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Interactions of N-desmethyl imatinib, an active metabolite of imatinib, with P-glycoprotein in human leukemia cells

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Abstract We measured intracellular accumulation of N-desmethyl imatinib (CGP 74588), the main pharmacologically active metabolite of imatinib (Gleevec or STI-571), in Bcr-Abl positive cells. Using a sensitive and robust non-radioactive *in vitro* assay, we observed that CGP74588 accumulates in significantly higher amount than imatinib in sensitive K562 cells. In contrast, the intracellular level of CGP74588 was significantly lower than that of imatinib in K562/Dox cells, which represent a multidrug resistant variant of K562 cells due to P-glycoprotein (*ABCB1*, *MDR1*) overexpression. An *in vitro* enzyme based assay provided evidence that CGP74588 might serve as an excellent substrate for P-gp. Accordingly, we found that CGP74588 up to 20μM concentration neither induced apoptosis nor inhibited substantially cell proliferation in resistant K562/Dox cells. In contrast, CGP74588 was capable to inhibit cell proliferation and induced apoptosis in sensitive K562 cells, although its effect was approximately 3 to 4 times lower than that of imatinib in the same cell line. Our results indicate that CGP74588 could hardly positively contribute to the treatment of CML where *ABCB1* gene overexpression represents a possible mechanism of resistance to imatinib *in vivo*.

Keywords MDR1, ABCB1, CGP 74588, intracellular drug level, cytotoxicity

Introduction

Imatinib mesylate (Gleevec or STI-571), a rationally developed inhibitor of the Bcr-Abl tyrosine kinase, is capable of blocking proliferation and inducing apoptosis in chronic myeloid leukemia (CML) cells both *in vitro* and *in vivo* [1,2]. In addition to Bcr-Abl tyrosine kinase, imatinib serves as an efficient inhibitor of other tyrosine kinases, including platelet-derived growth-factor receptor (PDGFR) tyrosine kinases [1], and c-Kit, a receptor tyrosine kinase structurally similar to PDGFR [3]. Later on, it was also approved for treatment of c-Kit positive gastrointestinal stromal tumors [4].

The pharmacokinetic properties revealed that imatinib is well absorbed after oral administration with high bioavailability exceeding 90% [5]. Imatinib becomes metabolized by cytochrome P450 (CYP), particularly by CYP3A4 and CYP3A5, to form several metabolites. The most abundant metabolite is N-desmethyl imatinib (CGP 74588), which represents 10-15% of administered imatinib [6]. Although CGP74588 has been reported to be pharmacologically active [6], little is known about its cellular distribution and possible interactions with ABC transporters, including P-glycoprotein (P-gp, *ABCB1*). To address this issue, we measured intracellular accumulation of imatinib and CGP74588 in Bcr-Abl positive cells which overexpressed P-glycoprotein.

Materials and methods

Patients

Only patients with chronic phase CML were included in the study that was conducted in accordance with the Declaration of Helsinki. All patients have signed informed consent approved by the local Ethics committee before sampling. 18 men and 7 women at median age 56 years (range 29-81) have already achieved complete cytogenetic response at the time of blood collection. All but four of them have achieved also major molecular response.

Drug administration

Imatinib was given to 7 patients as a front-line treatment, while in 18 patients was imatinib indicated due to the failure of interferon. Average duration of imatinib therapy was 54.5 and median 60 months (range 4-94). Imatinib was applied in a single daily dose of 400mg in all patients. Blood sample was collected in median 22 hours after drug ingestion (range 18-24).

Cell lines

A suspension of human chronic myelogenous leukaemia K562 cells, obtained from ECACC, was cultured in RPMI-1640 medium supplemented with a 10% calf foetal serum and antibiotics in 5% CO₂ atmosphere at 37°C. K562/Dox cells, which represent a MDR variant of K562 cells with overexpressed P-gp (*ABCB1*, *MDR1*), were kindly provided by prof. J.P. Marie (University of Paris 6, France). K562/Dox cells were cultured under the same conditions.

Chemicals

Imatinib mesylate (STI571; purity $\geq 98\%$) and N-desmethyl imatinib (CGP 74588; purity $\geq 98\%$) were kindly provided by Novartis (Basel, Switzerland).

Detection of P-gp mediated rhodamine 123 efflux

Cells at the density of 0.2×10^6 /ml were loaded with rhodamine 123 (Rh123; 2.0 μ M) in a growth medium containing 2 μ M Cyclosporin A (CsA) in the dark. After 30minute incubation cells were transferred into Rh123-free medium with or without 2 μ MCsA. Cell fluorescence, which reflects the Rh123 content and thus P-gp mediated dye efflux was analyzed in Rh123-free medium using FACSscan as described previously [7].

P-gp expression analysis

P-gp expression in K562 and K562/Dox cells was measured using UIC2 (Pgp, Beckman Coulter, USA) monoclonal antibody conjugated with phycoerythrin according to the manufacturer's instruction. The fluorescence of the cells was measured and analyzed by flow cytometry. Protein expression was quantified as the median fluorescence intensity shift.

Extraction of CGP 74588 and imatinib from plasma

Peripheral blood samples were taken by direct venipuncture. Plasma was separated by centrifugation (5000rpm/10min/4°C) and resulting supernatant was deproteinised with 4% formic acid (w/v) in 80% methanol/water (v/v)). CGP 74588 and imatinib contents in clarified samples were quantified using HPLC/MS/MS.

Extraction of CGP 74588 and imatinib from cells

Cells at density 5×10^5 cells/ml were incubated in growth medium with various concentration of CGP 74588 or imatinib for 5 hours at 37°C. Afterwards cells were centrifugated through silicone oil and resulting pellet was extracted with 1% formic acid (w/v) in 50% methanol/water (v/v)). CGP 74588 and/or imatinib contents in cell extract were quantified using HPLC/MS/MS.

Quantitation of CGP 74588 and imatinib by LC/MS/MS analysis

The HPLC system consisted of UltiMate 3000 RS pump, degasser, autosampler and column compartment (Dionex, Germering, Germany). Separations were performed at ambient temperature on a Polaris C_{18-A} 250 x 2.0mm (i.d.), 5 μ m particle size column (Varian Inc., Lake Forest, CA, USA) connected with a guard C₁₈ 4.0 x 2.0mm (i.d.) precolumn

(Phenomenex, Torrance, CA, USA). The mobile phase was composed of methanol-water (65:35, v/v) containing 7mM ammonium acetate. The HPLC flow rate was 300 μ l min⁻¹ and sample injection volume was set at 10 μ l.

An API 3200 triple quadrupole mass spectrometer (MDS SCIEX, Ontario, Canada) with electrospray ionization in positive ion mode was used for detection. The mass spectrometer was operated in the multiple-reaction monitoring (MRM) mode. Imatinib and CGP 74588 were monitored by MRM transitions 494.2 > 394.0 and 480.2 > 394.0 (dwell-time = 300 milliseconds), respectively.

Ion spray probe parameters were set to the following values: needle voltage 5500 V, declustering potential 71 V for imatinib and 66 V for CGP 74588, temperature 400°C, curtain gas (nitrogen) 1.38bar, nebulizer gas (zero air quality) 3.45 bar, turbo V-gas (zero air quality) 3.45 bar. The nitrogen pressure in the second quadrupole was measured at 3.7x10⁻⁸ bar, the collision energy and entrance potential was set at 33.0 eV and 5.0 V, respectively. The instrument was operated in unit resolution.

Measurement of drug-stimulated membrane ATPase

Principle of this assay: transport of substrates out of the cell by P-gp is coupled with hydrolysis of ATP, which serves as an energy source. Owing to the fact that these processes are stoichiometrically coupled, the detection of ATPase activity of P-gp is a measure of transporter activity. The assay is performed using purified membrane vesicles from *Sf9* (*Spodoptera frugiperda*) cells, expressing high level of P-gp (Sigma, Saint Louis, Missouri, USA). The ATPase activity was measured as described elsewhere [8].

Determination of cell survival and proliferation

The MTT assay was used for estimation of cell viability and growth as originally described by [9]. Cells were incubated in the presence of 0.5mg MTT/ml (final concentration) for 1 hour at 37°C, then pelleted by centrifugation and extracted by isopropanol acidified with 0.01M HCl. Absorbance was read at 570 nm in extracts clarified by centrifugation. The amount of formazane produced is proportional to the number of live and metabolically active cells.

Apoptosis assessment by flow cytometry

We adopted protocol published by [10]. Briefly, cells were washed in PBS and then fixed in ice-cold 70% ethanol in PBS. Fixed cells were washed in PBS and then stained for at least 30 minutes in PBS containing PI (10 μ g/ml) and RNase A (100 μ g/ml) prior to flow-cytometric

analysis on a Cytomics FC 500 System (Beckman Coulter). The apoptotic population was identified within 10 000 of analysed cells which DNA content was lower than in G1/G0 (hypoploid population) cell cycle phase [10].

Results and Discussion

We measured plasma levels of CGP74588, a main metabolite of imatinib, in 25 patients after administration of imatinib mesylate at daily dosage 400mg. Similarly to other researchers we found that conversion of imatinib into CGP74588 varied from 8 to 24% with the mean value approximately 16% of (Fig. 1). Our data are in a good agreement with those reported in the literature. Indeed, Gschwind and co-workers recently reported that 10 to 15% of imatinib become converted into CGP74588 in healthy volunteers [6].

Considering the fact that CGP74588 might represent even more than 20% of imatinib (Fig. 1), and it is pharmacologically active [6], we addressed the question whether it could efficiently affect cell survival of Bcr-Abl positive cells which expressed P-gp. Indeed, P-gp was identified as important factor that could confer resistance to imatinib [11,12]. However, to our knowledge nothing was reported about the cytotoxic properties of CGP74588 in sensitive, and more importantly in resistant, Bcr-Abl positive cells.

To address this issue, we measured the intracellular levels of CGP74588 and imatinib in K562 cells and their multidrug resistant (MDR) variant K562/Dox cells, which overexpress P-gp [13]. Expression of P-gp was verified in both cell lines using specific monoclonal antibody (Fig. 2). Function of P-gp, i.e., its ability to transport substrates across the cytoplasmic membrane, was confirmed using the rhodamine 123 efflux assay (Fig. 3). Analysis of cell extracts revealed that CGP74588 accumulated cells in significantly higher amount than imatinib in K562 (Fig. 4a). In contrast, the intracellular level of CGP74588 was significantly lower than that of imatinib in K562/Dox cells, which overexpress P-g (Fig. 4b). The finding that CGP74588 accumulated approximately two times more in sensitive K562 cells than imatinib was rather surprising as there is only a little structural difference between imatinib and its N-desmethyl metabolite. We can speculate that demethylation of the piperazine ring might lead to its higher sequestration within lysosomes due to the changed protonation. On the other hand, 3 to 4 fold decrease in content of CGP74588 in comparison to imatinib in resistant K562/Dox cells indicated that CGP74588 was more efficiently pumped out of the cells than imatinib. These results suggested that CGP74588 could serve as a “better substrate” for P-gp than imatinib.

To confirm this hypothesis we investigated whether CGP74588 could serve as a substrate for P-gp in an independent experiment. An *in vitro* enzyme based assay provided evidence that CGP74588 might serve as a substrate for P-gp (Fig. 5). Interestingly, CGP74588 seemed to be a more powerful stimulator of ATPase than imatinib (Fig. 5). Indeed, the stimulatory effect of CGP74588 on the ATPase activity was comparable to that found for verapamil (Fig. 5), which serves as a model substrate for P-gp in *in vitro* assays [8].

We further studied cytotoxic effects of CGP74588 in sensitive K562 and resistant K562/Dox cells. We found that CGP74588 inhibited cell proliferation and induced apoptosis in K562 cells although its antiproliferative and proapoptotic effect was 3 to 4 times lower than that of imatinib (Figs. 6, 7, Tab. 1). Not surprisingly, CGP74588 and also imatinib exhibited only weak antiproliferative and proapoptotic effects in resistant K562/Dox cells (Figs. 6, 7, Tab. 1). However, while cytotoxicity of imatinib was reduced in resistant K562/Dox cells approximately 10 times, cytotoxicity of CGP74588 was diminished more than 30 times (Fig. 6, Tab 1). Similar results were found for proapoptotic effects of CGP74588 in K562/Dox cells (Fig. 7). These results are consistent with the data found for intracellular levels of CGP74588 in K562 and K562/Dox cells (Fig. 4).

Our results indicated that CGP74588 exhibited only weak antiproliferative and proapoptotic effects in sensitive K562 cells. In contrast, CGP74588 could hardly influence cell proliferation and cell survival in Bcr-Abl positive cells that overexpress P-glycoprotein.

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Legends to Figures

Fig. 1. Analysis of plasma levels of CGP74588 and imatinib. Blood samples collected from 25 patients after a single oral dose of imatinib were assayed for imatinib and CGP74588 contents.

Fig. 2. Analysis of P-glycoprotein expression. Protein expression is quantified as the median fluorescence intensity shift. K562 cells (dotted line); K562/Dox (solid line).

Fig. 3. Detection of P-gp function. Cells were loaded with Rh123 and then transferred into a fresh medium with or without CsA. After 30min incubation fluorescence was measured by flow cytometry. Panel (a) K562 cells incubated with (solid line) or without (dotted line) CsA. Panel (b) K562/Dox cells incubated with (solid line) or without (dotted line) CsA.

Fig. 4. Determination of intracellular levels of CGP74588 and imatinib in sensitive K562 and resistant K562/Dox cells. Cells were incubated in growth medium with equal concentrations of CGP74588 and imatinib, as indicated. After 4h incubation cells were harvested and intracellular contents of both drugs were determined using LC/MS/MS. CGP74588 (triangles); imatinib (circles). Panel a) K562 cells; panel b) K562/Dox cells. The experimental points represent mean values from three replicate experiments, with standard deviations.

Fig. 5. Effect of CGP74588 and imatinib on ATPase activity in isolated membranes containing P-gp. No drug added (vehicle) - white circles; imatinib - black triangles; CGP74588 - black squares; verapamil - white hexagons. The experimental points represent mean values from three replicate experiments, with standard deviations.

Fig. 6. Effect of CGP74588 and imatinib on cell survival and cell proliferation in sensitive K562 and resistant K562/Dox cells. Cells were treated with CGP74588 (white triangles) or imatinib (black circles), as indicated. After 48hour incubation cells were subjected to the MTT assay. Cells cultured in standard medium without CGP74588 or imatinib were taken as a control. Panel a) K562 cells; panel b) K562/Dox cells. The experimental points represent mean values from three replicate experiments, with standard deviations.

Fig. 7. Effect of CGP74588 and imatinib on apoptosis induction in K562 and K562/Dox cells. Cells were cultured in a standard medium containing CGP74588 and imatinib, as indicated. After 48hour incubation cells were fixed in ice-cold 70% ethanol in PBS, stained in PI, and further processed for flow cytometric analysis using Cytomics FC 500 System (see Materials and Methods). Panel a, b, and c – K562 cells; panel d, e, and f, - K562/Dox cells. Untreated cells were taken as a control. Histograms represent typical results.

Fig. 1.

Fig. 2

Fig. 3.

Fig. 4a.

Fig. 4b.

Fig. 5.

Fig. 6a.

Fig. 6b)

Fig. 7.

Table 1

Effect of CGP74588 and imatinib on proliferation of K562 and K562/Dox cells

Compound	K562 cells IC ₅₀ (μM)	K562/Dox cells IC ₅₀ (μM)
Imatinib	0.17	1.9
CGP74588	0.58	> 20