Distinct Interaction of Nilotinib and Imatinib with P-Glycoprotein in Intracellular Accumulation and Cytotoxicity in CML Cell Line K562 Cells

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Nilotinib, a second-generation tyrosine kinase inhibitor (TKI), has been approved for first-line chronic myeloid leukemia (CML) treatment. The improved clinical response of nilotinib over that of the first-generation TKI, imatinib, has been thought to be a result of its high potency of inhibition of BCR-ABL kinase. This study aimed to characterize differences between nilotinib and imatinib in the intracellular accumulation and cytotoxic effect on the CML cell line K562. Accumulation of nilotinib in K562 cells was from 4.7- to 9.0-fold higher than that of imatinib. The cytotoxic effect of nilotinib on K562 cells was 14.2-fold higher than that of imatinib. Inhibition experiments in K562 cells, and examination of the cellular uptake using influx transporter-transfected human embryonic kidney (HEK) 293 cells, suggested that the influx transporters OCT1 and OATP1A2, which have been reported to mediate accumulation of imatinib in CML cells, contributed little to the uptake of nilotinib. Nilotinib was found to accumulate in imatinib-resistant K562 (K562/IM) cells overexpressing the efflux transporter P-glycoprotein (P-gp), although cytotoxic assays showed that K562/IM cells displayed 20000-fold greater resistance to nilotinib over the parent K562 cells. In conclusion, the present findings suggest that intracellular accumulation of nilotinib in CML cells contributes to its clinical response and efficacy in CML patients. Although nilotinib has been reported to be effective against imatinib-resistant ABL kinase mutants, the drug could not overcome imatinib resistance acquired by P-gp overexpression. These results imply that classification of mechanisms of drug resistance is important for suitable strategies to treat imatinib-resistant CML patients.

Key words nilotinib; imatinib; chronic myeloid leukemia; P-glycoprotein

Chronic myeloid leukemia (CML) is a hematologic malignancy initiated by the unregulated activity of the BCR-ABL tyrosine kinase. Nilotinib (Tasigna, Novartis Pharmaceuticals) was developed as a second-line BCR-ABL tyrosine kinase inhibitor for the treatment of CML following approval of imatinib (Glivec, Novartis Pharmaceuticals). Although imatinib has remarkable efficacy with minimal side effects in CML, not all patients benefit from imatinib because of resistance, including point mutations in the ABL kinase domain of BCR-ABL. In vitro profiling showed that nilotinib has higher potency in terms of BCR-ABL kinase inhibition, and nilotinib is effective against imatinib-resistant ABL kinase mutants with the exception of T315I. Despite the structural similarity between nilotinib and imatinib, their pharmacological or pharmacodynamic properties are different. Thus, which of these two agents is more suitable for the treatment of CML patients? Nilotinib was approved by the Food and Drug Administration (FDA) in June 2010 for first-line CML treatment through the ENESTnd trial. The data showed that nilotinib elicited superior rates of molecular response and complete cytogenetic response by comparison to imatinib. It was thought that the different pharmacological properties between nilotinib and imatinib were, at least in part, a result of differential interaction with the membrane transport system of the target tumor cells. Drug transporters involved in the disposition of imatinib have been well studied because the expression or activity of these transporters influences individual variability of imatinib pharmacokinetics and clinical response. It was reported that intracellular uptake of imatinib in CML cells is driven by the organic cation transporter (OCT) 1, and expression of OCT1 is important in determining clinical response to imatinib. Previously, we reported that organic-anion transporting polypeptides (OATP) 1A2 also mediates the uptake of imatinib into the CML cell line K562 cells. Additionally, our previous study demonstrated that imatinib is transported by the ATP-binding cassette (ABC) transporter, P-glycoprotein (P-gp), and that overexpression of P-gp in K562 cells caused them to acquire resistance to imatinib. Moreover, upregulation in the P-gp expression level in CML cells from patients has been reported to correlate with increased resistance to imatinib.

Very little information is available on the drug transporters involved in the disposition of nilotinib. In CML cells, the OCT inhibitor prazosin had no effect on the intracellular uptake of nilotinib. It has been reported that the uptake of nilotinib was unchanged in the CML cell line KCL cells overexpressed OCT1. Although nilotinib is considered to be a substrate for P-gp and breast cancer resistance protein (BCRP), the

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membrane transporters involved in the accumulation of this drug in CML cells have not been identified.

This study aimed to characterize differences in the intracellular accumulation and cytotoxic effect in the CML-derived cell line K562 between nilotinib and imatinib. We evaluated membrane transport pathways involved in the intracellular accumulation of nilotinib. Furthermore, we examined the intracellular accumulation and cytotoxic effect of nilotinib in the imatinib-resistant CML cells overexpressing P-gp.

**MATERIALS AND METHODS**

**Uptake of Nilotinib and Imatinib into K562 or K562/IM Cells** K562 cells were cultured in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) at 37°C under 5% CO₂. Cells were washed with incubation medium (145 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 5 mM d-glucose and 5 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) (pH 7.4)) prewarmed to 37°C. Next, 2.5×10⁶ cells were pre-incubated in incubation medium at 37°C for 30 min. Incubation medium containing nilotinib or imatinib (both from Selleck Chemicals, Houston, TX, U.S.A.) at 2.5–10 μM was then added to the cells. After incubation for 3 min, cells were washed once in 3 mL ice-cold phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and twice in 3 mL BSA-free ice-cold PBS. Cells were then lysed by incubation in 300 μL of 50% methanol/high performance liquid chromatography (HPLC) mobile phase (29:21 mixture of 10 mM phosphate buffer and acetonitrile for nilotinib; 4:6:0.1 mixture of acetonitrile, water, and phosphoric acid for imatinib) for 24 h. The resultant cell lysate was centrifuged at 12000×g and 4°C for 10 min. The concentration of nilotinib or imatinib in the supernatant was quantified using previously published HPLC method. A solution of 1 N NaOH was added to the cell pellet to solubilize the cells and the protein content was subsequently determined using a BioRad Protein Assay kit (Bio-Rad, Hercules, CA, U.S.A.).

The cellular uptake of nilotinib or imatinib was evaluated using Eadie–Hofstee plots. Experiments were conducted by incubating the cells in incubation medium containing nilotinib (1–10 μM) or imatinib (2.5–100 μM) for 3 min at 37°C. The influx of a substrate across cell membrane via transporter is characterized by saturability. The Kₘ and Vₘₐₓ for nilotinib and imatinib uptake were estimated according to the Eadie–Hofstee equation:

\[ V = -\frac{K_m \cdot V}{[S]} + V_{max} \]

where V is the amount of nilotinib or imatinib that is transported into K562 cells; Kₘ is the Michaelis–Menten constant; [S] is the extracellular concentration of the substrate; Vₘₐₓ is the maximum nilotinib or imatinib accumulation.

Inhibition studies were also conducted using K562 cells for the purpose of investigation of the major influx transporters for nilotinib in CML cells. In these experiments, K562 cells were incubated in incubation medium containing 5 μM nilotinib or 50 μM imatinib in the absence or presence of 100 μM tetraethylammonium (TEA) or naringin for 3 min at 37°C. TEA is reported to inhibit OCT, including OCT1, OCT2, OCT3 and OCTN2, while naringin is reported to inhibit OATP, including OATP1A2 and OATP2B1.

We previously established imatinib-resistant K562 (K562/IM) cells that acquired drug resistance, in part, due to the overexpression of P-gp. DNA sequencing showed no genetic mutations at the entire kinase domain of the ABL coding region in K562 and K562/IM cells. P-gp was overexpressed in K562/IM cells relative to K562 cells. Cyclosporin A (CsA), a P-gp inhibitor, restored both imatinib-sensitivity and the intracellular imatinib level. These findings indicated that P-gp was responsible for the development of imatinib-resistance in K562/IM cells. Incubation medium containing 2 μM (trough imatinib plasma levels in patients receiving standard-dose 400 mg/d imatinib) nilotinib or imatinib was added to 2.5×10⁶ K562 or K562/IM cells. After incubation for 10 min, cells were washed. Nilotinib, imatinib, and protein content were then quantified using the methodology described earlier.

**Cellular Uptake Study of Nilotinib and Imatinib in HEK293 Cells** The uptake of nilotinib and imatinib into cells overexpressing influx transporters was also evaluated. In these studies, genes encoding the transporters were transiently expressed in human embryonic kidney (HEK) 293 cells. HEK293 cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 10% FBS at 37°C under 5% CO₂. After 24 h cultivation in Poly-D-Lysine-Coated 6-well plates (Iwaki, Tokyo, Japan), 4 μg of pCMV6XL4, pCMV6XL4/OCT1, pCMV6XL5 or pCMV6XL5/OATP1A2 vector (Origene Technologies, Rockville, MD, U.S.A.) was transfected by adding 10 μL/well of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.). Medium was changed 6 h after transfection. At 48 h after transfection, cells were washed twice in prewarmed PBS at 37°C. Cells were incubated in 1 mL of incubation medium containing 2 μM nilotinib or imatinib. After an incubation time of 1 min at 37°C, cells were washed once in 5 mL ice-cold PBS containing 1% BSA and twice in 5 mL BSA-free ice-cold PBS. The cells were then lysed by incubating in 500 μL of 50% methanol/ HPLC mobile phase for 24 h. Nilotinib, imatinib, and protein content were then quantified using the methodology described earlier for the K562 cells. OCT1 and OATP1A2 protein expression was confirmed Western blot analysis in transfected HEK293 and K562 cells using the same methods in previous study.

**Cytotoxicity Assay** To quantify the cytotoxic effect of nilotinib and imatinib on cell growth, AlamarBlue™ was used in a semiautomated fluorescence method as described previously. Briefly, K562 and K562/IM cells were plated at 1.0×10⁴ cells/well in 96-well plates. Culture medium containing drug at the desired concentration was added, and the cells were then cultured for 48 h. AlamarBlue™ was added to each well and fluorescence was measured after 4 h incubation. Relative cell viability (%) was expressed as a percentage of the fluorescence observed using untreated cells. IC₅₀ (inhibitory concentration at 50%) was plotted using the following equation:

\[ IC_{50} = -10^{C \cdot \log (B)} \]

Two plot points that contain an inhibitory concentration at 50% were used. A is the concentration of drug that exceeded the amount required to give 50% cell viability. B is the concentration of drug lower than the amount required to give 50% cell viability. C is cell viability (%) at B. D is cell viability (%) at A.

**Statistical Analyses** Statistical analyses of transport ex-
Experiments for nilotinib and imatinib were adjusted for multiple comparisons using Dunnett’s method. Difference in uptake of nilotinib or imatinib between K562 and K562/IM cells was analyzed by using the Wilcoxon rank sum test. Two-tailed \( p \) values \(<0.05\) were considered statistically significant. Statistical analyses were performed using the R program v.2.7.1 (http://cran.r-project.org).

RESULTS

Uptake of Nilotinib and Imatinib into K562 Cells

Accumulation of nilotinib in K562 cells was markedly different from that of imatinib (Fig. 1A). Specifically, the accumulation of nilotinib (15.9–120.2 \( \mu \)M) was 4.7- to 9.0-fold higher than that of imatinib (3.4–13.3 \( \mu \)M) at each concentration of drugs (2.5–10 \( \mu \)M). Uptake of nilotinib in K562 cells increased with the substrate concentration, although the uptake profile did not fit the Eadie–Hofstee equation (Fig. 1B). By contrast, the uptake of imatinib into K562 cells increased in a concentration-dependent manner, which fitted the Eadie–Hofstee equation (Fig. 1C; \( V = -56.6 \cdot V/[S] + 83.3, R^2=0.90 \)). The estimated apparent \( K_m \) and \( V_{max} \) values for imatinib uptake in K562 cells were 56.6 \( \mu \)M and 83.3 nmol/3 min/mg protein, respectively.

Uptake of Nilotinib and Imatinib into HEK293 Cells Transfected with Influx Transporters

Nilotinib uptake was significantly enhanced in HEK293 cells expressing OCT1 compared with corresponding control cells transfected with pCMV6XL4 (Fig. 2A). Imatinib uptake was significantly enhanced in HEK293 cells expressing OCT1 compared with control cells transfected with pCMV6XL4 (Fig. 2B). Moreover, the uptake of imatinib was significantly enhanced in HEK293 cells expressing OATP1A2 compared with control cells transfected with pCMV6XL5. The accumulation of nilotinib or imatinib was decreased after incubation with the HEK293 cells expressing OCT1 in the presence of TEA. The accumulation of imatinib was decreased after incubation with the HEK293 cells expressing OATP1A2 in the presence of naringin.

Inhibition of Transporter-Mediated Uptake in K562 Cells

We evaluated the effects of inhibitors on uptake of nilotinib and imatinib in K562 cells (Fig. 3). OCT inhibitor TEA had no inhibitory effect on the uptake of nilotinib, whereas ni-
lotinib uptake was significantly decreased by OATP inhibitor naringin. Imatinib uptake was significantly decreased by the presence of either TEA or naringin.

**Uptake of Nilotinib and Imatinib into K562 or K562/IM Cells**

We compared the uptake of nilotinib or imatinib into K562 cells with that into K562/IM cells. There was no significant difference in the uptake of nilotinib between K562 and K562/IM cells (Fig. 4A). By contrast, uptake of imatinib into K562/IM cells was reduced to 77% compared to that into K562 cells (Fig. 4B).

**Cytotoxicity Assay**

We evaluated sensitivity of K562 and K562/IM cells to nilotinib or imatinib using the AlamarBlue™ fluorescence method (Table 1). The cytotoxic effect of nilotinib on K562 cells was 14.2-fold higher than that of imatinib. However, K562/IM cells were 2.0×10^4-fold more resistant to nilotinib compared to the parent K562 cells. K562/IM cells showed a 56.2-fold increase in resistance to imatinib over K562 cells. These cytotoxic effects of nilotinib and imatinib on K562 and K562/IM cells were enhanced by P-gp inhibitor CsA.

**DISCUSSION**

The present study demonstrates that nilotinib accumulates extensively in K562 cells than imatinib. The cytotoxic effect of nilotinib on K562 cells was much higher than that of imatinib, although this did not overcome resistance to imatinib acquired by P-gp-overexpression.

The accumulation of nilotinib in K562 cells was 4.7- to 9.0-fold higher than for imatinib (Fig. 1A). It was reported that the intracellular concentration of nilotinib in gastrointestinal stromal tumor cells was 7- to 10-fold greater than that of imatinib. Similarly, nilotinib is thought to accumulate at high levels in CML cells compared with imatinib. Previously, it has been assumed that good clinical response of nilotinib is brought about by potent inhibition of BCR-ABL kinase. Here, we report that robust intracellular accumulation of nilotinib in CML cells could contribute, at least in part, to good clinical response in CML patients.

We also addressed the question of why nilotinib accumulates at high levels in K562 cells. Specifically, we examined the concentration-dependent uptake of the two drugs into K562 cells. The uptake of imatinib fitted the Eadie–Hofstee equation (Fig. 1C), whereas the uptake of nilotinib uptake did not (Fig. 1B). This phenomenon might be caused by saturation of the efflux transporter by nilotinib. Low-affinity transport or low-expression of efflux transporter, including P-gp, was...

![Fig. 3. Inhibitory Effects of Transport Inhibitors on the Uptake of Nilotinib or Imatinib into K562 Cells](image)

The cells were incubated for 3 min with 2 µM nilotinib or 2 µM imatinib in the absence or presence of 100 µM inhibitor. TEA, tetraethylammonium. *p<0.05 versus control. Data are expressed as mean values±S.D. (n=3).

![Fig. 4. Uptake of Nilotinib (A) and Imatinib (B) into K562 or K562/IM Cells](image)

Two micromolar nilotinib or 2 µM imatinib was added to K562 or K562/IM cells, and the cells were incubated for 10 min. *p<0.05 versus control. Data are expressed as mean values±S.D. (n=3).

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<th>Nilotinib</th>
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<td>8.1×10⁻⁴</td>
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<td>16.75</td>
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CsA, cyclosporin A. Data are expressed as mean values (n=3).
assumed to account for the observed accumulation of nilotinib in K562 cells.

Key questions remain regarding details of the influx of nilotinib that contribute to high level intracellular accumulation of nilotinib in CML cells. It has been reported that OCT1 and OATP1A2 may be the principal transporters for mediating the uptake of imatinib into CML cells.\(^1\)\(^,\)\(^2\)\(^,\)\(^3\)\(^,\)\(^4\)\(^,\)\(^5\)\(^,\)\(^6\) Indeed, this conclusion would be consistent with the results of our present study (Fig. 2B). However, uptake of nilotinib was enhanced only by OCT1, but not by OATP1A2 (Fig. 2A). The finding that nilotinib is a transport substrate of OCT1 disagrees with the conclusions of a previous report of Davies et al., who found that nilotinib transport was not enhanced in KCL cells expressing OCT1 when the cells were incubated with nilotinib for up to 2h.\(^6\)\(^,\)\(^7\) However, the relatively long incubation period used in the study by Davies et al. might result in saturation of the nilotinib transport via OCT1. We used a shorter incubation period of 1 min, thereby resulting in a significant increased uptake of nilotinib.

Inhibition studies in K562 cells showed that TEA had no inhibitory effect on the uptake of nilotinib, whereas nilotinib uptake was significantly decreased by naringin (Fig. 3). By contrast, imatinib uptake was significantly decreased in the presence of either TEA or naringin. The latter result is consistent with the reports that suggested OCT1 and OATP1A2 are the major influx transporters for imatinib in CML cells.\(^1\)\(^,\)\(^2\)\(^,\)\(^8\)\(^,\)\(^9\) This study suggests that OCT1 has little effect on the cellular uptake of nilotinib into CML cells, even though OCT1 may be responsible for nilotinib uptake in OCT1 overexpressing cells. White et al. also found that the OCT1 inhibitor prazosin has no effect on nilotinib uptake in cells derived from CML patients.\(^8\) Though uptake of nilotinib was not enhanced in HEK293 cells expressing OATP1A2, an OATP inhibitor naringin significantly decreased nilotinib uptake into K562 cells. We believe other transporters that were not evaluated in this study may facilitate nilotinib uptake. Naringin is reported to be a typical OATP inhibitor that inhibits not only OATP1A2\(^8\) but OATP2B1.\(^2\) Further studies are required to establish whether other transporters are involved in the membrane transport process of nilotinib in CML cells.

Next, we attempted to determine the following: (i) does nilotinib accumulate in imatinib-resistant K562 cells overexpressing P-gp? (ii) does nilotinib overcome imatinib resistance acquired by P-gp-overexpression? There was no significant difference in uptake of nilotinib between K562 and K562/IM cells (Fig. 4A), though uptake of imatinib into K562/IM cells was significantly reduced compared to that observed for K562 cells (Fig. 4B). The cytotoxic effect of nilotinib on K562/IM cells, however, proved contrary to expectations (Table 1). The K562/IM cells were found to be 2.0×10^-4-fold more resistant to nilotinib than the parent K562 cells. The cytotoxic effect of nilotinib on K562/IM cells was partially restored by P-gp inhibitor cyclosporin A, suggesting that overexpression of P-gp might be associated with acquired resistance to nilotinib in K562/IM cells. We hypothesized that the influx of nilotinib was enhanced in K562 cells overexpressing P-gp when the cells were incubated with nilotinib for only 10min, whereas nilotinib was excreted from the cells via P-gp before nilotinib caused a cytotoxic effect. It is assumed that uptake of nilotinib into K562/IM cells is reduced via P-gp compared to that into K562 cells when the incubation period was longer. Several previous studies have demonstrated that the cytotoxic effect of nilotinib in K562 cells overexpressing P-gp is decreased compared with that in the parent K562 cells,\(^8\)\(^,\)\(^9\)\(^,\)\(^10\) and P-gp overexpression is one of the factors responsible for the development of nilotinib resistance.\(^10\) The present study was limited within the in vitro experiments in CML cell line, and focused on the interaction of nilotinib and imatinib with P-gp. Further investigation is required to reveal whether the interaction of nilotinib and imatinib with P-gp or unclear mechanism affects therapeutic effects or side effects in CML patients.

In conclusion, this study suggests that nilotinib markedly accumulates in K562 cells compared with imatinib. Our results indicate that the high levels of uptake of nilotinib might contribute to its potent cytotoxicity against CML cells. Nilotinib has been reported to be effective against imatinib-resistant ABL kinase mutants, but this could not overcome imatinib resistance acquired by P-gp-overexpression. These results imply that classification of mechanisms of drug resistance is important for suitable strategies to treat imatinib-resistant CML patients.

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