumor activity of other anticancer drug in resistant cell, over-expression of P-gp. In conclusions, the present study suggests that the pharmacokinetics/pharmacodynamics of drugs that interact with P-gp may be affected by combination treatment with imatinib mesilate, which is transported via human P-gp and inhibits P-gp function.

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Legends for Figures

Fig.1. Effects of imatinib mesilate on viability of K562 cells (A), K562/DXR cells (B) and K562/VCR cells (C). Each value represents the mean ± S.D. of three independent measurements.

Fig. 2. Cellular accumulation of 10 μM imatinib mesilate by LLC-PK1 cells and L-MDR1 cells. Each column represents the mean ± S.D. of three independent measurements.

Fig. 3. Effect of 1 μM cyclosporin on the accumulation of 10 μM imatinib mesilate by LLC-PK1 cells and L-MDR1 cells. Each column represents the mean ± S.D. of three independent measurements.

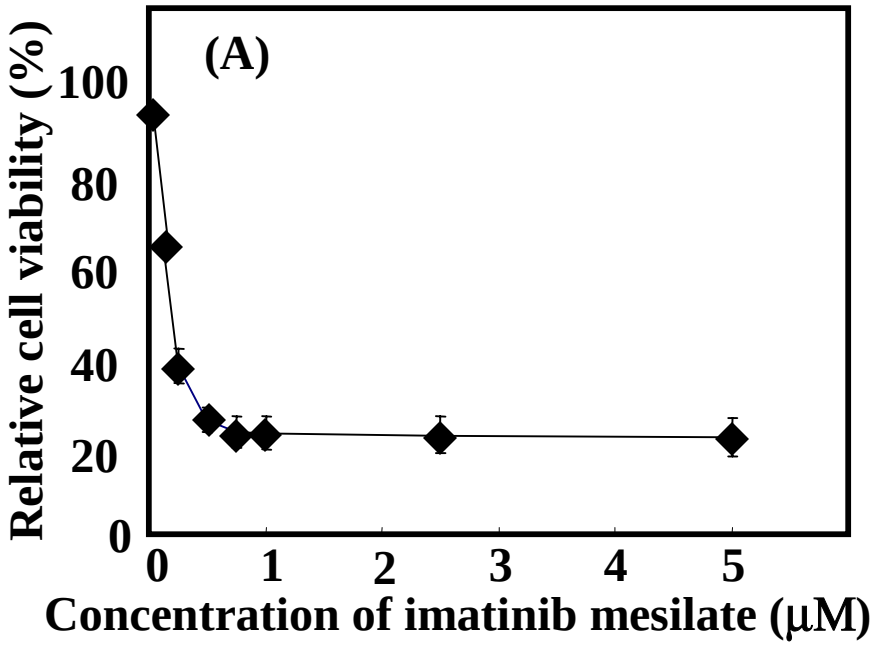
Fig. 4. Transcellular transport of 10 μM imatinib mesilate. Basal-to-apical transport (■) and apical-to-basal transport (◆) in LLC-PK1 cells (A) and L-MDR1 cells (B). Each point represents the mean ± S.D. of three independent measurements.

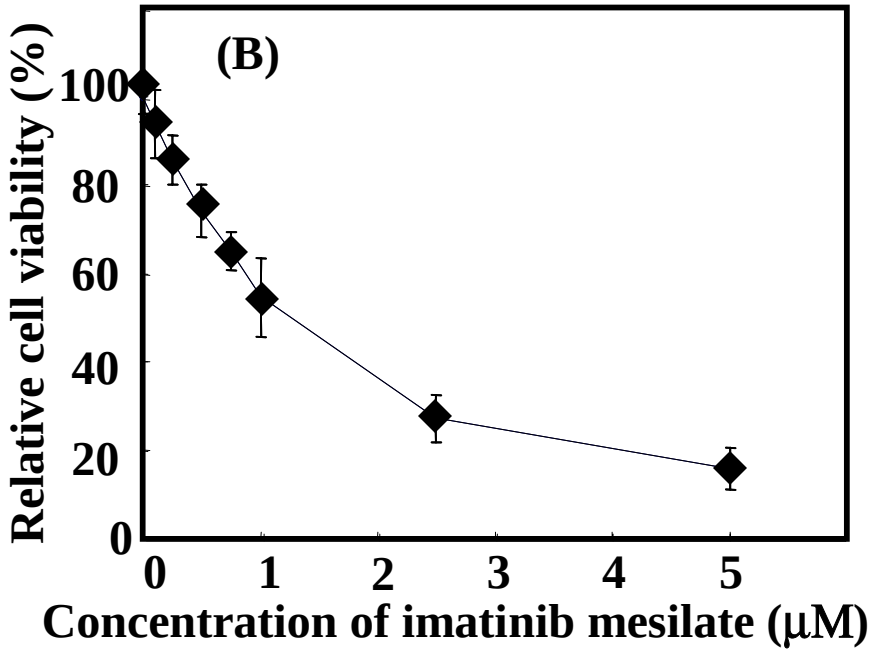
Fig. 5. Transcellular transport of 10 μM imatinib mesilate in cells treated with 10 μM Cyclosporin A. Basal-to-apical transport (■) and apical-to-basal transport (◆) in LLC-PK1 cells (A) and L-MDR1 cells (B). Each point represents the mean ± S.D. of three independent measurements.

Fig. 6. Effects of imatinib mesilate on cellular uptake of calcein-AM in LLC-PK1 and L-MDR1 cells monolayers. LLC-PK1 cells (□), L-MDR1 cells (△), L-MDR1 cells treated with 5 μM cyclosporin A (◆) and L-MDR1 cells treated with 5 μM imatinib mesilate (●). Each point represents the mean ± S.D. of three independent

measurements.

Fig. 7. Measurement of rhodamine 123 efflux as a functional test for evaluating the P-gp activity in K562 and K562/DXR cells in the presence of 10 μM cyclosporin (CyA) and 10 μM, 30 μM or 50 μM imatinib mesilate.





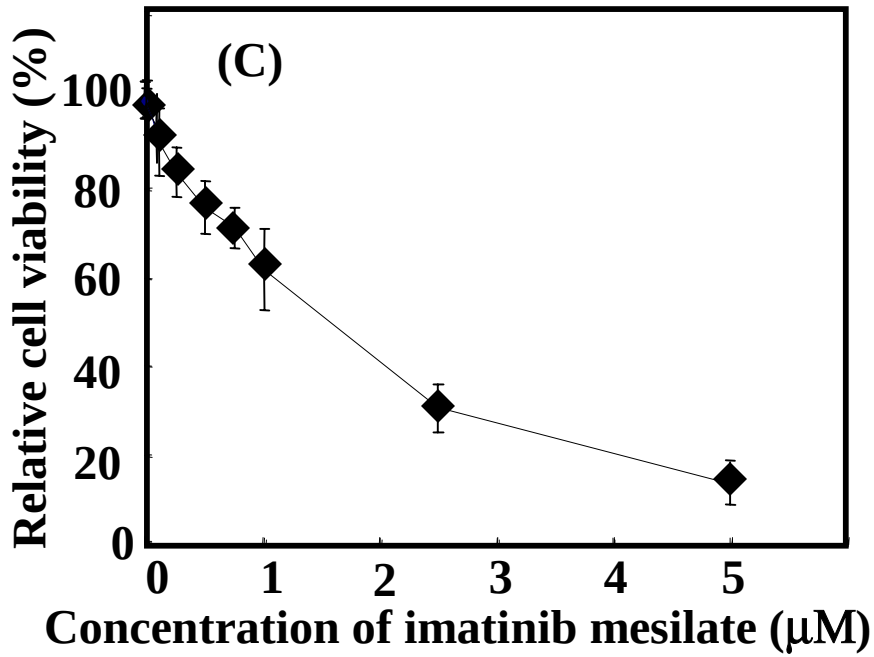


Fig.1

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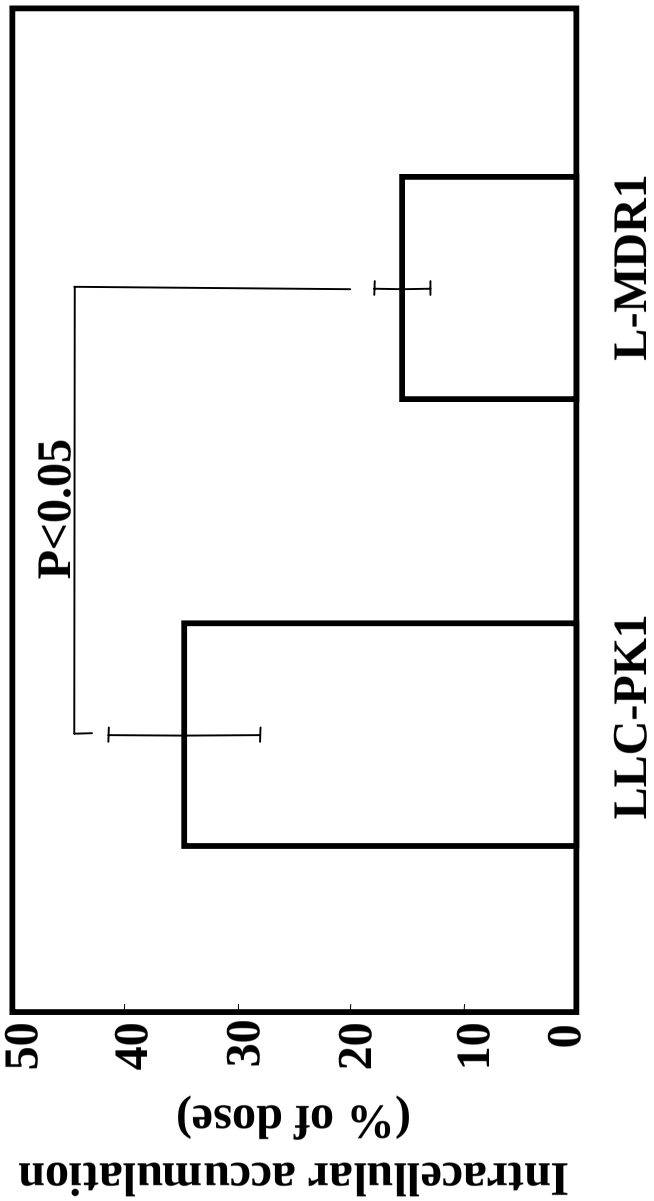

Fig.2

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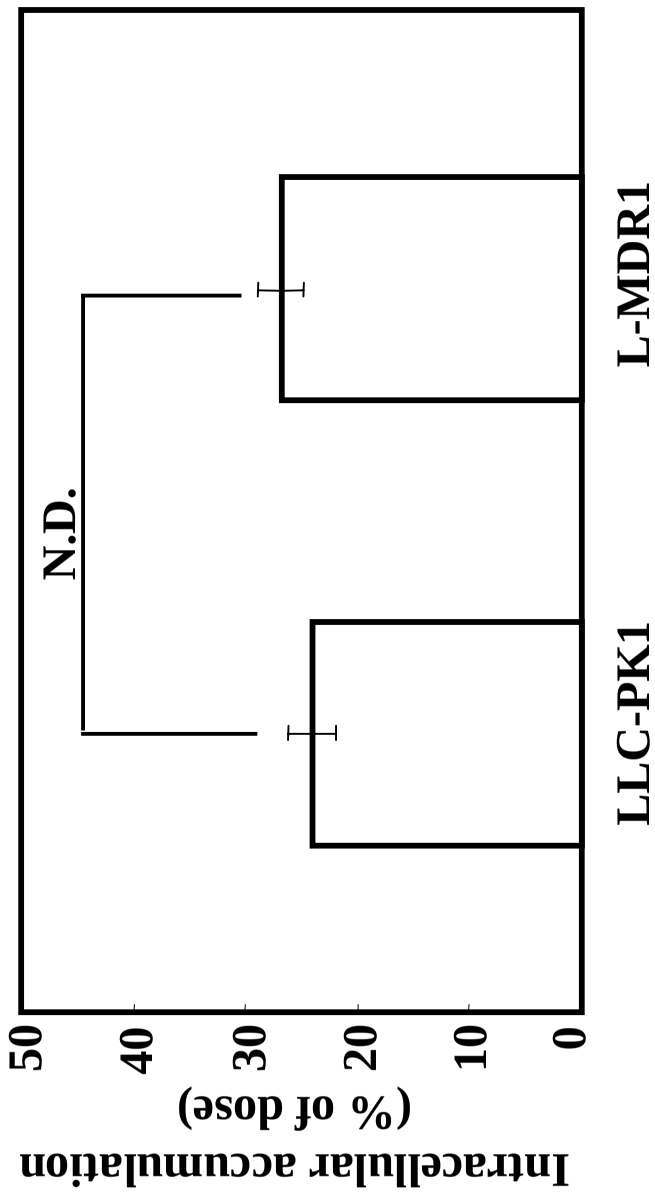

Fig.3

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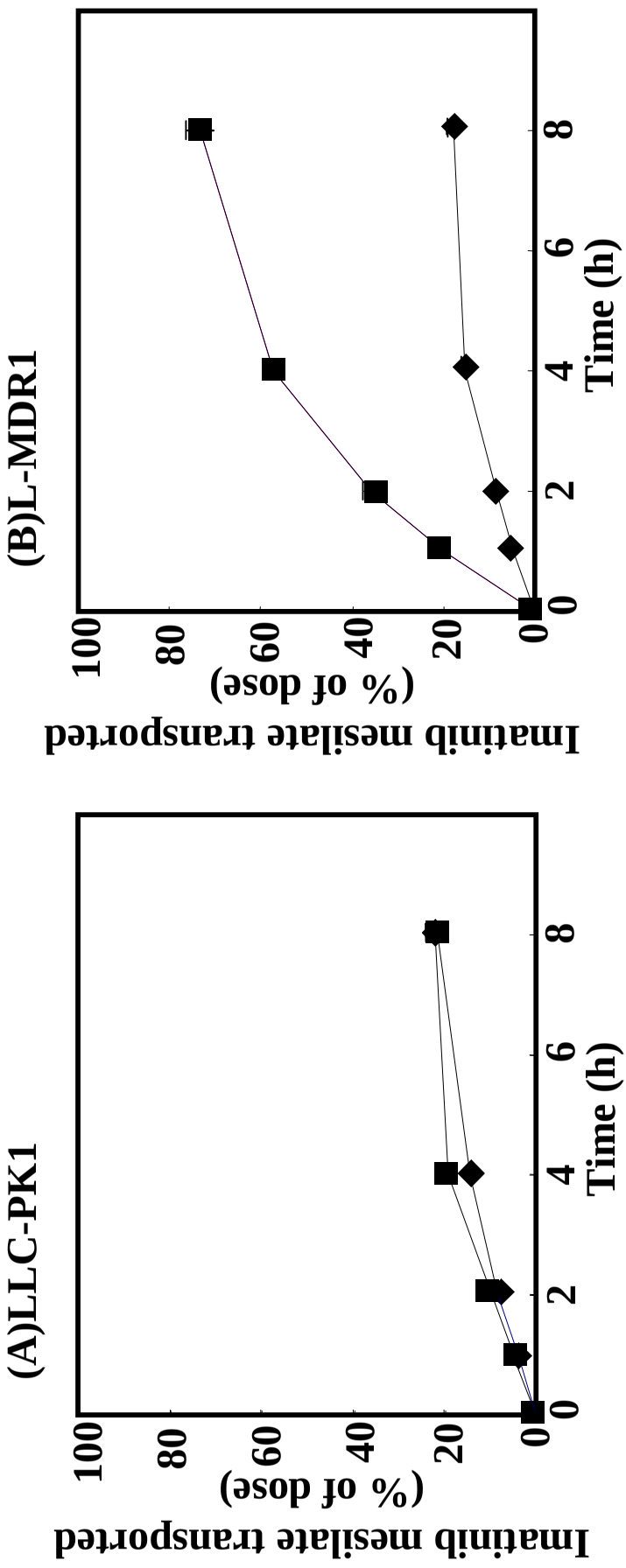

Fig.4

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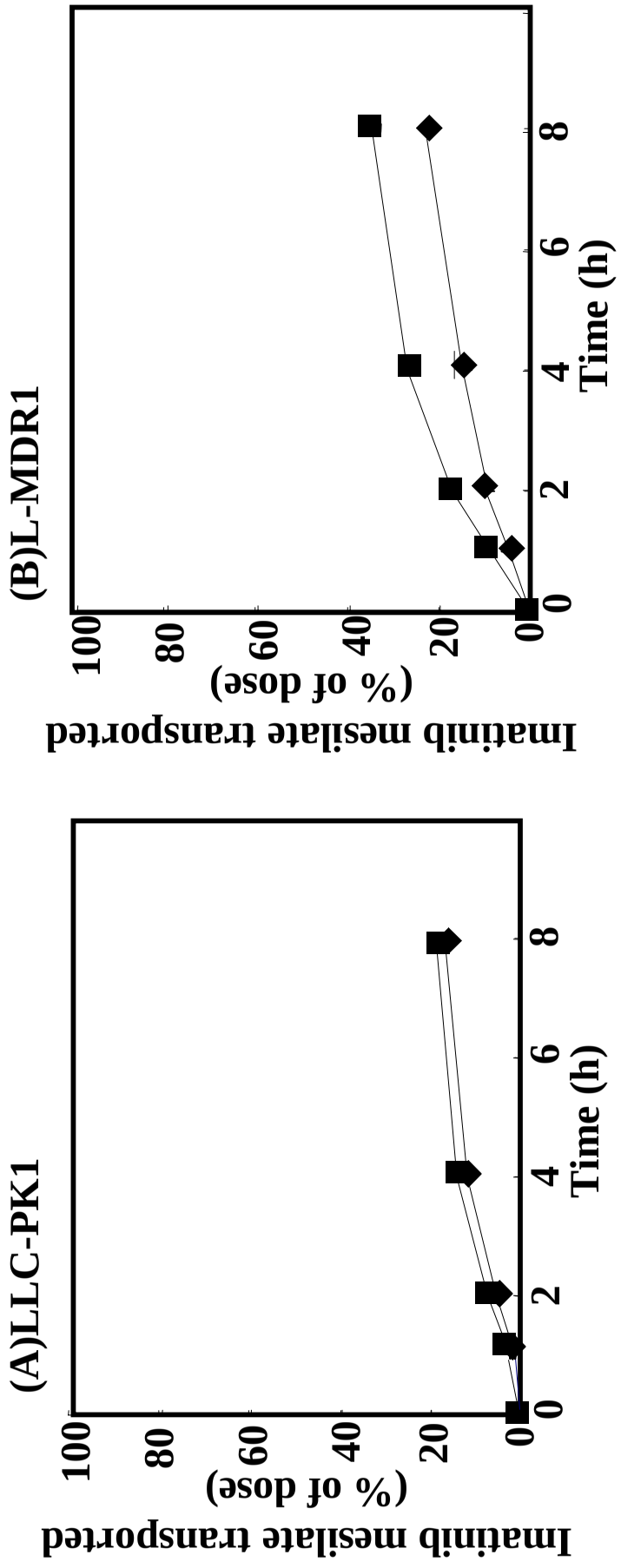

Fig.5

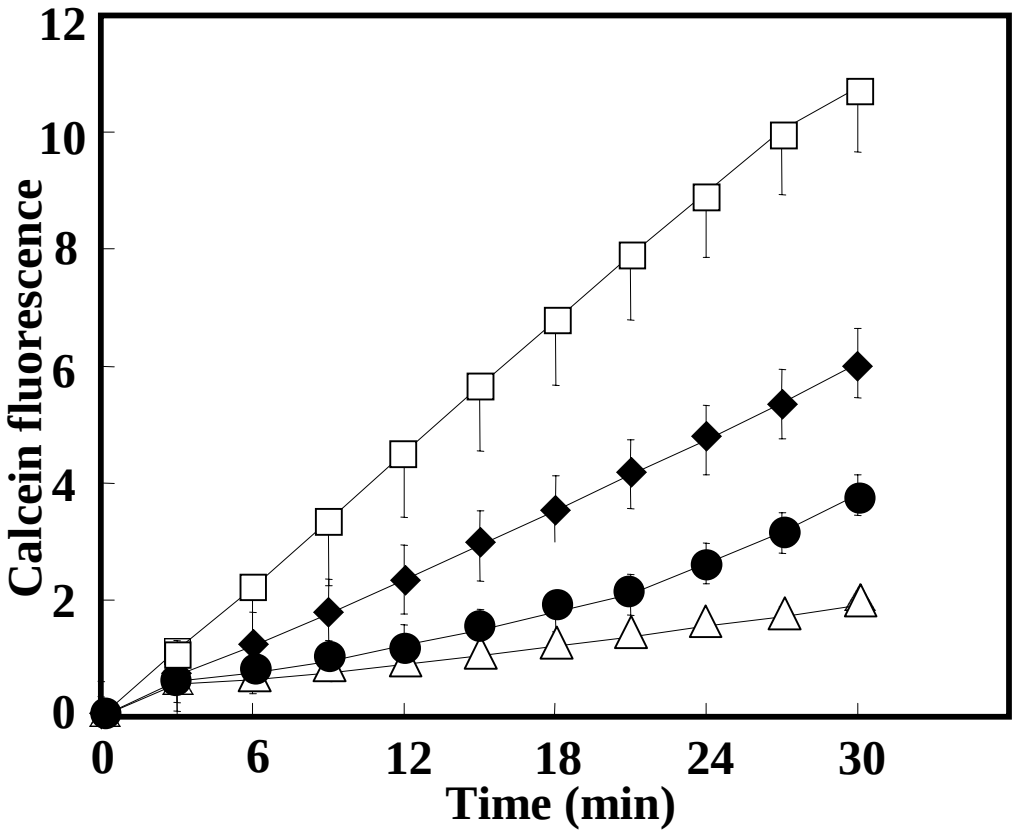

Fig.6

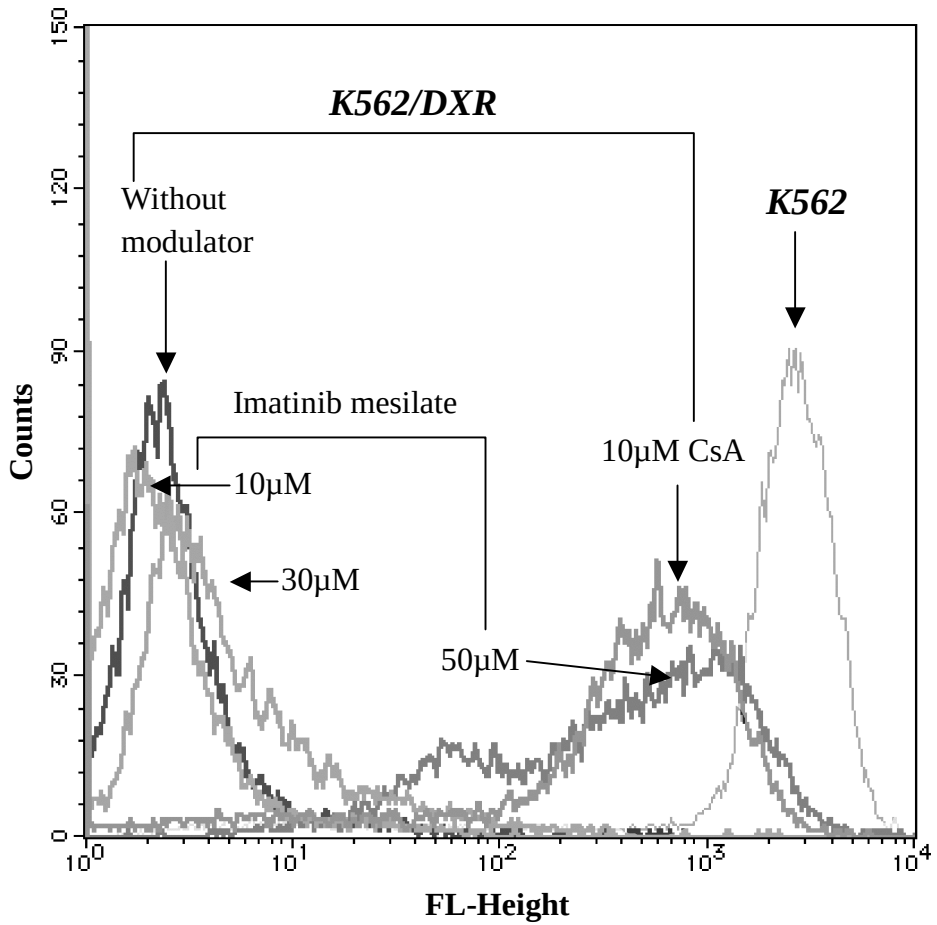

Fig.7