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Monitoring of imatinib targeted delivery in human leukocytes

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ABSTRACT

The success of imatinib therapy in chronic myeloid leukemia is highly influenced by its active transport into target cells. However, the methodology for analytical evaluation of intracellular drug concentration is rare and usually reliant upon the use of radioactively labeled drugs. More specifically, there is no published method available in the literature for the determination of imatinib concentration in granulocytes. To gain further insight into the intracellular drug uptake a very reliable two-stage sample concentration procedure was devised and coupled with a sensitive ultra-high performance liquid chromatography tandem mass spectrometry. The reliability of this sample preparation and sensitivity of the analysis was confirmed by a successful validation of all necessary method parameters to an impressive lower limit of quantification of 0.5 ng imatinib per 10^6 cells still at the signal to noise ratio of 670. The usefulness of the method is further improved with only 6 mL of blood being necessary for patient analysis. The method has been applied to blood samples of 13 CML patients treated with imatinib and all the measured intracellular drug concentrations were within the validated range. These and further measurements will enable the research of factors which may, besides blood plasma concentration, influence the individual's response to imatinib therapy. Furthermore, individualisation of dosing based on the directly measured targeted drug delivery could be possible.

1. Introduction

Imatinib mesylate (imatinib) is an orally administered highly specific pharmacologic inhibitor of the BCR-ABL1-tyrosine kinase – the fusion protein, which is the main cause of chronic myelogenous leukemia (CML). The drug binds to the active site of the BCR-ABL oncoprotein, thus blocking the phosphorylation of cellular proteins responsible for aberrant signal transduction and consequently for increased cell proliferation, reduced apoptosis and abnormalities in cell motility (Deininger and Druker, 2003).

Imatinib dose is modified according to the stage of CML (Peng et al., 2005). The majority of patients receive 400 mg a day, which is usually enough to give plasma concentration several times higher than the IC50 values needed for tyrosine kinase inhibition in vitro. This dose is recommended in therapeutic guidelines for the treatment of chronic phase patients (CP) (Deininger et al., 2003; Goldman, 2007). On the other hand, several studies have reported improved responses and longer time to progression with high dose imatinib treatment (Cortes et al., 2009; White et al., 2007). Due to higher risk of toxicity, higher doses are mostly reserved only for accelerated (AP) and blast crisis (BC) patients, where 600 mg/day and 800 mg/day imatinib is used, respectively (Goldman, 2007).

However, despite these clinical guidelines, each individual in CP, AP or in BC responds differently to the recommended treatment. Picard et al. first showed that the trough imatinib plasma levels vary largely in patients treated with standard-dose imatinib and highly correlate with both complete cytogenetic and major molecular response (Picard et al., 2007). In some CP patients higher doses could even overcome imatinib resistance. At that time therapeutic drug monitoring (TDM) was proposed as a possible means to ensure sufficient drug exposure, preventing severe side effects and checking adherence to therapy (Teng et al., 2012).

Because TDM is usually performed by plasma concentration measurement, little is known about the concentration of imatinib in other compartments of peripheral blood, i.e., peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs, granulocytes), the cells that are considered as target cells for imatinib treatment. The general assumption that the plasma concentration represents a good approximation of intracellular concentration may not always be true. Several studies have shown that imatinib enters the cell by active transport with organic cation transporter 1 (OCT1) (Thomas et al., 2004) and is being cleared out of the cell by two major efflux transporters: P-glycoproteine (P-gp) and breast cancer resistance protein (BCRP) (Thomas et al., 2004;

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Burger et al., 2004). While the role of P-gp and BCRP in imatinib resistance is still inconclusive, various studies have demonstrated that the functional behaviour of OCT1 transporter is highly variable among the CML patients and significantly correlates with patientś molecular response to imatinib treatment and their overall survival (White et al., 2006, 2007, 2010). OCT1 activity was reported to vary also with blood cell lineage and was found to be the highest in the neutrophil population, followed by monocytes and lymphocytes (Engler et al., 2011). Variable expression and function of transport and regulatory proteins, which are a consequence of polymorphic genes coding for these proteins, are probably important reasons for large inter-individual differences in plasma and intracellular levels of imatinib (Takahashi et al., 2010). Another factor contributing to lower uptake of imatinib into the target cell might be its binding to α1-acid glycoprotein, which diminishes the concentration of free drug in plasma available to penetrate intracellularly and exert its pharmacological action (Widmer et al., 2006).

Lipka et al. (2012) recently demonstrated that the intracellular imatinib retention crucially determines its biological activity as measured by induction of apoptosis. The correlation of imatinib intracellular concentration in bone marrow cells of CML patients with therapeutic response has also been shown by Zhong et al. (2012). Widmer et al. (2006) and D'Avolioa et al. (2012) observed in PBMĆs an intra/extracellular concentration ratio of approximately 8, confirming the active uptake of the drug into these cells.

Regarding the above information, it is obvious, that the specific active transport of imatinib into the target cells and its retention there, are just as relevant for the efficacy of imatinib therapy as its affinity for the BCR-ABL1.

No data exist in the literature regarding intracellular concentration of imatinib in neutrophil granulocytes, nor its relation to plasma concentration, although it was previously shown that these cells possess the highest OCT1 activity (Engler et al., 2011) and are therefore expected to have the highest intracellular drug concentration. Moreover, these cells are mature descendants of the myeloid line and could therefore in stable patients treated with imatinib be a relevant approximation of the CML stem cells, which are not accessible at the current state-of-the-art. The present paper thus describes the development and clinical application of a fast and sensitive HPLC–MS/MS method for granulocyte intracellular concentration determination.

2. Materials and methods

2.1. Chemicals and other material

Imatinib mesylate ⩾98% and stable isotope labelled internal standard [$^2H_8$]-imatinib were purchased from Sequoia Research, Pangbourne, UK and Alsachim, Illkirch, France, respectively. Ultrapure water was prepared with a Milli-Q® UF-Plus apparatus (Millipore Corp., Burlington, MA, USA). Ammonium formiate, hexane and ethyl acetate were purchased from Fluka (Buchs, Switzerland), Merck (New York, USA) and Carlo Erba (Val de Reuil, France), respectively. Formic acid (FA) (Suprapur®) and methanol for chromatography Lichrosolv® (MeOH) were obtained from Merck (Darmstadt, Germany).

Whole Blood CD15 MicroBeads, separation buffer (autoMACS® Running Buffer) and Whole blood column Kit were supplied by Myltenyi Biotech Gmbh (Bergisch Gladbach, Germany). Gibco® RPMI 1640 medium and Phosphate buffer saline (10×) (PBS) were purchased from Invitrogen Life Science Technologies (Carlsbad, CA, USA). Ficoll-Paque™ Plus was supplied by GE Healthcare (Buckinghamshire, UK).

2.2. Working solutions

Primary stock solution of imatinib was prepared by dissolving an accurately weighted amount of the drug in MeOH to yield 1 mg/mL drug concentration. Primary stock was diluted 10 and 100-times with 50% (v/v) EtOH in water to give secondary and tertiary stocks, respectively. Working solutions (WS) were prepared by diluting appropriate stock solution with 20% (v/v) EtOH to gain a desired concentration of imatinib in each WS.

Stock solution of isotope labelled [$^2H_8$]-imatinib was prepared in MeOH in 1 mg/mL concentration and was further diluted with the same solvent to 0.667 mg/L final internal standard concentration.

All the working and internal standard solutions were stored at −20 °C and brought to room temperature before use.

2.3. Calibration sample preparation

Calibration standards (CS) and quality control (QC) samples were prepared by diluting 50 μL of appropriate working solution with either 950 μL of granulocyte lysate or with plasma. CS and QC samples in cell lysate were prepared immediately before use, except when the sample stability was tested. Plasma CS and QC were used immediately or stored at −20 °C.

2.4. Patient sampling and cell isolation

The study protocol was reviewed and approved by the Slovenian National Medical Ethics Committee and an informed verbal and written consent was obtained from each patient included in the study.

About 6 mL of EDTA blood was collected from 13 CML patients on therapy with imatinib at times when other blood samples were being withdrawn for routine hematologic and molecular genetic analysis. Immediately after withdrawal, 2 mL of whole blood were centrifuged for 10 min at 660*g* to gain 1 mL of plasma, which was stored at −20 °C for imatinib plasma concentration determination. The remaining 4 mL of blood were used for granulocyte isolation with Miltenyi Whole blood column kit according to the slightly modified protocol provided by the manufacturer (http://www.miltenyibiotec.com). Briefly, 150 μL of CD15 Whole blood MicroBeads were added to 4 mL of whole blood, thoroughly mixed and incubated at 2–8 °C for 15 min. Afterwards, blood sample was centrifuged for 10 min at 470*g* and 4 °C in a swinging bucket rotor without break to separate plasma from the cell suspension. Plasma was removed, cell suspension, however, was diluted to 4 mL with ice cold separation buffer and transferred on the Whole blood column, which was previously placed into the Midi MACS magnet and rinsed with 3 mL separation buffer. The magnetically labelled CD15+ cells were retained within the column; the unlabelled cells, however, were rinsed by washing the column three times with 3 mL ice cold separation buffer. Finally, the column was removed from the magnet and granulocytes were immediately flushed out with 5 mL ice cold elution buffer into the collection tube. After centrifugation at 4 °C and 660*g* for 5 min, the cell pellet was resuspended in 1 mL of PBS and divided into three separate 2 mL Eppendorf tubes (each contained 300 μL of cell suspension). Cell concentration in the suspension was determined by Coulter Counter apparatus. Cells lysates were obtained by freezing the samples three times in liquid N_2.

Granulocytes used to prepare the blank cell lysate for CS in QC samples and to test the drug loss during isolation were isolated from the blood of 10 healthy volunteers after Ficoll density gradient centrifugation and red blood cell lysis. The homogenized cell lysate was prepared by ultrasonification of granulocyte cell