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Continuous cytostatic effects of BCR-ABL tyrosine kinase inhibitors (TKIs) after washout in human leukemic K562 cells

Tsuyoshi Aoyama,a Yoshihiko Shibayama,b Tatsuhiko Furukawa,c Mitsuru Sugawaraa and Yoh Takakuma*,a

aLaboratory of Pharmacokinetics, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060–0812, Japan.
bDepartment of Drug Formulation, Faculty of Pharmaceutical Sciences, Health Sciences University, Tobetstu 061-0293, Japan.
cDepartment of Molecular Oncology, Graduate School Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan.

Corresponding author’s e-mail address: y-kuma@pharm.hokudai.ac.jp
[Abstract]

Tyrosine kinase inhibitors (TKIs) are used as the first choice for chronic myeloid leukemia (CML) pharmacotherapeutics. Some patients taking these drugs showed good therapeutic reactivity despite the disappearance of drugs from blood. We investigated whether these drugs have sustained effects even after their disappearance and whether their effects depend on their amounts of intracellular accumulation. Cell proliferation after exposure of K562 cells or Multidrug resistance-1 (MDR-1)-transfected K562 cells was determined by a cell counting kit-8 assay. The intracellular accumulation amount of the drug showing a sustained cytostatic effect was measured by ultra performance liquid chromatography-mass spectrometry. Cell viability decreased in a culture time-dependent manner after washing out nilotinib and dasatinib. The sustained cytostatic effect of dasatinib, but not that of nilotinib, correlated with the intracellular accumulation level. In contrast, imatinib showed continuous a cytostatic effect after drug washout for long-term exposure but not after drug washout for short-term exposure. These results suggest that a good response in patients with a low serum concentration of imatinib, nilotinib or dasatinib may be due to the cytostatic effect of that drug continues even after its disappearance in plasma.

Keywords: Tyrosine kinase inhibitors, Cytostatic effect, Multidrug Resistance protein-1, Chronic myeloid leukemia, P-glycoprotein
Chronic myelogenous leukemia (CML) is a type of blood cancer in which primarily neutrophils proliferate randomly and form blasts due to tumorigenesis of pluripotent stem cells. An abnormal chromosome called Philadelphia chromosome (Ph+ chromosome) is present in cases of CML.1,2) As a mechanism of CML onset, it has been proposed that the Ph+ chromosome, which is formed by mutual translocation of chromosomes 9 and 22th, is triggered and that the BCR-ABL fusion protein, which is the final product of it, has persistent tyrosine kinase activity and causes abnormal proliferation of leukemia cells such as blasts, and resulting in the development of CML.3) For CML drug therapy before the 2000s, interferon-α (INF-α) alone or in combination with chemotherapy was mainly used. However since the side effects were greater than the therapeutic effect of these therapies, long-term treatment has been difficult. Imatinib, a BCR-ABL tyrosine kinase inhibitor (TKI), became available in 2001. Not only the therapeutic effect but also the safety of treatment with this new drug was increased dramatically.4, 5)

BCR-ABL TKIs are now used as first-line drug treatment of CML in Japan. The development of TKIs such as imatinib has dramatically improved the effectiveness and safety of treatment, but inter-individual variation in the therapeutic effect has been observed in about 30% of patients.5,6) Since these drugs are oral formulations, therapeutic reactivity is often affected by the compliance situation. In fact, previous studies showed that there was a correlation between the compliance with imatinib treatment and therapeutic effect of imatinib.7, 8) On the other hand, many clinical trials are currently being carried out to determine whether the therapeutic effects of imatinib, nilotinib and dasatinib are sustained after administration of the drugs has been discontinued in patients in whom therapeutic effects of the drugs were achieved for a certain period of time. However, the results of those trails showed that relapse occurred in about 40-60% of the subjects within those trials.9-11) Those clinical trials were based on the theory that the therapeutic effect is sustained after discontinuation of drug administration in cases in which a therapeutic effect has been achieved by drug treatment for a certain period of time, but the therapeutic effect was not sustained and treatment had to be started again in many of participants in those trials.9, 11) Therefore, the current strategy for maintaining the therapeutic effect is continuous treatment in most cases. Although it has been shown that it is difficult to maintain a therapeutic effect by discontinuous of treatment, it has not been investigated whether it is possible to maintain a therapeutic effect by dose reduction or by administration every other day after obtaining certain effects.9,11,12) Therefore, we considered that it is important to determine whether the cytostatic effect of TKI can be maintained not by complete discontinuous of drug administration but by intermittent administration of the drug. The intracellular concentration of a TKI is important for its cytostatic effect, and TKIs are substrates of MDR-1 (P-glycoprotein), which is also a cause of resistance to various drugs. Previous studies have
also shown a relationship between therapeutic effect and MDR-1 overexpressions.\textsuperscript{13-17} Therefore, it is also necessary to consider the possibility that MDR-1, which is responsible for tolerance of treatment with TKIs, affects maintenance of the therapeutic effect by administration every other day.

In this study, we examined whether TKIs have cytostatic effects in the plasma concentration range of TKIs observed clinically.

Regarding the therapeutic effect of TKIs, we investigated whether K562 cells derived from human chronic myelogenous leukemia were exposed to TKIs for a short time and whether the cytostatic effect of the TKI persisted after removing the drug by washout.

[Materials and Methods]

**Inhibitors**

Imatinib mesylate, nilotinib and dasatinib were purchased from Ark Pharm, Inc (USA). Imatinib and nilotinib were prepared in 500 μg/mL stock solution in DMSO and dasatinib was prepared in 50 μg/mL stock solution and stored at -20 °C. Dilution of the preservation solution was carried out immediately before each experiment.

**Cell lines**

Cell lines used in this study were purchased from RIKEN (Ibaraki, Japan). Human chronic myeloid leukemia K562 cells (parent K562 cells) and hMDR-1-transfected K562 cells were cultured in RPMI 1640 medium containing 10% FBS and 500 μg/mL penicillin sodium and streptomycin at 37 °C in a 5% CO\textsubscript{2} atmosphere. Expression of MDR-1-mRNA was confirmed by qPCR.

**RNA isolation and qPCR**

Total RNA was extracted using RNAiso Plus (Takara Bio Inc, Shiga, Japan). cDNA synthesis was carried out from the extracted total RNA using a ReverTra Ace qPCR R Kit (Toyobo Co. Ltd, Osaka, Japan). Primer sequences were 5’ TTGTTCAGGTGGCCTCTGGAT’3 and 5’CTGTAGACAAACGATGAGCTATCA’3 for hMDR-1. PCR products were separated on 2% agarose gels in TAE buffer and stained with ethidium bromide.

**Rhodamine123 (Rh123) accumulation assay**

Two hundred µL of a cell suspension (1×10\textsuperscript{7} cell/mL) was added to a microtube, and Rh123 solution (at a final concentration of 5 or 10 µM) was added. After exposure to Rh123, cell suspension was centrifuged at 1000 rpm for 3 minutes at 4 °C, and the supernatant was aspirated. The Rh123 in the sample was then washed out using Transport buffer (25 mM HEPES, 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\textsubscript{2}, 0.8 mM MgSO\textsubscript{4}, 5 mM glucose,
pH7.5). Following complete removal of the supernatant, cells were lysed with 0.1 M NaOH. Fluorescence intensity was measured with a microplate reader at a wavelength of Em 488, Ex 530 nm. The measured intracellular accumulation was expressed as a numerical value (nM/mg protein) corrected by the protein amount calculated by the Lowry method.

Cell viability assay

Cell viability was assessed using a Cell counting kit-8 (Dojindo, Kumamoto, Japan). Briefly, cells were seeded at 1 × 10^4 cells in a 96-well microplate and the drug solution or drug-free medium was added. After drug exposure for 24, 48, 72 and 96 hr, CCK-8 assay solution was added to each well and incubated at 37 °C for 3 hr. Then the absorbance was measured with a microplate reader at a wavelength of 450 nm. Finally, a growth curve was drawn.

Washout methods

A cell suspension was seeded at 1 × 10^4 cells in a 96-well microplate. A drug solution or drug-free medium was added and the cells were exposed to the drug solution or drug-free medium 0.5 or 24 hr. Then the cell solution of each well was washed with a fresh medium at 37°C to a concentration not affecting cell proliferation. Finally, the cells after washing and removal were again cultured for a certain period of time, and a cell viability assay was performed.

Intercellular accumulation of TKIs by UPLC/MS/MS

A cell suspension was seeded in a 48-well microplate at 1 × 10^6 cells. A drug solution was added, and the cell suspension was exposed to drug solution for 0.5 or 24 hr. Then the cell suspension was collected and centrifuged at 1000 rpm for 3 minutes at 4°C, and the supernatant was aspirated. The drug in the medium was then washed out using phosphate buffered saline (PBS). Following complete removal of the supernatant, the residue was resuspended with phosphate buffer and then frozen and crushed at -80 °C. After thawing the cell sample, the cell sample was added to imatinib (or dasatinib) as an internal standard (at a final concentration of 100 ng/mL), 2 mM Tris and ethyl acetate: 1-butanol (4:1) and the mixture was stirred for 30 seconds. The mixture was then centrifuged at 21500 × g for 20 minutes at 4 °C. The supernatant was separated and dried under reduced pressure at 45 °C for 1 hr. Then the sample was resuspended in the mobile phase and measurement was carried out by UPLC/MS/MS (Waters Corporation, USA). Compounds were quantified with MRM. The measured intracellular accumulation was expressed as a numerical value (ng/mg protein) corrected by the protein amount calculated by the Lowry method.
Statistical analysis

All statistical analyses were carried out using Origin. Quantitative data are presented as means ± SD. Welch's t-test was used to compare the difference between the two groups. \( p < 0.05 \) was considered statistically significant.

[Results]

Long-term exposure of TKIs for K562 cells

The viability of K562 cells after long-term exposure to TKIs within clinically observed plasma concentration ranges was evaluated in order to confirm the concentrations of TKIs that have no inhibitory effect on the proliferation of cells. Imatinib completely inhibited cell proliferation at 100 ng/mL, whereas it did not affect cell proliferation at low concentrations of 10 ng/mL or less (Fig.1A). The cell proliferation observed at these concentrations was maintained from 24 hr to 96 hr and the time dependence of the effect was also confirmed.

Unlike Imatinib, Nilotinib and dasatinib, which are second-generation TKIs, completely inhibited cell proliferation at low concentrations (Fig.1B, nilotinib: 10 ng/mL; Fig.1C, dasatinib: 0.1 ng/mL). Concentrations that did not affect cell proliferation were also lower than that of imatinib (\( \leq 0.01 \) ng/mL). It was confirmed that the concentrations at which these two agents do not affect cell proliferation were both 0.01 ng/mL.

Continuous cytostatic effect of TKIs after washout

K562 cells were exposed to each of the TKIs for 0.5 hr, and each of the drugs was washed out from the culture medium until it reached a concentration that was shown in long-term exposure experiments to have no effect on cell proliferation. It was then examined whether the inhibitory effect of each TKI on cell proliferation was maintained when the cells were cultured again.

When cells were exposed to imatinib at the indicated concentration for 0.5 hr and cultured after washout, there was no exposure concentration-dependent effect on cell viability (Fig. 2A). However, when cell were exposed to imatinib at a high concentration of 1,000 ng / mL or more for 24 hours to drug washout, a continuous cytostatic effect was observed for up to 48 hours of culture (Fig.3A and B).

There was a cell viability of almost 100% when cells were exposed to nilotinib at concentrations in the range of 0.01 to 10 ng / mL, but cell proliferation was inhibited at 1000 ng/mL or higher (Fig. 2B). The effect was maintained at all incubation time points observed.

Cell exposed to dasatinib showed no decrease in cell viability with time over the exposure concentration range of 0.01 to 12.5 ng/mL. At exposure concentrations of 50 and 100 ng/mL, cell viability decreased until 48 or 72 hr after washout, but cell proliferation was observed at a later time. It was confirmed that dasatinib maintained its cell proliferation inhibitory effect
Next, in order to determine whether MDR-1 contributes to the persistent cytostatic effect observed with short-term exposure to each of the TKIs, similar experiments were carried out using hMDR-1-transfected K562 cells and parent K562 cells. Before evaluating the effect of MDR-1 on cell viability, the levels of hMDR-1 mRNA expression and the transport activities of K562 cells and hMDR-1-transfected K562 cells were examined by qPCR and by the intracellular amounts of Rh123 (Supplemental Figs.1 and 2). There was no significant difference in cell viability between parent K562 cells (wild type) and hMDR-1-transfected K562 cells when the cells were exposed to nilotinib at 1,000 ng/mL (Fig.3A). When the cells exposed to nilotinib at a concentration of 2,500 ng/mL, a significant difference was observed between the two groups at 24, 72 and 96 hr of culture (Fig.3B, *p < 0.05).

On the other hand, when the cells were exposed to dasatinib at a concentration of 50 ng/mL exposure, a significant difference was observed between the two groups of cells until 48-72 hr of culture, but no difference was observed between the two groups at 96 hr of culture.

When the cells were exposed to dasatinib at a concentration of 100 ng/mL, a significant difference was observed between the two groups of cells until 72-96 hr of culture, and the cell viabilities at 96-hr culture were 53.7 ± 7.9% in the wild type group and 92.7 ± 6.9% in hMDR-1-transfected K562 cells group (Fig.3C and D).

**Intercellular accumulation of second-generation TKIs**

The intracellular accumulation of nilotinib and dasatinib at the exposure concentration at which the effect was confirmed even after washing was evaluated. The intracellular accumulation after exposure of parent K562 cells to nilotinib for 0.5 hr increased with increase in the exposure concentration, and the amount of accumulation was 13.5-fold larger in the 2,500 ng/mL-exposed group than in the 100 ng/mL-exposed group. The intracellular accumulation in the 2,500 ng/mL exposure group tended to be greater than that in the 1,000 ng/mL exposure group, but the difference was not significant (Fig. 4A).

In the cell viability assay, a sustained cytostatic effect was not found when the cells were exposed to 100 ng/mL of nilotinib, but a sustained cytostatic effect was found when the cells were exposed to higher concentration of 1,000 and 2,500 ng/mL.

The intracellular accumulation of dasatinib increased with exposure concentration dependency and the amount of dasatinib that accumulated longer (by 7.5-times longer) in the 100 ng/mL-exposed group than in the 10 ng/mL-exposed group (Fig. 4B). A sustained cytostatic effect was observed when the concentration of dasatinib was 100 ng/mL but not at lower concentrations such as 12.5 ng/mL (Fig.2C).

The accumulation amount of each of the TKIs in hMDR-1-transfected K562 cells was measured and compared with the maximum intracellular accumulation amount of each of the TKIs in parent K562 cells to confirm the contribution of MDR-1 to intracellular
accumulation. When cells were exposed to nilotinib at a C\text{max} equivalent concentration of 2,500 ng/mL, intracellular accumulation in hMDR-1-transfected K562 cells was 87.4 ± 19.9% of that in wild-type cells, and the difference was not significant (Fig. 4A).

On the other hand, when the cells were exposed to dasatinib at a C\text{max} equivalent concentration of 100 ng/mL, intracellular accumulation in hMDR-1-transfected K562 cells was 38.1 ± 11.5% of that in wild-type cells, and the difference was significant (Fig. 4B, \(p<0.05\)). The results suggested that the contribution of MDR-1 to intracellular accumulation is greater for dasatinib than nilotinib.

[Discussion]

The development of TKIs was important for treatment of CML. The first-generation tyrosine kinase inhibitor imatinib showed greater efficacy and safety than those of conventional therapy with INF-α.\textsuperscript{4-6} Moreover, the second-generation TKIs dasatinib and nilotinib showed a stronger BCR-ABL tyrosine kinase inhibitory effect and a greater therapeutic effect than those of imatinib.\textsuperscript{18-20}

Although these TKIs dramatically increased the therapeutic effect, it is necessary to take them daily for maintaining the therapeutic effect. Clinical trials in which the medications were discontinued in patients who had reached a major molecular response (MMR), have been carried out, but about half of the participants in those trials relapsed.\textsuperscript{9-11} The results of those trials suggested that there is a problem with the test concept of the therapeutic effect being maintained with complete of administration. We therefore hypothesized that a method of dose reduction or intermittent administration would be more effective than the current treatment method. Our experimental results in fact support the hypothesis. TKIs other than imatinib were shown to have a strong cytostatic effect even after drug washout with short-term exposure.

Administration of imatinib is currently the first-line therapy for CML, and the effective plasma concentration of imatinib has been established.\textsuperscript{5, 6} The cases in which treatment failed increased when trough concentrations were 1,000 ng/mL or less.\textsuperscript{5, 21} It has been shown that poor compliance with imatinib treatment is correlated with a reduced therapeutic effect and that it is necessary to maintain the plasma concentration of imatinib to obtain a sufficient therapeutic effect.\textsuperscript{22} In fact, our data showed that exposure time was important for a continuous cytostatic effect of imatinib than intracellular amount (Fig. 3A, B and Supplemental Fig. 3). In addition, a previous study showed that imatinib induced more cell death as the exposure time became longer.\textsuperscript{23} Therefore, these results suggest that not only exposure concentration but also exposure time is important for imatinib to exert a continuous cytostatic effect.

On the other hand, although there have been studies showing correlations between the therapeutic effects of nilotinib and dasatinib and their plasma concentrations, there have been
no results that can provide standards for CML treatment.\textsuperscript{23, 24} Dasatinib has a remarkably short half-life compared with those of other TKIs and the plasma concentration of dasatinib can not be maintained for a long time. However, the therapeutic effect of dasatinib has been reported to be superior to those of other TKIs.\textsuperscript{20, 24, 25} Previous studies showed that dasatinib not only inhibited its therapeutic target BCR-ABL but also strongly inhibited other tyrosine kinases involved in cell proliferation such as SRC, whereas imatinib inhibited only BCR-ABL.\textsuperscript{27, 28} Thus, the mechanisms underlying the therapeutic effects of imatinib and dasatinib are different. In fact, our results demonstrated that even after K562 cells had been exposed to dasatinib at a final concentration of 100 ng/mL and then the drug was washed out, the cytostatic effect of dasatinib was sustained for up to 96 hrs. These results indicated that there is possibility not to maintain a constant plasma concentration of dasatinib (Fig. 2C).

Vainstein et al. reported that dasatinib-treated patients with an inhibitory potential at peak concentration (IPP), calculated from the integrated IC$_{50}$ slope and $C_{max}$ above the threshold value had a significantly higher CCyR ratio than that in patients with IPP below the threshold value.\textsuperscript{28} Their results suggested that $C_{max}$ is important as well as CCyR for the therapeutic effect of dasatinib. However, in their study, IC$_{50}$ and IPP were calculated from the results of exposure of cells to TKIs for 72 hrs. The conditions used in their study are not a good reflection of the clinical conditions. On the other hand, the results of our study in which CML cells were exposed for a short time to a TKI at a concentration corresponding to its $C_{max}$, a condition that reflects the clinical condition, showed that the cytostatic effect of dasatinib is sustained even with exposure for a short time. Thus, our results partially support the results obtained by Vainstein et al. suggesting that exposure to a concentration corresponding to $C_{max}$ is important.

Since previous studies showed that TKIs including imatinib, nilotinib and dasatinib were substrates of MDR-1, the therapeutic effects of TKIs are greatly affected by MDR-1 expression.\textsuperscript{13-17} Indeed, our study showed that dasatinib completely suppressed proliferation of parent K562 cells after short-term exposure at a final concentration of 100 ng/mL, but did not suppress proliferation of hMDR-1-transfected cells (Fig. 3D). On the other hand, nilotinib showed a strong cytostatic effect after short-term exposure at a high concentration, suggesting that its cytostatic effect was not affected by MDR-1 (Fig.3A, B). Furthermore, when we investigated the influence of MDR-1 expression on the intracellular concentrations of nilotinib and dasatinib, we found that the amount of intracellular accumulation in cells exposed to dasatinib for a short time at a final concentration of 100 ng/mL was significantly larger (3.4-fold larger) in hMDR-1-transfected K562 cells group but that there was no difference between the amounts of intracellular accumulation of nilotinib in parent K562 cells and hMDR-1-transfected K562 cells after short-term exposure of the cells to nilotinib at a high concentration. These results are consistent with results of previous studies showing that nilotinib was weaker than imatinib or dasatinib as a substrate for MDR-1.\textsuperscript{13, 14, 30, 31} Our
results also showed that nilotinib has a stronger and longer continuous cytostatic effect than that of dasatinib. Nilotinib has a longer half-life than that of dasatinib, and MDR-1 is thought to have a lower affinity than dasatinib as a substrate. Therefore, it is thought that nilotinib has a stronger suppressive effect than that of dasatinib on cell proliferation due to the greater intracellular accumulation of nilotinib than that of dasatinib.

Effects of ABC transporters (MDR-1 (P-gp), BCRP) on the cytostatic effects of TKIs used clinically for CML therapy were observed in previous studies, but sufficient consideration was not given to clinically confirmed plasma concentrations and half-lives of drugs in most studies. Our results showed that the experimental conditions of our study closely reflect clinical conditions and that dasatinib and nilotinib had sustained cytostatic effects after drug washout with short-term exposure. We also investigated how MDR-1 expression affects the continuous cytostatic effects of TKIs and we found that dasatinib had an attenuated inhibitory effect on cell proliferation due to decreased intracellular accumulation but that nilotinib did not.

In conclusion, we have shown that imatinib has a continuous cytostatic effect after drug disappearance in the case of long-term exposure to a high concentration of imatinib and that nilotinib and dasatinib have a sustained cytostatic effect after drug disappearance with constant intracellular accumulation even after short-term exposure. Our results suggest that the therapeutic effects of TKIs including imatinib, nilotinib and dasatinib can be maintained by dose reduction or intermittent administration if conditions such as our findings are met.

However, in order to realize this, consideration should be given to problems such as the possibility of point mutations including T315 occurring, and further investigation is necessary.

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[Conflict of Interest]
The authors declare no conflict of interest.

[Supplementary materials]
The online version of this article contains supplementary materials (Supplementary Fig.1 and 2).
[REFERENCE]


Fig. 1 Cell viability after long-term exposure of K562 cells to (A) imatinib, (B) nilotinib and (C) dasatinib. K562 cells were exposed to imatinib mesylate, nilotinib and dasatinib at the concentrations indicated above. The cytotoxic effects of the TKIs on K-562 cells were determined by CCK-8 assays at 24, 48, 72 and 96 hr after exposure to the TKIs. Each point represents the mean ± S.D. of at least three measurements.
Fig. 2 Continuous cytostatic effects of (A) imatinib, (B) nilotinib and (C) dasatinib after exposure for 0.5 hr and drug washout. After exposure to TKIs for 0.5 hr, the cell suspension was collected and thoroughly washed with the culture medium warmed at 37°C. The cell suspension was then seeded in a 96-well microplate and cell viability was determined by a CCK-8 assay after 24, 48, 72 and 96 hr. Each point represents the mean with S.D. of at least
three measurements.
Fig. 3. Exposure time dependence of a continuous cytostatic effect of imatinib at concentrations of (A) 1,000 ng/mL and (B) 2,500 ng/mL. After exposure to TKIs for 0.5 or 24 hr, the cell suspension was collected and thoroughly washed with the culture medium warmed at 37°C. The cell suspension was then seeded in a 96-well microplate and cell viability was determined by a CCK-8 assay after 24, 48, 72 and 96 hr. Each point represents the mean with S.D. of at least three measurements.
Fig. 4 Contribution of hMDR-1 to the continuous cytostatic effects of nilotinib (A. 1000 ng/mL, B. 2500 ng/mL) and dasatinib (C. 50 ng/mL, D. 100 ng/mL) for K562 cells. After exposure to TKIs for 0.5 hr, each cell suspension was collected and thoroughly washed with the culture medium warmed at 37°C. The cell suspension was then seeded in a 96-well microplate and cell viability was determined by a CCK-8 assay after 24, 48, 72 and 96 hr. Each point represents the mean with S.D. of at least three measurements.

* $p < 0.05$ for the comparison with wild type.
Fig. 5 Contribution of hMDR-1 to intracellular accumulation of (A) nilotinib and (B) dasatinib in K562 cells. After exposure to TKIs for 0.5 hr, each cell suspension was collected and thoroughly washed with the culture medium warmed at 37°C. The cell suspension was then frozen and crushed at -70 °C, and the sample was cryopreserved as a cell sample until measurement. Intracellular samples were analyzed for levels of TKIs by UPLC/MS/MS. The results are shown as ng/mg protein. Columns represent means of at least three independent experiments, each done in duplicate. Bar represent S.D. * $p < 0.05$ for the comparison with wild type.