PALACKÝ UNIVERSITY OLOMOUC

Faculty of Medicine and Dentistry

Significance of intracellular and plasmatic levels of new tyrosine kinase inhibitors

Ph.D. Thesis

Mgr. Kateřina Mičová

Supervisor: RNDr. David Friedecký, Ph.D.

Consultant: prof. RNDr. Tomáš Adam, Ph.D.

Acknowledgements

Declaration

I hereby declare that this thesis has been fully written by me under the expert guidance of my supervisor RNDr. David Friedecký, Ph.D. All the used literature is cited in chapter 4.

In Olomouc, 8th August, 2014   …………………………………

                                                          Mgr. Kateřina Mičová

List of contents

4

6

1. Introduction

Leukaemia is a type of cancer, one of the most extensive causes of death in the world. This type of malignant disease is developed from cells in the bone marrow, bloodstream, and lymphatic system. Rapid production of abnormal and immature white blood cells that are not able to fight infection is the characteristic hallmark of the disease. Unregulated proliferation of white blood cells is responsible for more inconveniences associated with impairment of other blood elements production. In general, leukaemia subtypes are divided into lymphoid and myeloid category. Names are derived from the white blood cells which they develop from. Leukaemia can be either acute or chronic depending on the natural course of the disease. Progression of chronic leukaemia is slower and less aggressive compared to the acute form requiring immediate treatment initiation. Thus, leukaemia is classified to four major groups: acute lymphocytic leukaemia (ALL), acute myelogenous leukaemia (AML), chronic lymphocytic leukaemia (CLL), and chronic myelogenous leukaemia (CML), (Harmon, 2011; http://www.hematology.org/).

1.1. Chronic myeloid leukaemia

Chronic myeloid leukaemia (CML) is cancerous disease characterized by unregulated growth of myeloid cell line in bone marrow. Increased number of mature granulocytes (basophils, eosinophils and neutrophils), their precursors and frequently also platelets in blood is typical primary finding at the time of disease diagnosis. Incidence of CML (1-2/100000) rise with age and relation between affected men and women is 1.3 : 1 (Brincker, 1982). The majority of the patients are diagnosed in the chronic phase of the disease that if untreated progresses to an accelerated phase and further to the terminal phase called blast crisis. Leukaemic cell line originates in one progenitor hematopoetic stem cell characterized by the presence of Philadelphia chromosome with *BCR-ABL1* fusion gene responsible for expression of constitutively active tyrosine kinase Bcr-Abl (also known as p210$^{BCR/ABL}$). This protein triggers downstream activation of critical pathways that are essential for the transformation potency of Bcr-

Abl (Nowell *et al*, 1960; Lugo *et al*, 1990). Advanced understanding of CML molecular biology have led to the development of compounds with the potential to selectively block its action biochemically, so called tyrosine kinase inhibitors (TKIs). Discovery of these agents evoked revolution in the cancer therapy especially by the high rate of treatment response, tolerable side effects and improvement of life quality of the patients.

1.1.1. Characteristics and diagnosis of CML

Previously, CML was described only by presence of abnormal myeloid cell line expansion in the bone marrow, leukocytosis and splenomegaly. In 1960 the cytogenetic hallmark of this disease – Philadelphia (Ph) chromosome was revealed. This typical genetic abnormality of CML is present in bone marrow cells in more than 90 % of patients with CML and 20-30 % of patients with acute lymphoblastic leukaemia. Ph chromosome rise in consequence of reciprocal translocation t(9;22)(q34;q11). Gene fusion of *BCR* ("breakpoint cluster region") at chromosome 22 with *ABL* ("Abelson tyrosine kinase gene") at chromosome 9 gives the rise to oncogene *BCR/ABL1* (Fig. 1) which expression product is chimeric oncogenic protein Bcr-Abl, constitutively active cytoplasmic form of Abl kinase. Activity of this fusion-protein does not undergo the regulation control mechanism typical for Abl; therefore it evokes malignant disease by various cytoplasmic and nuclear signal transduction pathways activation that affects the growth and survival of hematopoetic cells. This kinase also has distinct effect on DNA reparation which could play a role in the development of other chromosomal changes and mutations involved in disease progression and also could play role in aggressive nature of CML at later stages (Nowell *et al*, 1960; Lugo *et al*, 1990; Rowley *et al,* 1973).

In most cases the disease is diagnosed in chronic phase. Accelerated phase and blast crisis diagnoses are detected only in 5-10 % of patients (Kantarjian *et al*, 1993). Fatigue, weight loss, bleeding, sweats, abdominal fullness related to splenomegaly, purpura, leukocytosis, anemia, and thrombocytosis can be included among common physical and laboratory symptoms (Savage *et al*, 1997).

Diagnosis of CML is based on complete blood count, including a differential and a platelet count, aspiration of bone marrow for percentage of blasts and basophils determination, marrow biopsy and cytogenetic analyses for the presence of Ph chromosome and/or *BCR-ABL1* fusion gene (Faderl *et al,* 1999).
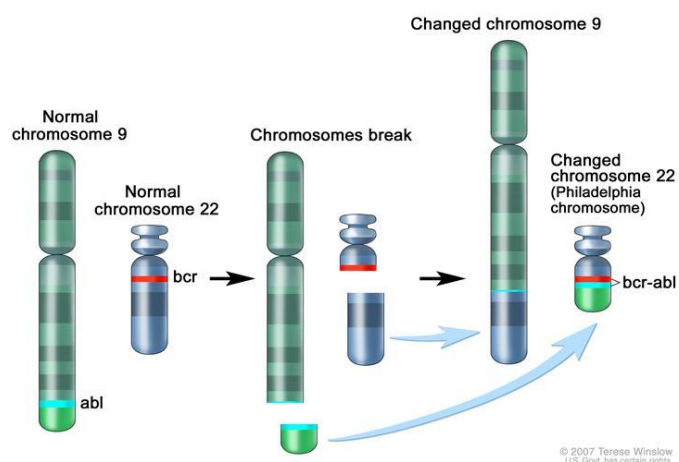
Figure 1: Reciprocal translocation t(9;22)(q34;q11) giving the Philadelphia chromosome (adapted from http://www.cancer.gov).

Cytogenetic analysis is widely used method for CML diagnosis valuable for a fast Ph chromosome identification by classical G-karyotypic examination but also for the possibility to demonstrate additional karyotypic abnormalities which often occur in advanced disease such as trisomy of chromosome 8 and 19; isochromosome i(17q) and accessory Ph chromosome, so called "double Ph" (Mitelman, 1993). Nevertheless cytogenetic analysis for Ph chromosome demonstration is not always sufficient for diagnose assessment and confirmation. In this case molecular methods are useful and currently widely used tools for diagnosing and also for monitoring the disease. Exact breakpoints of fusion genes can be detected by genomic polymerase chain reaction (PCR) and Southern blot assay, *BCR-ABL* transcript at the RNA level is determined by reverse transcriptase PCR (RT-PCR) and northern blot analysis. Presence of

p210[BCR/ABL] by monoclonal antibodies against Bcr and Abl is demonstrated by western blot analysis and immunoprecipitation. Other molecular method except RT-PCR used for disease monitoring during various treatment approach is fluorescence in situ hybridization (FISH) that allows easy detection and quantification of metaphase and non-dividing interphase cells in peripheral blood. Main advantage of this method is the rapidity and possibility to analyse more cells than with conventional cytogenetics (Faderl *et al*, 1999; Guo *et al*, 1991; Hochhaus *et al*, 1998; Muhlmann *et al*, 1998).

1.1.2. Molecular biology of Bcr-Abl

Oncogene *BCR-ABL1* localized on Philadelphia chromosome can be expressed in three different forms, size of fusion protein Bcr-Abl spans from 190 to 230 kDa depending on the breakpoint in *BCR.* The majority of the patients with CML express the protein of 210 kDa, so called p210[BCR/ABL] or Bcr-Abl1. In rare cases of CML, but in most cases of Ph positive acute lymphoblastic leukaemia (ALL) patients (60-80 %), the result of *BCR/ABL1* gene is the translation into smaller 190 kDa oncogenic protein, so called p190[BCR/ABL] which shows higher tyrosine kinase activity than p210 [BCR/ABL]. The protein p230[BCR/ABL] is found in a part of patients with chronic neutrophilic leukaemia (CNL), (Lugo *et al*, 1990; Melo, 1996; Voncken *et al*, 1995).

Bcr-Abl oncogenes differ in amount of Bcr included in an oncoprotein originates in a fusion of 5' end of *BCR* joining to 3' end of the *ABL1* sequence (Melo, 1996; Frank *et al*, 1996). The 5' untranslated region of *BCR* is important because *BCR-ABL1* is regulated by the *BCR* promoter (Shah *et al*, 1991). The breakpoints within the *ABL1* gene are localized at the 5' end and are spread over 300 kb in a region containing two alternative spliced exons (Ib and Ia), whereas the breakpoints within *BCR* lie within clustered regions (Melo, 1996; Rumpold *et al*, 2011). Splicing in Bcr breakpoints M (major), m (minor), or μ (micro) leads to three distinct protein products. Abnormal fusion protein p210[BCR-ABL] rises from the major breakpoint cluster region localized between exons 12 and 16 (also called b1-b5) with the size about of 5.8 kb. Different forms of p210 (namely b2a2

10

or b3a2 junction) with latter encoding sequence are also produced due to alternative splicing (van Rhee *et al*, 1996). Larger protein p230$^{BCR-ABL}$ is produced via more distal breaks between exons 17 and 20 (μ-*BCR* region) and is characteristic for Bcr-Abl positive CNL with e19a2 transcript (Pane *et al*, 1996). The minor breakpoint region of p190$^{BCR-ABL}$ spreads out about 54.4 kb between exons e2' and e2 (e1a2 junction). Due to alternative splicing, low number of p190 transcript can be detected by RT-PCR in up to 90 % of patients with typical CML transcript p210; high levels of both transcripts were even found in patients in blastic phase of CML (van Rhee *et al*, 1996).
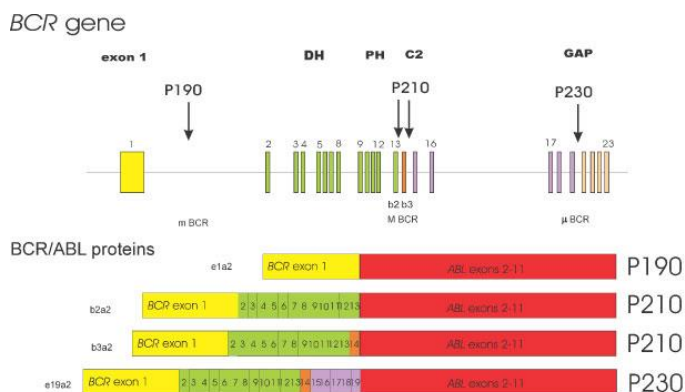
Figure 2: Schematic representation of the *BCR* gene and Bcr-Abl translocation products (adapted from Heisterkamp *et* Groffen, 2002).

The Bcr-Abl fusion protein evokes an oncogenic transformation by deregulation of the tyrosine kinase activity leading to the phosphorylation of signalling molecules and activation of several downstream signalling pathways like Myc, Ras, c-Raf, MAPK/ERK, SAPK/JNK, Stat, NFKB, PI-3 kinase and c-Jun (Pane *et al*, 1996). Structurally, Abl1 is non-receptor cytoplasmic tyrosine kinase that transduces signals from the cell surface growth factor and the adhesion receptors. The sequence encodes Src-homology (SH3 and SH2) domains, the tyrosine kinase domain, the DNA-binding domain, the actin-binding domain, the nuclear localization and the export signals (Advani *et al*, 2002; Wang, 1993). The Bcr region contains a

coiled-coil oligomerization domain, a serine/threonine kinase domain, a pleckstrin homology (PH) domain, a Dbl/cdc24 guanine nucleotide exchange factor homology domain, several serine/threonine and tyrosine phosphorylation sites, and the binding sites for the Abl SH2 domain, Grb2, and 14-3-3 proteins (Advani *et al*, 2002; Raitano *et al*, 1997; Ghaffari *et al*, 1999).

1.1.3. Course of the disease

The "natural course" of CML can be divided into initial chronic phase and following transformation to more aggressive and acute stadium. In the majority of the patients the disease is diagnosed in the chronic phase, that generally persists long time from several months up to a couple of years. This stadium is fairly responding to the treatment. Increased number of leukocytes and thrombocytes is characteristic, the number of blasts in bone marrow achieve at maximum of 10 %. Splenomegaly is other characteristic mark which can be as well as leukocytes amount suppressed by the medicaments. Within unpredictable period the disease can transform into the more aggressive stage, the accelerate phase. This could be characterized by obscure fever, bone and joint ache, weakness, night sweating, weight loss and abdominal pain caused by splenomegaly or infarcts in spleen. Leukocytosis characterized by increased amount of the basophils (≥20 %) and the blast (15-30 %) in the peripheral blood and/or the bone marrow and also the thrombocytopenia is usually observed in laboratory tests. If the progress is not suppressed by the appropriate treatment the disease usually proceeds to the blastic phase with clinical symptoms and the laboratory finding resembling advanced acute leukaemia with short survival probability in the range of a few months. The manifestation and the course of this phase are very similar to the acute leukaemia; myeloid precursors can form tumours in the lymph nodes, skin and bones. The amount of immature blast cells in the bone marrow and also in peripheral blood exceeds 30 %. These immature blood cells can have lymphoid structure with the lymphoid markers expression as well as the myeloblastic or undifferentiated leukaemia-like phenotype. In some

cases the blast crisis could appear without an intervening accelerated phase (Druker *et al*, 2006; Faber *et al*, 2010b; Griffin *et al*, 1983; Kantarjian *et al*, 1988).

Patients are usually diagnosed in the chronic phase of CML and early after the treatment initiation the symptoms are usually retreated with optimal treatment response. Complete hematologic remission is achieved over three months, immature granulocytes disappear from differential peripheral blood count and splenomegaly also shrinks back. After 6-12 months of imatinib treatment the most of patients achieve the complete cytogenetic response (response to treatment of CML that occurs in the marrow, rather than just in the blood, characterized by Ph+ cells reduction under detectable limit by either conventional or FISH cytogenetic testing) and the number of them major molecular response (characterized by 3-log reduction of *BCR-ABL* transcript or a *BCR-ABL1* (international scale) = 0.1 %). The survival of patients with CML under imatinib treatment after the achievement of an optimal molecular response has been estimated to be prolonged up to 20-25 years. In IRIS (International Randomized Study of Interferon vs STI571) study the 5-year overall survival in 89 % of patients treated by imatinib in first line in chronic phase of CML was achieved, 83 % of treated patients continue in imatinib therapy without progression (Druker *et al*, 2006; Faber *et al*, 2010b). Transformation to accelerate phase under TKI therapy is often associated with additional cytogenetic changes and mutations of fusion gen *BCR-ABL*. Even in the era of TKI, the blastic phase of CML cannot be cured by any of known conservative treatment and the results of the bone marrow transplantation are also poor (Faderl *et al*, 1999; Faber *et al*, 2010b; Kantarjian *et al*, 1988).

1.1.4. Treatment of CML

The main goal of the treatment in the pre-TKI era was the reduction of tumour cells, Ph chromosome elimination and prevention of disease progression to the advanced stage. Today, achievement of the major molecular response has been introduced as the most important target. The treatment success is evaluated by the achievement of hematologic, cytogenetic and molecular response at the pre-determined time intervals

that is characterized by the blood count normalization and retreat of disease-related symptoms, elimination of Ph^+ bone marrow cells and a significant (at least 3-log) reduction of *BCR-ABL1* transcript level measured by real-time PCR, respectively (Tab. 1) (Faber *et al*, 2010b).

The radiation therapy of the spleen or total body eventually splenectomy were previously the first and the only one treatment possibility till the discovery of chemotherapy. This approach had minimum success with no effect to prolonged overall survival.

First steps in the development of leukaemia treatment were awarded by the Nobel Prize in Physiology or Medicine in 1988 for Gertrude Belle Elion and George Herbert Hitchings. In 1950s they have synthesized antimetabolites - diaminopurine, thioguanine, and 6-merkaptopurine (also known as 6-MP and trade name Purinetol) that were proved to be effective for leukaemia treatment. But with all of these new chemotherapeutic treatments, the disease was only suppressed not primarily cured. After chronic phase period patients went into remission but then relapsed and died (http://www.chemheritage.org/).

Also in 1950s the chemotherapeutic agent busulfan was introduced to induce myelosuppression and relatively efficacious blood count control. The mechanism of the cytotoxicity of this alkylation agent is via hydrolyzation, the release of methansulfonate groups and alkylation causing DNA disruption. The narrow therapeutic window and distinct interindividual variability in the absorption and the metabolism of this drug were the most significant complications in the busulfan therapy management. Currently this drug still has a role in the conditioning before allogenous human stem cell transplantation (HSCT). Larger biological availability resulting in higher plasmatic levels cause severe risk of organ toxicity ("busulfan lung") but on the other hand frequent relapses were achieved in patients with lower busulfan plasma levels. Long-term drug usage is also associated with infertility in both gender and irreversible haematopoiesis aplasia (Hehlmann *et al*, 1994; Slattery *et al*, 1997; Faber *et al*, 2010b).

In 1970's busulfan was replaced by a cell cycle-specific inhibitor of DNA synthesis in S-phase of cell cycle – hydroxyurea that rapidly, but transiently evoke the hematologic response with fewer adverse events (nausea,

14

vomiting, diarrhoea, mucosal ulcers and skin manifestation). In the comparison to the busulfan, patients treated by hydroxyurea show longer median survival (44 months compared to 56 months, respectively), (Hehlmann *et al*, 1994; Hochhaus et al, 1997). These two chemotherapeutic agents induced complete hematologic response in 50 % to 80 % of patients but cytogenetic remission was very rare. No Ph chromosome elimination was achieved and the progression to advanced blast phase of the disease was postponed only (Kantarjian *et al*, 1993, 1998).

Immunotherapy using interferon-α, introduced a new era of CML management. This human naturally forming protein with antiviral function has a wide action on immune system contributing to antileukaemic, antiproliferative and antiangiogenic effect. Not only complete hematologic but also significant cytogenetic response was reached in treated patients, even 20-25 % achieving a complete cytogenetic response (Kantarjian *et al*, 1995). Long discussions were held about the ability of complete CML cure with interferon. Only a small group of patients (5 %) was shown with undetectable *BCR-ABL* gen by RT-PCR after this therapy. Over two years of molecular response duration there is a possibility to the sequential interferon dose reduction and the treatment termination but only in rare cases the minimal residual disease level resembled to the condition after allogeneic stem cell transplantation (Faber *et al*, 2010b; Hochhaus *et al*, 1995). Adverse events appear in the majority of patients mainly in the beginning of the therapy. This includes elevated temperature up to fevers, flu-like syndrome, shakes, weakness, fatigue, headache, and muscles, joints and bones pain. The medium-frequent events are lack of appetite, nausea, emesis, diarrhoea, abdominal pain, skin rash, emotional lability up to the depression, increased hair loss, myospasm, hepatic and hematologic toxicity manifested by neutropenia and thrombocytopenia, anaemia is less frequent. In most of cases the events are in the long term quite well tolerated, but in 10 – 25 % of patients tend to early therapy interruption (Faber *et al*, 2010b).

Allogenous HSCT or the bone marrow transplantation (BMT) has been traditionally regarded as the only chance for the complete cure of CML but the applicability is limited by suitable bone marrow donor and also by the

age of the patient and the disease stage. HSCT enables long-term and disease-free survival in 50-80 % and 30-70 % of patients, respectively; however relapses occur in 15-30 % of patients and late relapses rarely occurs years after the transplantation (Horowitz *et al*, 1996).

Table 1: Definitions of response (adapted from Ramirez *et* DiPersio, 2008).

Response	Criteria
Haematological	Check every 2 weeks until CHR achieved, then monitoring every 3 months.
Complete (CHR)	Complete normalization of peripheral blood count: white blood cells count $< 10 \times 10^9$ L^{-1} with normal differential, platelet count $< 450 \times 10^9$ L^{-1}; ≤ 1 % of circulating immature cells (only if consisting of metamyelocytes); disappearance of all signs and symptoms of disease including palpable splenomegaly.
Partial	White blood cells of $10\text{-}20 \times 10^9$ L^{-1} or normal count with >1 % of persistent immature cells (blasts, myelocytes, or metamyelocytes); platelet count < 50 % of pre-treatment count, but $> 450 \times 10^9$ L^{-1}; splenomegaly < 50 % of pre-treatment extent, but persistent, or other signs of disease.
Cytogenetic	Check every 6 months until CCR achieved then every 12-18 months. At least 20 marrow metaphases must be examined.
Complete (CCR)	No Ph^+ metaphases.
Major (MCR)	0-35 % Ph^+ metaphases (complete + partial).
Partial (PCyR)	1-35 % Ph^+ metaphases..
Minor	36-90 % Ph^+ metaphases.
Molecular	Check every 3 months.
Deep (MR$^{4.5}$ or MR$^{5.0}$)	BCR-ABL mRNA undetectable by RT-PCR or 4.5/5.0 log reduction of BCR-ABL transcript level.
Major (MMR)	Bcr-Abl/Abl ratio < 0.10 % or $\geq$ 3-log reduction of *BRC-ABL* mRNA

In general, younger patients in chronic phase of CML, with HLA identical sibling, and HSCT performed during first year after the diagnosis have better prognosis to disease-free and overall survival than older patients in advanced stage of CML, with unrelated HLA matching donor of the bone marrow (Gratwohl *et al*, 1988). The chemotherapy pre-treatment before the transplantation also show significant influence on disease-free survival that was higher among patients pre-treated by hydroxyurea (61 %) than among those with busulfan medication (45 %), (Goldman *et al*, 1993).

Advanced understanding of malignant diseases molecular biology and pathogenesis of CML led to the development of novel specific therapeutics

targeted especially to the protein responsible for the disease inception, tyrosine kinase Bcr-Abl – tyrosine kinase inhibitors (TKIs).

1.2. Tyrosine kinase inhibitors

Many of fundamental cellular actions including the cell development, differentiation, proliferation, survival, growth, apoptosis, cell shape, adhesion, migration, T-cell and B-cell activation, angiogenesis, responses to extracellular stimuli, neurotransmitter signalling, platelet activation, transcription, and glucose uptake are regulated by tyrosine kinases (TKs). Due to their key role in the cell metabolism and signaling, insufficient or abnormal function of TKs can lead to several disorders including the developmental abnormalities, immunodeficiency, non-insulin-dependent diabetes mellitus, atherosclerosis, psoriasis, renal disease, neurological disorders, leukaemia, and solid tumours. Using the X-ray crystallographic studies, the tertiary structure of tyrosine kinases was revealed and understanding of "ATP-binding site" regions facilitated accelerated new drug development (Hunter, 1998; Madhusudan *et* Ganesan, 2004; Fabbro *et al*, 2002). During the last 20 years, a number of preclinical and clinical studies with various TKIs proceeded and the most promising imatinib (STI571, IM) was the first Bcr-Abl tyrosine kinase inhibitor used for the treatment of CML. Even if the IM therapy is very successful, some patients do not respond to IM or relapse after long-term usage. This led to the second generation of TKIs introduction (Faber *et a*l, 2010b).

1.2.1. Imatinib

The development of this small molecule represents large improvement and main success for targeted chemotherapy in the cancer treatment and break-through in CML treatment. Formally, the imatinib is derivate of phenylaminopyrimidine with chemical formula (4-[(4-methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[{4-(pyridinyl)-2-pyrimidinyl}amino]phenyl]benzamide methanesulfonate), (Fig 3).

For the first time, IM was synthesized in 1992, called CGP57148 or STI571, and very early its outstanding favourable effect was evident in the mouse model of CML, the drug operated uncytostatically and very

selectively. The inhibition activity was proved for several tyrosine kinases e.g. c-Abl, PDGFR-α, PDGFR-β, and c-KIT. The imatinib inhibits the autophosphorylation of Bcr-Abl (IC$_{50}$ 188 ± 18 nM) and suppresses proliferation and tumour formation of myeloid cells expressing *BCR-ABL* (Druker *et al*, 1996; Buchdunger *et al*, 1996; Manley *et al*, 2002). The crystallographic studies have shown that the high selectivity and the efficacy of imatinib are based on its ability to bind and lock Bcr-Abl in its inactive, auto-inhibited conformation. It competitively inhibits the kinase by occupying ATP-binding site; phosphorylation in various pathways that lead to malignant transformation of cells is blocked. Bcr-Abl inhibition via IM results finally in apoptotic death of CML cells (Martinelli *et al*, 2005; Roskoski *et al*, 2003; Druker *et al*, 1996).
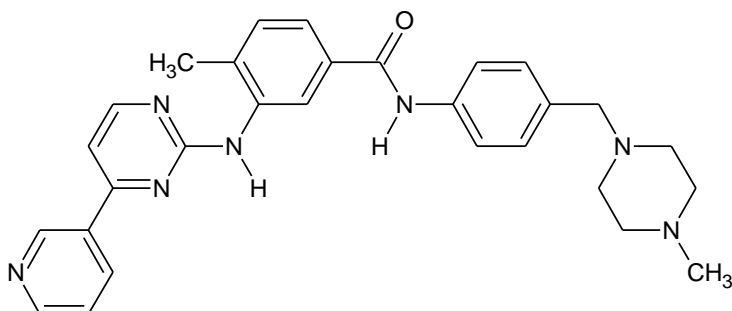
Figure 3: Structure of imatinib molecule.

The clinical trials started in 1998, one year later the second phase of clinical studies were ongoing and the third phase trails continued in 2000. In May 2001, based on the excellent results, the U.S. Food and Drug Administration (FDA) approved IM use for CML patients in the dose 400 mg/day in chronic phase and 600 mg/day in advanced phase. Since July 2002 full payment by insurance companies is approved in Czech Republic (Faber, 2005).

Currently IM is the first line treatment in newly diagnosed patients in chronic phase of CML. It is also used for the treatment of Ph positive acute lymphoblastic leukaemia, gastrointestinal stromal tumour, chronic eosinophile leukaemia or hypereosinophilic syndrome, and systemic

18

mastocytosis positive for FIP1L1-PDGFRα or ETV6-PDGFRβ (Faber *et al*, 2010b).

1.2.1.1. Mechanism of action

Imatinib is a competitive inhibitor of ATP binding to the catalytic domain of Abl kinase. Based on the crystal structure of the protein it is known, that IM binds to A-loop of Abl in the inactive conformation, contacting 21 amino acids residues. IM forms a number of hydrogen bonds with the kinase domain that enable fix and stable binding. Any change of single amino acid can influence this binding, e.g. substitution of threonine 315 by methionine (T315I) in the insulin receptor kinase causes complete insensitivity to IM (Nagar *et al*, 2002; Deininger *et al*, 2003). The mechanism of action is well studied in *BCR/ABL* positive cells. IM interacts with p210$^{BCR/ABL}$ in nucleotide binding place to prevent from ATP linkage and thus stabilize the enzyme in inactive conformation. In this manner the phosphorylation of tyrosine kinase is blocked and numbers of signal pathways that participate on the cell leukaemic phenotype origin are suppressed. Hence, IM does not inhibit the origin of the leukaemic gene *BCR-ABL* that plays a key role in CML formation, but defends the effect on the protein level (Fig. 4) (Faber *et al*, 2010b).

According to the current results, IM action leads to the apoptosis of leukaemic cells and the inhibition of pathologic clone proliferation. IM also does not inhibit leukaemic progenitor cells hence it is not able to completely cure but "only" suppress the leukaemic clone proliferation to clinically safety level of disease minimal. Therefore IM is recommended to permanent usage even if the stable and long-term molecular response expressed by negative *BCR-ABL* detection in RT-PCR is achieved (Faber *et al*, 2010b; Graham *et al*, 2002).
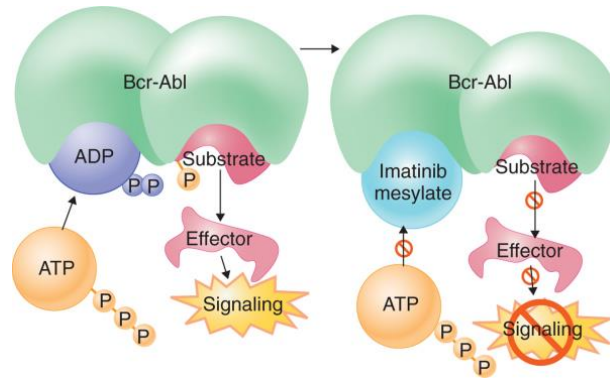
Figure 4: Imatinib mechanism of action. Molecule of IM occupies the ATP binding pocket of the ABL kinase domain, prevents binding of ATP molecule and thereby inhibits phosphorylation of substrate and downstream activation of signal pathways leading to leukaemic cell transformation. ADP = adenosine diphosphate; ATP = adenosine triphosphate; P = phosphate group (adapted from http://www.cixip.com)

1.2.1.2. Clinical studies

Phase I trials with STI571 began in June 1998. The main purpose was to set maximally tolerated dose with optimal clinical benefit. Patients with chronic phase of CML who had failed the therapy with the interferon-α were taken to the study group. Favourable results were reached for the dose of 300 mg of daily oral therapy or higher. CHR was achieved in 53 of 54 patients (98 %) and CCR in 7 (13 %) of these patients. Side effects have been mostly mild and dose limiting toxicity was not observed. Nausea, vomiting, diarrhoea, fluid retention, periorbital edema, muscle cramps, arthralgias, and skin rashes were included among the most common side effects. Frequently monitored myelosuppression of grade 2 or 3 at the dose 300 mg in 21 % and 8 % of patients respectively was probably related to the therapeutic effect due to the suppression of cells positive for Ph chromosome occurrence (Ph+) haematopoiesis (Druker *et al*, 2001a). Due to STI571 efficiency in Phase I clinical trial the study was expanded to include patients with myeloid blast crisis (n = 38) and patients with relapsed or refractory Ph+ ALL or lymphoid blast crisis of CML (n = 20). Daily doses of STI571 ranged from 300 to 1000 mg. In 21 patients (55 %)

with a myeloid blast crisis response was achieved, CHR was proved in 4 of these responding patients. In lymphoid blast crisis or ALL patients response was achieved in 14 cases (70 %), 4 had complete response. Seven patients (18 %) with a myeloid blast crisis continued with IM treatment and remained in remission from 101 to 349 days after the treatment beginning. Unfortunately, all but one patient with a lymphoid blast crisis or ALL has relapsed, thus the activity of STI571 in CML blast crisis and Ph+ ALL was proven but response is not durable (Druker *et al*, 2001b).

The treatment success of the phase I studies prompted immediately the beginning of phase II studies, where STI571 was used as single agent for all stages of CML. Results from a group of patients with the advanced stages of CML (accelerated phase, blast crisis) and Ph+ ALL corresponded to phase I trial. Overall hematologic response (HR) and CHR was achieved in 52 % and 15 % of myeloid blast crisis patients (n=229), respectively and in 31 % of patients hematologic response was maintained more than 4 weeks. However only 16 %, and 7 % reached MCR and CCR, respectively. In Ph+ ALL patients (n=56) overall HR and CHR was achieved in 59 % and 22 %, respectively and hematologic response remaining longer than 4 weeks in 27 % of patients. The treatment response was higher in patients in the accelerated phase (n=181), where 82 %, and 69 % of patients achieved overall HR, and sustained HR remaining longer than 4 weeks (complete in 34 %), respectively. MCR and CCR were reached in 24 % and 17 % of patients. The most promising results were observed in a group of patients in CML chronic phase after the failure of the interferon therapy; in 95 % of patients remaining CHR was reached and also achieved MCR and CCR in 60 %, and 41 % of patients. The durability of achieved responses was demonstrated; progression free survival appeared in 89.2 % at 18 month. Both of these studies of phase I and phase II confirmed the distinct efficacy of IM for the CML treatment and allowed the FDA to approve IM for the treatment of CML in advanced phase and after IFN therapy failure (Sawyers *et al*, 2002; Ottmann *et al*, 2002; Kantarjian *et al*, 2002; Talpaz *et al*, 2002).

In the phase III clinical studies IM (n=553) was compared also with the combination of IFN and cytarabine (n=553). During the study crossover between therapies was allowed in the case of the treatment failure or

intolerance. The obtained results evidently demonstrated imatinib superiority to IFN and cytarabine in CHR (95.3 vs. 55.5 %), MCR (87.1 vs. 34.7 %), CCR (76.2 vs.14.5 %), and progression-free survival during 18 months (96.7 vs. 91.5 %), respectively (O'Brian *et al*, 2003). Due to better tolerance and more efficient treatment response achievement under IM therapy the drug was approved for first-line treatment of CML in the United States and Europe.

The number of extensive studies had been in progress aimed at imatinib treatment efficiency, safety and improvement (Kantarjian *et al*, 2004; Deininger *et al*, 2014; Thielen *et al*, 2013; Baccarani *et al*, 2014; Mahon *et al*, 2010). One example is the comparison between the standard and high-dose IM therapy in Ph+ chronic phase chronic myeloid leukaemia. In the group of 114 patients receiving IM 400 mg twice daily, 96 % (n=109) had MCR (Ph<35 %), CCR (Ph 0 %) was achieved in 90 % of patients (n=103) and no patient has progressed to accelerated or blastic phase during median follow-up of 15 months. The estimated two-year survival rate was 94 %. The molecular analyses using quantitative PCR in overall 112 patients showed BCR-ABL/ABL reduction to < 0.05 % in 63 % (n=71) and to undetectable levels in 28 % (n=31). The treatment response in high-dose IM administration reached significantly better results (CCR: p = 0.0005; MMR: p = 0.00001 and CMR: p = 0.001). Surprisingly, high-dose IM was also well tolerated only with more frequent myelosuppression but it was manageable with dose reduction or with growth factor support (erythropoietin, filgrastim), (Kantarjian *et al*, 2004).

In other group of 153 patients receiving IM 400 mg once or twice daily, molecular response was deeper in 800 mg/day arm (4-log reduction of *BCR-ABL1* mRNA: 25% vs. 10% of patients; 3-log reduction: 53% vs. 35%). CHR was similar, CCR but with grade 3-4 most commonly hematological toxicities was higher in IM800 (85% vs. 67%; 58% vs. 31%, respectively). Even if a few patients have relapsed, progressed or died, both progression-free and relapse-free survival were superior for IM800 (Deininger *et al*, 2014).

High-dose IM in the combination with cytarabine did not prove higher treatment efficacy and the MMR rate during overall follow-up (Thielen *et al*, 2013). Final update from the Tyrosine Kinase Inhibitor Optimization and

22

the Selectivity (TOPS) study that evaluate high- (800 mg/day; n=319) versus standard-dose (400 mg/day; n=157) IM in newly diagnosed patients with Ph+ chronic phase CML shows close similarity of MMR, event-free survival (EFS), progression-free survival (PFS), or overall survival (OS) in both groups of patients. The estimated rates of PFS on the treatment and OS at 42 months were significantly higher in patients with MMR at 6, 12, and 18 months compared with those without MMR. Rates of CCR and MMR was observed higher in patients with 1 or no sustained treatment interruptions during the first 12 months compared with patients who had more than 1 interruption during the same period, but no improvement in EFS or PFS. The results show that the adherence to prescribed dose without interruption may be more important than the initiation of the therapy with higher doses of imatinib (Baccarani *et al*, 2014).

Another issue in IM treatment was the discontinuation of the therapy in patients with CML who have maintained complete molecular response (CMR; no detectable Bcr-Abl protein by PCR). The Multicentre Stop Imatinib (STIM) trial included patients from 19 participating institutions in France with more than 2 years of documented CMR while on IM therapy attempted the treatment discontinuation. A total of 69 patients were followed-up for more than 12 months. Forty-two patients (61 %; 19 of 34 with prior interferon therapy and 23 of 35 with IM first-line therapy) relapsed (40 before 6 months, one patient at month 7, and one at month 19). The Sokal scores showed that 17 of 35 patients (48.6 %), 15 of 23 patients (65.2 %), and 7 of 8 patients (87.5 %) who relapsed were at low, intermediate, or high risk, respectively. All patients who relapsed responded to re-introduction of imatinib: 16 of the 42 patients who relapsed showed decreases in their *BCR–ABL* levels, and 26 achieved CMR that was sustained after imatinib re-administration. Results of this study show that IM can be safely discontinued in patients with a CMR of at least 2 years duration. The imatinib discontinuation under these conditions yields promising results for the molecular relapse-free survival, raising the possibility that, at least in some patients, CML might be cured with tyrosine kinase inhibitors (Mahon *et al*, 2010).

1.2.1.3. Resistance

Increased risk of resistance to the treatment is the most serious problem associated with IM therapy. Primary resistance is defined as the failure to achieve a complete hematologic response within 3 months, any cytogenetic response within 6 months, a partial cytogenetic response within 12 months and a complete cytogenetic response within 18 months. However, the occurrence of secondary (or acquired) resistance, defined as the loss of response in patients who initially responded to the treatment, plays important role. The mechanism of the resistance could be further divided into the two basic groups – Bcr-Abl dependent and Bcr-Abl independent (Druker *et al*, 2006). In the first group the formation of specific point mutations within several critical regions of the *ABL* kinase domain is the most frequent mechanism of resistance. These mutations either intercept the contact between the drug and the kinase domain or change the steric form of the protein, thereby usually causing the switching from the inactive conformation to the active form (Quintás-Cardama *et al*, 2009).

Crystallographic studies have shown the high selectivity and the efficacy of IM due to its ability to bind and lock Bcr/Abl in its inactive, auto-inhibited conformation. Mutations can be divided into four groups based on the localization of their development. The first group of mutations (e.g. G250E, Q252R, Y253F/H and E255K/V) includes amino acids which form the phosphate-binding loop for ATP (also known as the P-loop). P-loop mutations are more frequent in the advanced CML - accelerated phase or a blast crisis. They are 70-100-fold less sensitive to imatinib than native Bcr/Abl and have higher transforming activity than the wild-type of p210. A second group (e.g. V289A, F311L, T315I and F317L) is situated in the IM binding site and reacts directly with inhibitors via hydrogen bonds or Van der Waals interactions. Probably most serious of all mutations is the T315I that is resistant to imatinib and also to all second-generation Bcr/Abl inhibitors (e.g. dasatinib, nilotinib and bosutinib). The third group (e.g. M351T and E355G) clusters close to the catalytic domain and the fourth

group of mutations (e.g. H396R/P) is located in the activation loop, whose conformation is a molecular switch controlling kinase activation/inactivation (Fig. 5), (Druker *et al*, 2006; Martinelli *et al*, 2005). Another mechanism dependent on Bcr-Abl is the gene amplification of *BCR-ABL1*, the subsequent overexpression of p210 and therefore significantly less efficacy of IM (Quintás-Cardama *et al*, 2009). The first mediator of the Bcr-Abl independent mechanism of resistance is the serum α1-acid glycoprotein (AGP). IM binds to AGP, therefore the plasmatic and intracellular concentration of the drug decreases and the inhibition of Abl kinase is reduced (Widmer *et al*, 2006). Another mechanism independent from Bcr-Abl is the constitutive activation of downstream signalling molecules such as Src family kinases (SFKs) which activate the pathways regardless of Bcr-Abl inhibition. SFKs regulate cell proliferation and survival and are also involved in the development of late-stage CML (Danhauser-Riedl *et al*, 1996).

1.2.1.4. Transport mechanism

IM is also a substrate for numerous drug transporters which play a key role in the drug absorption, distribution, elimination *in vivo*, transport of the drug across the cell membrane and hence in the determination of plasmatic and intracellular concentrations. The actual amount of IM in the target cells is a direct function of the balance between the influx and efflux. The influx of IM is mediated via *hOCT1* (human organic cationic transporter), therefore the lower expression of this transporter results in a lower intracellular concentration of the drug. Low activity of *hOCT1* leads to poor response on the part of the patient to the treatment (Thomas *et al*, 2004; White *et al*, 2006, 2007). The *ABCB1* transporter (also known as P-glycoprotein), coded by the *MDR1* (multi drug resistance) gene, supports the efflux of the drug out of the cells (Ni *et al*, 2010; Dohse *et al*, 2010). These transport mechanisms could be one of the reasons for the Bcr/Abl independent mechanism of resistance.
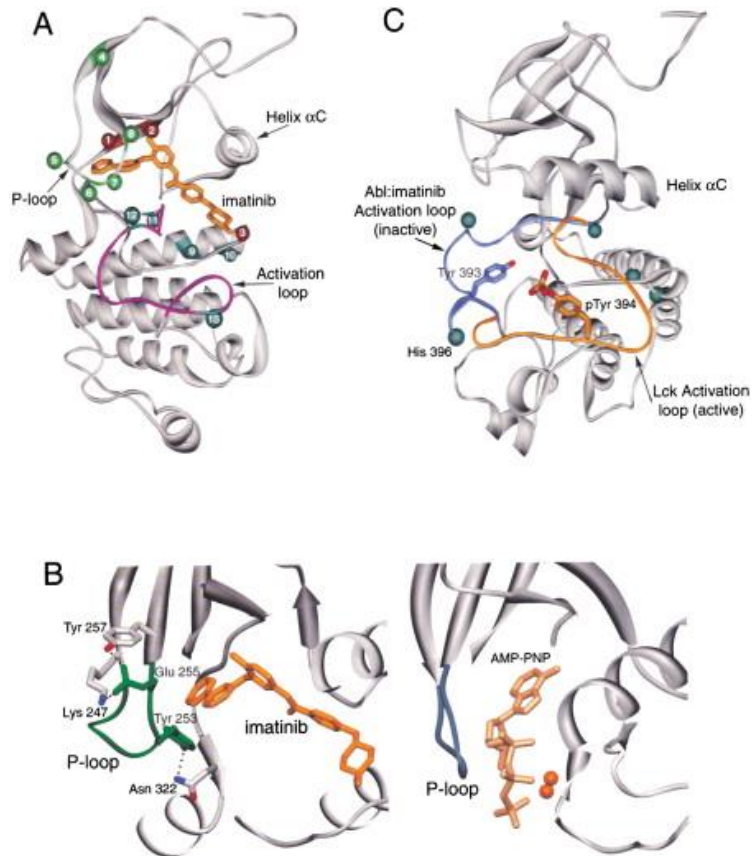
Figure 5: Proposed mechanisms of imatinib-resistant mutations action based on the crystal structure of ABL complexed with imatinib. **A:** Ribbon representation of the kinase domain of ABL complexed to imatinib, depicting resistant mutations. Imatinib is shown in gold. Positions 1–3 (red) are mutations that directly affect imatinib binding. All other positions are those that likely affect the ability of the kinase to achieve the conformation required to bind imatinib, including those in the P loop (4–8; green) and those in the vicinity of the activation loop (9–13; cyan). The activation loop is coloured purple. The positions of amino acids found mutated are depicted by spheres: 1, F317; 2, T315; 3, F359; 4, M244; 5, G250; 6, Q252; 7, Y253; 8, E255; 9, M351; 10, E355; 11, V379; 12, L387; 13, H396. **B:** Comparison of the P loop in the ABL: imatinib structure (left) with that of active insulin receptor kinase (IRK) (right). Glu 255 in the P loop (green) of ABL makes strong interactions with Tyr 257 and Lys 247. The P loop is distorted, with Tyr 253 swung into the ATP binding site, forming a hydrophobic face against which imatinib packs. In IRK, the P loop (blue) is folded out over the nucleotide. Orange spheres indicate Mg^{2+} ions associated with nucleotide. Representation constructed using a

previously described program. **C:** Mutations located at sites that could influence conformational changes undergone by the activation loop. For comparison, the activation loop of Lck (an active tyrosine kinase) is shown in gold, including the phosphorylated Tyr residue (pTyr 394). Tyr 393, the site of activating phosphorylation in ABL, is shown in blue. Positions of mutations M351T, E355G, V379I, L387M, and H396R are depicted as spheres (adapted from Shah et al, 2002).

1.2.1.5. Imatinib metabolism

Imatinib has an extremely favourable clinical pharmacokinetics profile. The plasma peak concentration is achieved at 2 h after oral administration; the bioavailability is ~98 % irrespective of oral formulation or dosage while the absorption is not influenced by food or concomitant antacid use (van Erp *et al*, 2009). Despite equal dosages of the drug (e.g. 400 mg/day), relatively high interindividual variability in the drug exposure has been observed (Picard *et al*, 2007; Faber *et al*, 2012; Larson et al, 2008; Peng et al, 2004). This could be related to several factors including the activities of the metabolizing enzymes, influx/efflux transporters such as *hOCT1* and P-glycoprotein, along with poor patient adherence to the treatment (Roth *et al*, 2010). A significant portion of pharmacokinetics variability also originates in α1-acid glycoprotein plasma concentration (Widner *et al*, 2006).

The general principle of the xenobiotics metabolism in human body is to enhance their excretion from body by altering them to more hydrophilic and less toxic forms. Biotransformation is set of metabolic pathways that modify chemical structure of the drug with the assistance of various enzymes activity. Metabolic pathways are divided into phase I and phase II reactions. In phase I, reactive and polar groups are introduced into the molecule via variety of enzymes. The most common biotransformation phase I reactions are dealkylation, oxidation, hydrolysis, reduction, and some less frequent reactions like chiral inversion (Holcapek *et al*, 2008). In some cases, phase I metabolites are more active in comparison with parent drug, as an example tamoxifen versus its metabolite endoxifen (Stearns *et al*, 2003), codeine versus morphine (Dayer *et al*, 1988), and others; therefore monitoring of not only a parent drug but also of its metabolites could be important. Phase I metabolites are most often preliminary point

for further biotransformations via conjugation reactions that produce phase II metabolites. These activated metabolites are conjugates with glutathione (GSH), glucuronic acid, sulphate, and glycine and other amino acids. Most of these reactions are catalysed by number of enzymes such as cytochrome P450 isoforms in phase I reactions (Fig. 6) and uridine diphosphate glucuronyltransferase (UGT) or sulfotransferase (SULT) in phase II metabolism (Holcapek *et al*, 2008).

Since imatinib is metabolized by cytochrome P450 3A4 (CYP3A4), drugs which influence CYP3A4 may affect the effect of imatinib (Peng *et al*, 2004). The first step in IM metabolism is the formation of N-demethylated product (CGP74588) which is also pharmacologically active. This is followed by a series of oxidative processes. The second phase of the metabolic pathways includes imatinib, and its metabolites conjugation mainly with glucuronic acid. Imatinib is largely excreted in faeces and a minor portion in urine (67.8 % of a dose in 11 days and 13.2 % in 7 days, respectively), (Gschwind *et al*, 2005).
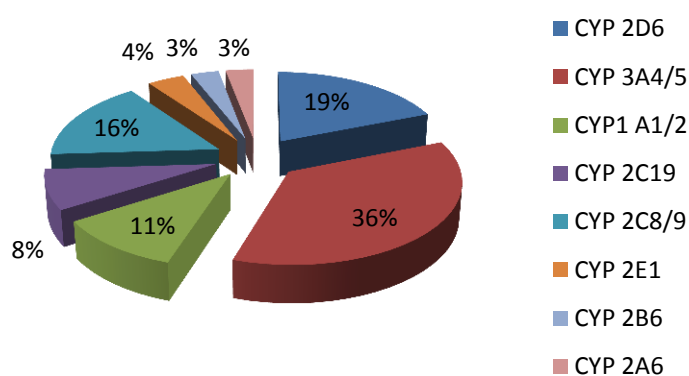
Figure 6: Proportion of drugs metabolized by P450 enzymes (adapted from Wrighton et al, 1992)

Currently, only a few studies about IM metabolism were published (Gschwind *et al*, 2005; Marull *et al*, 2006; Rochat *et al*, 2008; Ma *et al*,

2009). In a study of Gschwind *et al,* biotransformation of IM was studied in four male healthy volunteers after a single oral dose of 14C-labeled imatinib mesylate. Fourteen main metabolites including glucuronides in human plasma were found using RP-HPLC coupled with Q-TOF mass spectrometer. Major observed metabolic transformations were N-demethylation, piperazine ring oxidation with lactam formation, piperazine-N-4 oxidation, benzylic hydroxylation, the loss of piperazine moiety by oxidative deamination and rapid further oxidation of the intermediate aldehyde to carboxylic acid metabolite. Phase II metabolism included direct conjugation of IM and the N-desmethyl metabolite with glucuronic acid, and glucuronidation of oxidative metabolites (Fig. 7), (Gschwind *et al*, 2005).

Next study by Marull *et* Rochat was aimed at imatinib fragmentation and new metabolite identification from microsomal incubations containing CYP isozymes. The fragmentation study was carried on triple-quadrupole and linear ion trap tandem mass spectrometers (MS^n). Accurate mass determination was performed by enhanced mass resolution with delta mass errors below 20 ppm. Hydrophilic interaction liquid chromatography was used for resolving one demethylated-, two hydroxy- and three N-oxide metabolites. Various rates of metabolite formation were observed between CYP isozymes. Structure elucidation of metabolites was performed with deuterium oxide containing mobile phase or by incorporation of ^{18}O from $H_2^{18}O$ added in the incubations (Marull *et* Rochat, 2006). Similar study by Rochat *et al* was aimed at IM metabolite profiling in parallel to IM quantification in plasma of patients. The metabolite identification by LC-MS/MS was performed using either in vivo incubation of human liver microsomes or patient plasma samples; for simultaneous determination of imatinib plasma level (IPL) and 14 metabolites, plasma samples of 38 patients were examined. In the comparison of metabolite profile in microsomal incubations and patients plasma samples some differences were observed such as induction of some plasma metabolites as results of sequential transformations. The correlation studies between IPL or its metabolites and patients' outcome were not allowed because only a few data were recorded (Rochat *et al*, 2008).

deamination + oxidation
to carboxylic acid
(M42.2)

N-oxidation
(CGP72383)

lactam formation
(APG049, APG050)
(M23.7, M25.6, M27.6, M28.8, M29.6)

N-oxidation
(CGP71422)

N-demethylation
(CGP74588, M20.0a, M25.6, M28.8, APG049

hydroxylation
(AFN911)

N- and/or O-glucuronidations
(M20.0a, M21.0, M23.7, M25.5, M27.6)
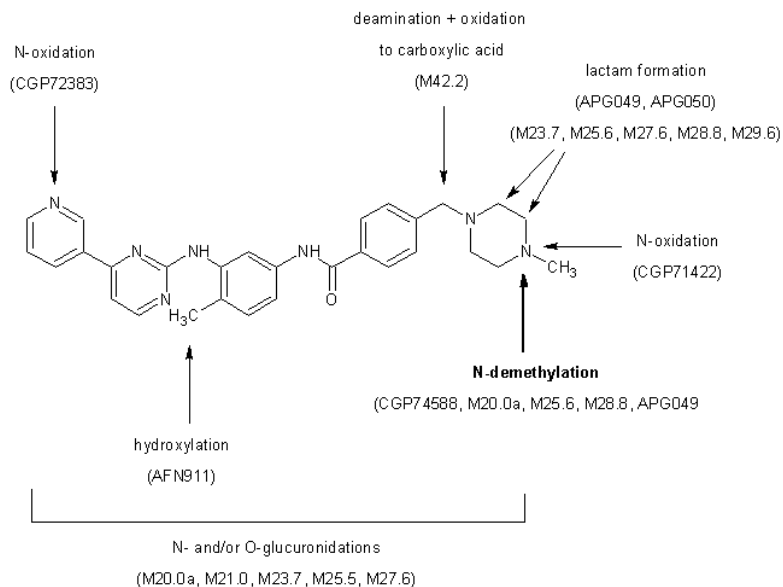
Figure 7: Chemical structure of imatinib and identified metabolic processes in human. The main metabolic pathway was the N-demethylation to CGP74588 (adapted from Gschwind et al, 2005).

Further study dealing with imatinib metabolite characterization and differentiation of hydroxylation and N-oxidation was performed by Ma *et al* by LC/APCI-MS. Experiments were performed with rat liver microsomes in the presence of NADPH and nine metabolites were found in the UV chromatogram: four oxidative metabolites, N-desmethyl metabolite, N-demethylation followed by oxidation metabolite and three novel conjugates of adenine dinucleotide phosphate (ADP$^+$) with IM, N-desmethyl IM, and N-oxide IM (Ma *et al*, 2009).

1.2.2. The second generation of TKIs

The first possibility for overcoming IM resistance is the dose escalation of the drug. A high-dose (600 or 800 mg) may be more effective than standard doses of IM in patients with CML resistance to IM or also as the first-line therapy in newly diagnosed chronic phase CML (O'Brian *et al*,

2003; Kantarjian *et al*, 2003; Cortes *et al*, 2003; Baccarani *et al*, 2009). In the last years, two other oral Bcr-Abl TKIs, dasatinib (Sprycel[®]) and nilotinib (Tasigna[®]) have been approved not only for the treatment of CML in patients who have failed or have been intolerant of imatinib therapy but also for the first-line treatment based on recently accomplished randomized studies (Cortes *et al*, 2010a,b; Kantarjian *et al*, 2006, 2007, 2009; le Coutre *et al*, 2008; Apperley *et al*, 2009; Hochhaus *et al*, 2007, 2008).

Nilotinib (Fig. 8) is a derivate of IM with a similar structure but allowing better topographic fit to Bcr-Abl. It results in 20-fold greater potency and less likelihood of disruption by mutations (Cortes *et al*, 2010a; Kantarjian *et al*, 2006, 2007; le Coutre *et al*, 2008).

Dasatinib (Fig. 9) is structurally unrelated to IM but inhibits wild-type Bcr-Abl with a 325-fold higher efficacy. Unlike other TKIs, dasatinib binds to the active as well as inactive conformations of p210. Dasatinib also potently inhibits members of the Src family of kinases and is known as a "dual" Src and Abl inhibitor (Cortes *et al*, 2010b; Apperley *et al*, 2009; Hochhaus *et al*, 2007, 2008; Kantarjian *et al*, 2009).
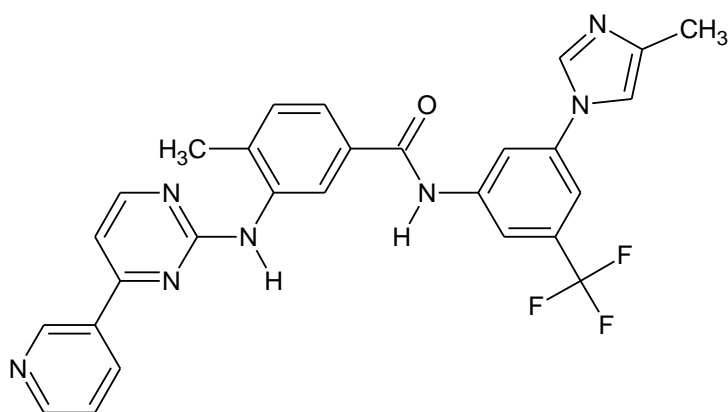
Figure 8: Structure of nilotinib molecule.

31
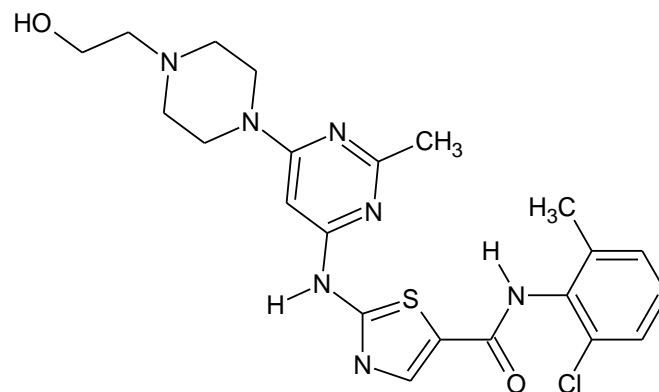
Figure 9: Structure of dasatinib molecule.

Both nilotinib and dasatinib induce high rates of hematologic and cytogenetic responses in patients with all phases of imatinib resistant CML. During in vitro studies, both agents inhibited almost all imatinib resistant mutation-positive cell lines apart from T315I (Pavlovsky *et al*, 2009; McFarland, 2009). Both of the drugs are generally well tolerated and after dosage reduction, adverse events may be resolved. The most frequently occurring nonhaematologic toxicities during dasatinib treatment include diarrhoea, headache, haemorrhage, musculoskeletal pain, pyrexia, fatigue, skin rash, infection, nausea and dyspnea. The most common complication was fluid retention, which occurred in 50 % patients and was presented in most cases as superficial edema in patients treated with imatinib, but in case of dasatinib as pleural effusions in 10-20% of patients or very rarely as pericardical effusions. In addition, certain hematologic adverse events such as neutropenia, thrombocytopenia and anaemia were observed (Cortes *et al*, 2010b; Apperley *et al*, 2009; Hochhaus *et al*, 2007, 2008; Kantarjian *et al*, 2009; McFarland, 2009). Nilotinib toleration and toxicity is very similar, the most common adverse events were rash, pruritus, nausea, fatigue, headache, constipation, diarrhoea, and vomiting. The most frequent hematologic events included neutropenia, anaemia and thrombocytopenia. Additionally, certain laboratory abnormalities were observed, such as elevated bilirubin, liver transaminases,

32

hypophosphatemia, elevated lipase or amylase and hyperglycaemia (Cortes *et al*, 2010a; Kantarjian *et al*, 2006, 2007; le Coutre *et al*, 2008; McFarland, 2009).

1.3. Using mass spectrometry in therapeutic drug monitoring

In general, therapeutic drug monitoring (TDM) practiced usually in clinical or toxicological laboratory is defined as a determination of chemical parameter like plasma or blood drug concentration that serves as an indicator for appropriate therapy adjustment. Based on the knowledge of pharmacokinetics, pharmacodynamics and pharmaceutic properties, the main goal of TDM is to set the most effective and safe particular treatment and achieve the optimal benefit for each patient. Performing TDM requires excellent communication among the team of scientists, clinicians, nurses and pharmacists. The reason for the indication of drug blood/plasma level include especially monitoring of compliance, treatment efficacy (diagnosing under treatment or avoiding toxicity caused by high plasma levels), individualizing therapy during early therapy or dosage changes, monitoring and detecting drug interactions and guiding withdrawal of therapy (Kang *et* Lee, 2009).

For several years TDM was performed using immunoassay but this technique is known for its broad unreliability caused by non-specific interferences from related compounds, metabolite interferences or matrix effects. Little over last decade, important progress was registered in the development of the technique liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). This technique became superior to immunoassay due to better specificity and sensitivity and also it enables measurement of analytes without natural chromophores and fluorophores. The main disadvantage of LC-MS/MS is still high instrument cost, higher technical complexity, expensive consumption expenditures and highly-specialized staff.

Currently, quickness and simplicity of the drug determination is basic requirement for the majority of clinical and toxicological laboratories where sample high throughput is needed. Some companies offer (not only) LC-MS/MS kits for fast, reliable, and standardised analysis with tandem

33

mass spectrometry. RECIPE (Munich, Germany) is one of the leading companies in HPLC and LC-MS/MS diagnostic today. For advantageous TDM they offer ClinCal®Matrix Calibrators, ClinCheck®Matrix Controls, ClinMass®Optimisation Mixtures and ClinMass®Internal Standards for determination of many groups of drugs (benzodiazepines, antiepileptics, immunosuppressants, psychoactive drugs, antimycotics, and many others (www.recipe.de). Chromsystems offers similar complete reagent kits and also separately available controls and calibrators for laboratory diagnostics with HPLC and LC-MS/MS. As an example the kit MassTox® Immunosuppressants for determination of cyclosporin A, everolimus, sirolimus, tacrolimus and MassTox® Series A reagent kit with possibility to analyse up to 150 drugs can be mentioned (http://chromsystems.com).

LC-MS/MS is continually evolving and wide-spreading technique in routine clinical and toxicological laboratories all over the world. Methods for any analyte determination by LC-MS/MS include three necessary fundamental steps: sample preparation, chromatographic separation (if needed) and mass spectrometric detection (Adaway *et* Keevil, 2012).

1.3.1. Sample preparation

Sample processing is dependent on the analyte character, protein-bounding strength and sample type. Essential requirement in sample preparation is to get rid of matrix (everything present in the sample that does not contain the analyte of interest). More complex samples like blood need extensive sample treatment in contrast for instance to urine where only the sample dilution is sufficient.

Preparation of sample for HPLC-MS/MS analysis is based on removal of macromolecules like proteins that could cause HPLC column clogging and removal of other co-eluting substances like salts and phospholipids causing impaired analyte detection, sensitivity loss, and reduced assay specificity and accuracy due to reduced ionization efficiency (Adaway *et* Keevil, 2012; Strathmann *et* Hoofnagle, 2011; Vogeser *et* Seger, 2008).

The most popular sample treatment strategies include solid phase extraction (SPE), immunoaffinity purification, liquid-liquid extraction (LLE), dilution or protein precipitation (PP). SPE is an affinity based method using

a liquid mobile phase and solid stationary phase. Sample cleaning is based on analyte trapping on immobilized media, washing proteins and other interfering substances to waste and final analyte elution usually by organic solvent (methanol, acetonitrile). Currently, SPE offers several modes of principles based on stationary phase-analyte interaction (hydrophobic, ion exchange and mixed mode) and therefore MS compatible extracts free of majority of interferences can be obtained. The typical offline setting is characterized by sample preparation away from the LC-MS system but thanks to automatization online setting has spread widely. In this manner analyte is trapped by small column, washed and after switching the valve eluted onto analytical column. This sample preparation is less time-consuming but more demanding on instrumentation. Prior on line SPE protein precipitation is usually required to prevent instrument blockage but also commercial systems performing SPE without previous PP are available. These systems use either disposable extraction cartridges (Symbiosis, Spark Holland, Netherlands) or turbulent flow technology (Aria, Thermo Fisher Scientific, USA). Immunaffinity purification (or immunoaffinity extraction) is based on antibodies bound to solid phase allowing separation of the antibody-bound analyte from other non-bound matrix components. This method offers significant reduction of unwanted compounds included in matrix but on the other hand some non-specific interference from related compounds or metabolites often occurs.

LLE is based on mixing the sample (usually aqueous) with immiscible organic solvent such as diethyl ether, ethyl acetate, methyl tert-butyl ester, and hexane. Separation of analyte from matrix is based on the solubility in these immiscible solvents. After the separation of two solvent layers, analyte enriched extract is used for the analysis. This method is characteristic for quite poor extraction recovery therefore further subsequent extractions may be carried out to increase analyte quantity and improves extraction recovery. LLE is very effective way how to remove unwanted compounds but it is very difficult to automate and can be time-consuming (Adaway *et* Keevil, 2012; Strathmann *et* Hoofnagle, 2011; Vogeser *et* Seger, 2008). For instance several acidic and neutral pharmaceuticals (warfarin, ibuprofen, paracetamol, phenobarbital, diclofenac, thiopental and many others) can be extracted from whole

blood by LLE based on mixing the blood sample with acidified ethyl acetate (Simonsen *et al*, 2010).

The simplest technique for sample preparation is PP. It can be easily automated and can be performed in 96-well plates to avoid transfer steps. Rapidity of this clean-up procedure is negatively compensates by relatively dirty sample extract that is also diluted by solvent used for precipitation. This leads to problem with sensitivity and careful method validation of whole sample procedure must be performed. In less complicated matrices and analyte of interest in high concentration, sample treated only by dilution and centrifugation can be used for analysis (Adaway *et* Keevil, 2012; Strathmann *et* Hoofnagle, 2011; Vogeser *et* Seger, 2008).

1.3.2. Chromatographic separation

Liquid chromatography has become very popular technique for separation of wide range of possible analytes in complex matrices. Chromatography is essential tool for effective analyte separation and elimination of matrix components often causing inaccuracy related to the ionization process generally called "matrix effects". In general, molecules present in the matrix or eventually in solvent, coeluting compounds and *m/z* isobars can be cause of ion suppression or enhancement. Signal reduction of the target analyte in complex matrix sample compared to signal of target analyte dissolved in pure solvent is the result of the ion suppression. Less volatile or non-volatile compounds can reduce or impede ionization process and decrease ion formation. Ion enhancement, on the other hand, describes the increase of ionization yield of analyte in complex matrix sample compared to pure analyte solution. Variable ionization or precision of the extraction procedure could be eliminated by effective sample clean-up technique, optimization of chromatographic method, enrichment of mobile phase by ion-generating supportive substances or addition of internal standard (IS) to the sample. IS should have similar structure, properties and behavior like analyte of interest; most commonly it is isotopically labelled analyte or structural analogue. In liquid chromatography sample is delivered through LC system to analyser by MS compatible mobile phase that consists of aqueous and organic phase

(methanol or acetonitrile) with common buffers (ammonium formate or acetate) and pH modifiers (formic acid, trifluoroacetic acid, ammonia etc.). Organic/aqueous ratio is maintained or changed during analysis to alter the distribution of analyte and matrix components between the mobile and stationary phase (Adaway *et* Keevil, 2012; Strathmann *et* Hoofnagle, 2011; Vogeser *et* Seger, 2008, 2010). A variety of analytical columns are used with different stationary phase depending on analyte of interest character: normal phase – NP (Deng *et al*, 2005), reverse phase – RP (Mičová *et al*, 2010b; Haouala *et al*, 2009), hydrophilic interaction liquid chromatography – HILIC (Rochat *et al*, 2008; Marull *et* Rochat, 2006) with various chemical properties and particle size from 5 μm (Haouala *et al*, 2009; Velpandian *et al*, 2004) to 3 μm (Honeywell *et al*, 2010) can be used for separation. Small particles enable better resolution but increasing back pressure generated by the system is the limiting factor for the choice of column. Currently, LC systems, so called ultra-high performance liquid chromatography, enable separation under higher pressure using LC columns filled with stationary phase with very small particles (sub 2 μm), (Mičová *et al*, 2010b). This provides fast and effective method for subsequent MS analysis with increased peak resolution and sensitivity.

1.3.3. Mass spectrometry

Detection by mass spectrometry is possible for charged analytes in gas phase; therefore ionization technique must convert analyte in liquid phase to gaseous ions. Ionization is operated in positive or negative mode depending on analyte structure and chemical properties. The most used ionization technique in clinical laboratories is electrospray (ESI) and atmospheric pressure chemical ionization (APCI).

ESI is the most universal soft ionization technique especially suitable for polar/less polar compounds with molecular size from tens up to several thousands Daltons (for multiple charged molecules). In ESI ionization small droplets from liquid mobile phase containing analyte molecules continually evaporate and concentrate. Thanks to combination of voltage, temperature, air and increasing charge in shrinking droplets, ions from droplet surface released from liquid to gas phase and enter the mass

spectrometer (Fig. 10). These processes are called desolvatation and desorption. ESI is the most favorite technique for drug analysis in TDM (Adaway *et* Keevil, 2012; Strathmann *et* Hoofnagle, 2011; Vogeser *et* Seger, 2008).
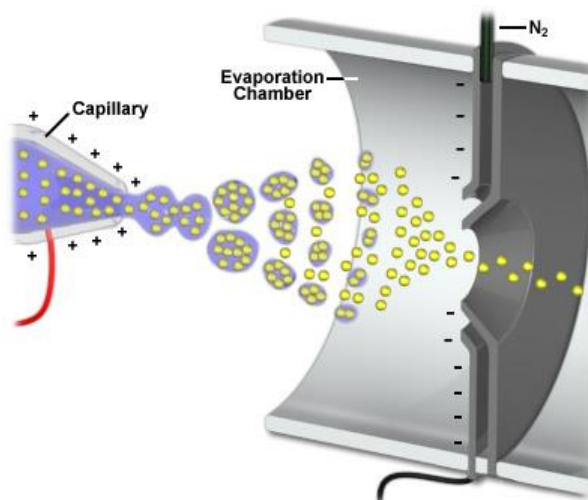
Production of ions in APCI is based on hot ionization source with a corona discharge region, where the sample is completely vaporized and plasma is produced by a corona discharge. Ions are formed through physical interactions between the evaporated solvent ionized by corona discharge and gaseous sample components including analysed sample (Adaway *et* Keevil, 2012; Strathmann *et* Hoofnagle, 2011; Vogeser *et* Seger, 2008).

Mass spectrometry analysis is based on separation of multiple ions from the sample according their specific mass-to-charge ratio (m/z) and recording their relative abundances. In general, mass analyser can be divided into two groups: unit and high resolution mass spectrometers depending on their resolving power. Magnetic sector instruments, single or multiple quadrupole mass filters, and linear or three-dimensional quadrupole ion traps offers unit resolution. Time-of-Flight (TOF), Fourier

transform ion cyclotron resonance (FTICR) mass spectrometer and Orbitrap belong to the high resolution instruments. Various types of mass spectrometers diverge in parameters of mass range limit, analysis speed, sensitivity, mass accuracy and resolution, and therefore can be used for different analysis type. The most common mass spectrometers in clinical laboratories are quadrupole analysers. They are constructed of four parallel rods in square formation. One diagonal pair is positively charged and the second negatively charged in any time of analysis. The charge of rods alters continually depending on set frequency that enables stable flight of analysed ion trough the rods by balanced attraction and repulsion of the ion. Frequency is optimized for every ion and can change immediately during analysis which enables subsequent detection of several analytes. Period in milliseconds of set voltage and frequency, when one *m/z* is detected, is so called as the dwell time. Particular analysers have limited speed of switching voltage and frequency thus amount of possible monitored ions is also limited. Quadrupole analysers have quite narrow range of *m/z* therefore molecules with high molecular weight is possible only with their multiple recharge. For higher specificity multiple set of quadrupole analysers in tandem ordering are used very often in clinical laboratories. A common analyser is triple quadrupole mass spectrometer (or "triple quad") that consists of three quadrupoles. The first (Q1) and the third (Q3) quadrupole serve as mass filters and the second (q2) operate as collision cell. Ions transferring Q1 enter in q2 where collision with inert gas such as nitrogen proceeds and fragments subsequently pass through Q3 to detector (Fig. 11).

With single or multiple mass detectors, there is a possibility to use several methods, most often it is full scanning, selected ion monitoring (SIM) or multiple reaction monitoring (MRM). Full scan provides complex information about sample via broad *m/z* range and it is useful for unknown compounds identification by determining the retention time and mass fragment fingerprint.

SIM mode is useful for determination of selected molecule on the base of known structure/mass. Before analysis instrument voltage and frequency is tune to set optimal levels for analyte of interest transition

through mass analyser that serves as mass filter (Adaway *et* Keevil, 2012; Strathmann *et* Hoofnagle, 2011; Vogeser *et* Seger, 2008).

In MRM mode Q1 serve as mass filter for precursor ion of interest that passes to collision cell (q2) where fragmentation take a place. Based on instrument parameters tuning selected specific fragment of parental molecule (product ion) is transmit via Q3 to detector. Precursor ion and product ion couple is so called "MRM transition". This mode of detection is very specific and selective, minimizing nonspecific interactions (Adaway *et* Keevil, 2012; Strathmann *et* Hoofnagle, 2011; Vogeser *et* Seger, 2008).
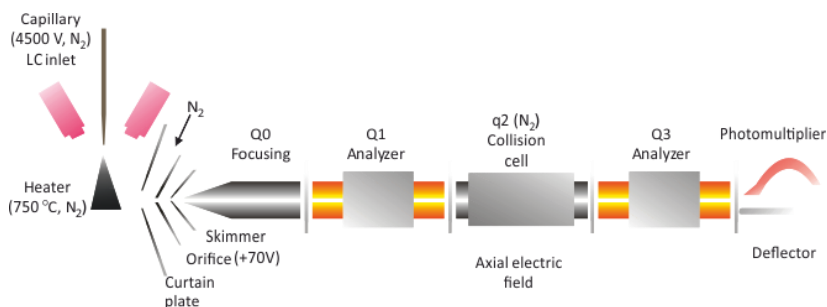
Figure 11: Triple quadrupole analyser scheme.

In recent years, thanks to advances in techniques and instrumentation, mass spectrometry with high resolution (TOF, FTICR and Orbitrap) became important tool for identification and determination of analytes especially for distinguishing of compounds with very close mass-to-charge ratio *(m/z)*. Mass resolution is defined as the difference ($\Delta m = m_2 - m_1$) between two mass spectral peaks. However, mass analyser performance is usually expressed in terms of mass resolving power defined as $r = m/\Delta m$ and higher resolving power is required to distinguish the same mass difference for ions of higher mass. Mass measurement accuracy is usually expressed as the "rms" difference (in ppm) between measured mass and exact mass (based on elemental composition), (Xian *et al*, 2012).

Orbitrap mass analyser invented by Alexander Makarov consists of three electrodes. The cut-outs represent both the standard trap and the so-called high-field compact trap. Outer electrodes have the shape of cups

facing each other and electrically isolated by a hair-pin gap secured by a central ring made of dielectric. A spindle-like central electrode holds the trap together and aligns it via dielectric end-spacer. When voltage is applied between the outer and central electrodes, the resulting electric field is strictly linear along the axis and thus oscillations along this direction will be purely harmonic. At the same time, the radial component of the field strongly attracts ions to the central electrode (Figure 12), (Zubarev *et* Makarov, 2013). We used Orbitrap analyser for metabolisation study of IM metabolites based on their exact mass measurement.
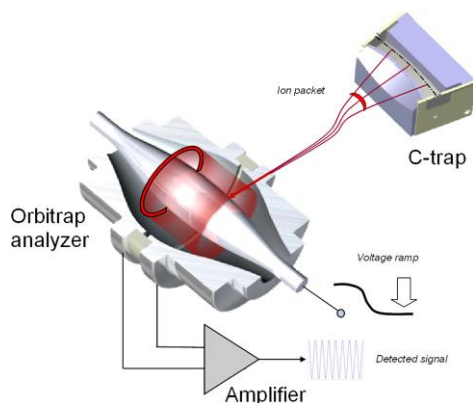
Figure 12: Cross section of the C-trap and Orbitrap analyser. Ions are stored in the rf-only bent quadrupole of the C-trap, then the rf is ramped down and a high-voltage pulse is applied across the trap, each m/z being ejected in a short packet. The packet from the C-trap enter the analyser during the voltage ramp and spread into oscillating rings that induce current detected by the differential amplifier (adapted from Zubarev *et* Makarov, 2013).

1.3.4. Frequently monitored drugs in clinical laboratories

Therapeutic monitoring is usually required for drug with narrow therapeutic index, pharmacokinetics variability, severe side-effects, high risk of toxicity due to overdosage and drugs simply subjected to another drug interaction. To improve efficiency and benefits of the treatment, number of drugs are prescribed and used in parallel; therefore the

development of LC-MS/MS methods for simultaneous determination of several compounds is required.

Immunosuppressants are widely routinely monitored group of drugs used mainly in post-transplantation patient care to prevent the rejection of transplanted organs and tissues, for treatment of autoimmune diseases or diseases with autoimmune origin (systemic lupus erythematosus, Crohn's disease, rheumatoid arthritis, multiple sclerosis, etc). They can be categorized several classes: the calcineurin inhibitors (cyclosporine, tacrolimus), the mTor (mammalian targets of rapamycin) inhibitors (sirolimus, everolimus) and inosine monophosphate dehydrogenase inhibitor (mycophenolic acid). First two groups, calcineurin inhibitors and mTor inhibitors are usually determined in whole blood with anticoagulant (EDTA) addition because of their binding to red blood cells. By contrast mycophenolic acid is preferably monitored in plasma (Taylor, 2004; Meinitzer *et al*, 2010; Bogusz *et al*, 2007).

Antifungal drugs are the group of widely monitored therapeutics used for the prophylaxis and treatment of fungal infections. The most common monitored groups are azole antifungal drugs (e.g. voriconazole, itraconazole, fluconazole, posaconazole, iodiconasole) and echinocandins (e.g. echinocandin B, cilofungin, caspofungin, micafungin), (Chachbouni *et al*, 2010; Decosterd *et al*, 2010; Baietto *et al*, 2010).

Antiviral drugs are designed to cure viral infections like hepatitis B and C, some types of influenza, herpes viruses and not least HIV infection. Mechanism of action is based on interrupting viral replication in host organism. They have been classified into three groups: nucleoside reverse transcriptase inhibitors (NRTIs – e.g. zidovudine, zalcitabine, lamivudine, emtricitabine, abacavir), non-nucleoside reverse transcriptase inhibitors (NNRTIs – e.g. efavirenz, nevirapine, delavirdnine, rilpivirine) and protease inhibitors (PIs – e.g. ritonavir, indinavir, lopinavir, atazanavir, saquinavir, darunavir). The drugs are commonly used in combination to improve efficacy (Gu *et al*, 2007; Dickinson *et al*, 2005; Rouzes *et al*, 2004).

Anticolvusants (gabapentin, carbamazepine, lamotrigine, levetiracetam, oxcarbazepine, phenobarbital, phenytoin, topiramate, valproate) used for controlling of epileptic seizures are often also indicated in combination but interactions between them occur frequently. Therefore

TDM of these antiepileptic drugs especially in patients maintained on multiple medicaments is important (Hori *et al*, 2006; Zhang *et al*, 2008; Park *et al*, 2008).

In recent years, antidepressants have become widely prescribed drugs all over the world. Main groups include tricyclic antidepressants (TCAs – clomipramine, nortriptyline, loferamine, dosulepin, doxepin), monoamine oxidase inhibitors (MAOIs – isoniazid, nialamide, toloxaton, selegiline, phenoxypropazin, caroxazone) and selective serotonin re-uptake inhibitors (SSRIs – citalopram, fluoxetine, paroxetine, sertraline), (Jiang *et al*, 2010; Moraes *et al*, 1999; Bhatt *et al*, 2006).

Antibiotics are widely used for the treatment of bacterial infections. Antibiotics represent many compounds divided into several classes and the classification is based on mechanism of their action and also on their target: aminoglycosides (gentamycin, kanamycin, neomycin), ansamycins (streptomycin), cephalosporins (cefaclor, cefixime, cefepime, ceftobiprole), glycopeptides (vancomycin), macrolides (erythromycin, azithromycin, spiramycin), nitrofurans (nitrofurantion), oxazolidonones (linezolid, torezolid), penicillins (amoxicillin, ampicillin, dicloxacillin, oxacillin, penicillin G or V), polypeptides (bacitracin), quinolones (ciprofloxacin, gemifloxacin, levofloxacin, ofloxacin, grepafloxacin), sulfonamides (sulfamethizole, sulfadiazine), tetracyclines (doxycycline, oxytertacycline), drugs against mycobacteria (rifampicin, cycloserine, streptomycine) and many others (de Velde *et al*, 2009; Xiao *et al*, 2005; Catena *et al*, 2009).

Anticancer/cytotoxic drugs are used in the treatment of several types of cancer. Monitoring of this type of medicaments is very important and necessary due to non-specific target tissue. Chemotherapeutic drugs can be divided into alkylating agents (ifosfamide, melphalan, busulfan, cyclophosphamide, thiotepa), vinca alkaloids (vinblastine, vincristine, vindesine), antimetabolites (methotrexate, permetrexed, raltitrexed, tegafur, 5-fluorouracil) and cytotoxic antibiotics (anthracyclines – doxorubicin, daunorubicin; actinomycin, bleomycin, plicamycin, mitomycin). Other types of cytotoxic drugs are selective inhibitors of onocogenous tyrosine kinases (imatinib, dasatinib, nilotinib, bosutinib, lapatinib, erlotinib), immune modulating drugs (lenalidomide,

43

thalidomide), carboplatin and others (Bunch *et al*, 2010; Gjerde *et al*, 2005; Haouala *et al*, 2009).

Among drugs influencing cardiovascular system anti-arrythmics (procainamide, phenytion, encainide, metoprolol, amiodarone, verapamil), diuretics (hydrochlorothiazide, furosemide), anti-thrombotic drugs (antiplatelet drugs, anticoagulants, thrombolytic drugs), and positive inotropes (dopamine, epinephrine, digoxin, eicosanoids, theophylline) can be included. These drugs variously influence cardiovascular system: suppress abnormal rhythms of the heart such as atrial fibrillation or flutter, ventricular tachycardia and fibrillation, reducing thrombus formation or alter the force of muscular contractions (Hashimoto *et al*, 2008; Gonzalez *et al*, 2011; Gao *et al*, 2010).

1.4. Therapeutic monitoring of imatinib

1.4.1. Methods for IM detection

Imatinib levels are widely measured in human plasma from blood samples taken usually in the morning before subsequent dosing at steady state; determination is performed preferably by simple and fast technique. The most frequent technique is liquid chromatography coupled with UV detection (Widmer *et al*, 2004; Davies *et al*, 2010; Vivekanand *et al*, 2003; Pursche *et al*, 2007; Velpaldian *et al*, 2004), simple mass spectrometry (Parise *et al*, 2003) and, more often, with tandem mass spectrometry (TMS), (Chahbouni *et al*, 2009; Titier *et al*, 2005; De Francia *et al*, 2009; Haouala *et al*, 2009; Mičová *et al*, 2010a,b).

The sample preparation is usually based on protein precipitation by an organic solvent (De Francia *et al*, 2009; Haouala *et al*, 2009; Parise *et al*, 2003; Velpaldian *et al*, 2004; Bakhtiar *et al*, 2002), acidic solution (Roth *et al*, 2010) or the use of extraction to organic solvent (Titier *et al*, 2005). With regard to similar structural properties of IM and other TKIs, several methods for their simultaneous determination were developed (De Francia *et al*, 2009; Haouala *et al*, 2009).

Separations are performed on a short C18 RP column with a particle size to the 5 µm. Analyses take up to 20 minutes with column wash and re-

equilibration. Analytes are eluted by a mobile phase consisting of an acidic buffer and an organic solvent (mostly acetonitrile or methanol), (Titier *et al*, 2005; Mičová *et al*, 2010a; De Francia *et al*, 2009; Haouala *et al*, 2009). We have developed an ultra high performance liquid chromatography method using C18 RP column filled by 1.7 μm particles without a specific sample preparation with a separation time of 2.2 min (Mičová *et al*, 2010b).

The electrospray ionization in the positive mode is the most frequent method for mass spectrometry in TDM practice. In the case of TMS, the drugs are detected in the MRM mode (Chahbouni *et al*, 2009; Titier *et al*, 2005). The quantification of the drug by LC-TMS is usually based on an isotopic dilution, using the internal standard imatinib-D8 (Titier *et al*, 2005; Haouala *et al*, 2009; Bakhtiar *et al*, 2002) or another structurally similar compound e.g. quinoxaline (De Francia *et al*, 2009).

Capillary zone electrophoresis coupled usually with a diode array detector is another technique used for imatinib and its main metabolite determination in human urine and plasma (Rodríguez-Flores *et al*, 2003; Rodríguez-Flores *et al*, 2005; Mičová *et al*, 2009).

Currently, we have developed a new method for determination of IPL using a flow injection analysis (FIA) coupled with MS/MS. Due to the exclusion of the separation step an excellent sample throughput was achieved with high sensitivity through the placement of up to 200 samples per hour. The method reduces the analysis time significantly and declines requirements for consumable expenditures (Mičová *et al*, 2010b).

Current attempts to determine IM levels directly in cells lead to endeavour to find a new marker for correlation with clinical response and also a better understanding of IM distribution in cells. However, there are several problems that make intracellular IM level measurement difficult for the use in routine practise. Fast and considerate isolation of white blood cells from whole blood and preparation of these samples represent complex problem due to time limitation due to the fast drug efflux by MDR protein ABCB1 (P-glycoprotein pump), (Klawitter *et al*, 2009; Potěšil *et* al, 2010; Kutsev *et al*, 2009; Mlejnek *et al*, 2011).

1.4.2. The significance of IM therapeutic monitoring

Monitoring of IPL could be helpful in identification of patients with poor compliance to therapy in the case of low plasma levels, drug-drug and food-drug interactions (via cytochrome P450 3A4) and to avoid severe side effects or toxicity. In addition IPL is used for individualization of therapy by dosage adjustment. In several studies pharmacokinetics and the correlation between IPL and clinical response was examined (Picard *et al*, 2007; Larson *et al*, 2008; Singn *et al*, 2009; Awidi *et al*, 2010; Zhong *et al*, 2012; Ishikawa *et al*, 2010; Takahashi *et al*, 2010; Ohnishi *et al*, 2012; Forrest *et al*, 2009).

A study by Picard *et al* demonstrated significantly higher mean plasmatic concentrations in a group of patients with a CCR than in a group without CCR (n = 56; 1123 ± 617 ng/mL versus n = 12; 694 ± 556 ng/mL, respectively). In addition, the median plasmatic levels of IM in patients with a MMR (n = 34; 1452 ± 649 ng/mL) were higher than in those without MMR (n = 34; 869 ± 428 ng/mL; P<0.001), (Fig. 13). The results of the experiment suggested that trough IPL are associated with both CCR and MMR to standard-dose IM in CML and the efficient plasma threshold for trough IM levels should be set above 1002 ng/mL in vivo (Picard *et al*, 2007).

In a study by Larson *et al* the correlation between IPL with clinical responses was reported as well. The IM trough levels among patients who achieved CCR (n = 297) were significantly higher than those for patients who did not achieve a CCR (n = 54); the mean values were 1009 ± 544 ng/mL and 812 ± 409 ng/mL, respectively. A similar tendency was observed between MMR and IPL. Except IPL correlation with CCR and MMR, relationship between IPL and other parameters like body surface area (BSA), body weight (BW) and age was examined. IPL in steady state was slightly higher in females than males (1078 ± 515 ng/mL vs 921 ± 53 ng/mL; respectively) but this could be the cause of differences in BW in sexes. Weak correlation was observed between IPL and both BW ($r^2 = 0,015$) and BSA ($r^2 = 0.038$) but due to large patients intervariability it is not consider to be clinically significant. However, as in the study of Picard *et al,*

on the base of obtained results maintaining the threshold value above 1000 ng/mL may be important for achieving CCR (Larson *et al*, 2008).
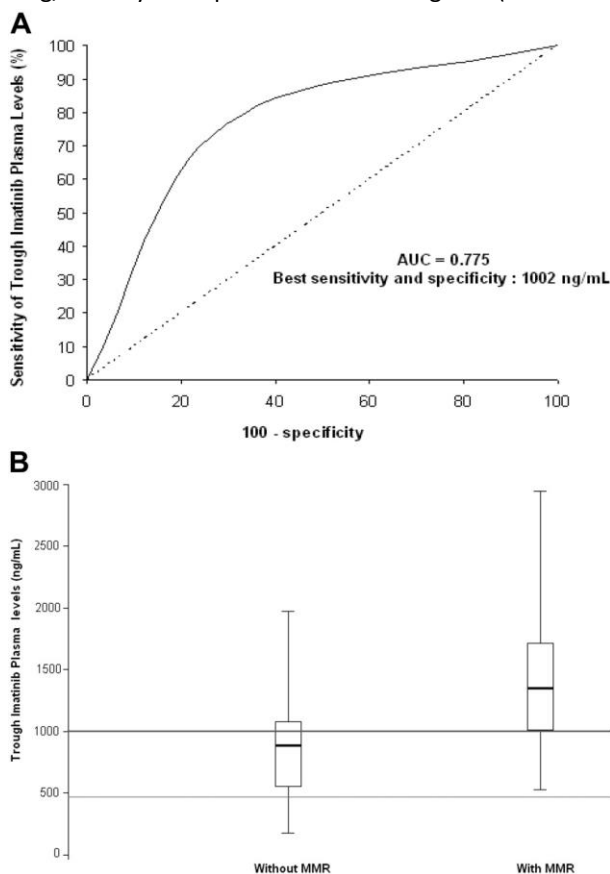
Figure 13: Trough plasma imatinib threshold for MMR. (A) Receiver operating characteristic (ROC) curve analysis. Regarding trough imatinib plasma levels and their discrimination potential for MMR, the area under the ROC curve (AUC) was 0.775, with best sensitivity (77%) and specificity (71%) at a plasma threshold of 1002 ng imatinib/mL. (B) Box plots of trough imatinib plasma levels. The graph shows the dispersion around the median for patients with MMR (n = 34; median = 1350 ng/mL) and those without (n = 34; median = 885 ng/mL). The line across each box is the median; the bottom edge is the first quartile and the top edge is the third quartile; the error bars represent minimal and maximal values; the bottom line shows the 493.6 ng/mL (i.e., 1 µmol/L) target concentration required to result in BCR-ABL–positive cell death in vitro; the top line shows the 1002 ng/mL efficient plasma threshold for trough imatinib levels in vivo. Of the 34 patients with MMR, 26 (76%) had trough imatinib plasma levels exceeding the 1002 ng/mL threshold. Of the 34 patients without MMR, 24

(71%) had trough imatinib plasma levels below the 1002 ng/mL threshold, whereas 27 patients (79%) had trough imatinib plasma levels exceeding the initially described target concentration (493.6 ng/mL) required to result in BCR-ABL–positive cell death in vitro (Peng *et al*, 2004; Buchdunger *et al*, 1996; Druker *et al*, 1996). This initial target was validated in phase 1 to 2 studies on the basis of cytogenetic criteria, but it is not always sufficient to achieve MMR in vivo. Indeed, using a sharper criterion than CCR such as a 3 log reduction of BCR-ABL transcript levels, the efficient plasma threshold for trough imatinib levels should be set above 1002 ng/mL in vivo (adapted from Picard *et al*, 2007).

Mean plasma levels of IM in responders (n=20) were significantly higher than those in non-responders (n=20), (2.34 ± 0.52 vs. 0.69 ± 0.15 µM) in a study by Singh *et al* All patients responding to IM had achieved a haematological and cytogenetic response, and their corresponding plasma levels were found to be higher than 1 µM. As well as in French study groups, inter-patient variability in IPL in both responder and non-responder was observed (Singn *et al*, 2009).

Similar study was performed in Jordanian CML patients and results corresponding to previous studies were published. The mean IPL for patients with MMR or better and for those without MMR were 2766 ± 811 and 1777 ± 624 ng/mL, respectively (P < 0.001). ROC analysis yielded a threshold value of 2158 ng/mL that is once so high like in other studies (Awidi *et al,* 2010).

In Chinese patients after a median follow up period of 4 month for 84 CML patients treated with IM, 62 (73.81 %) achieved CCR, 12 (14.29 %) achieved PCyR, 10 (11.90 %) were drug-resistant and 19 (22.62 %) achieved MMR. The IPL were significantly higher in the MMR group than in the PCyR (1531.88 ± 634.09 vs 812.75 ± 480.29 ng/mL; p = 0.002) or drug resistant group (1531.88 ± 634.09 vs 875.17 ± 243.09 ng/mL; p = 0.011) and the CCR group than in the PCyR (1288.39 ± 498.23 vs 812.75 ± 480.29 ng/mL; p = 0.027). There were no significant differences between the CCR and MMR groups in IPL (1288.39 ± 498.23 vs 1531.88 ± 634.09 ng/mL; p = 0.136). In this study the intracellular IM concentration in bone marrow of 28 CML patients was also determined with a median rate of 8.22 (2.38 – 111.00) ng/mL. The IPL in this group of patients was 1636.04 ± 976.75 ng/mL and there was no significant correlation between IM intracellular and plasma concentration (r = 0.10, p = 0.478). IPL was positively correlated with AGP

48

(r = 0.443, p < 0.001) and IM daily dose (r = 0.422, p < 0.001). There were no significant correlation between IPL and height (r = 0.001, p = 0.992), weight (r = 0.002, p = 0.984) or BSA (r = -0.008, p = 0.945), (Zhong *et al*, 2012).

Several studies were performed in Japanese CML patients. In study by Sakai *et al* by Nagasaki CML study group, patients in the optimal-response group showed a significantly higher IPL than those in the suboptimal or failure categories (P = 0.0087). Significant relationship was also found between dose/BSA and the response (P = 0.01) and the dose and response (P = 0.01), (Sakai *et al,* 2009).

In BINGO study by Ishikawa *et al,* IPL in patients who achieved MMR (n = 38) were significantly higher than in those who did not achieve MMR (n = 22), (Fig. 14). The median values were 1092.5 ng/mL (range 562 – 2150 ng/mL) and 853 ng/mL (range 361 – 1205 ng/mL), respectively (P = 0.0022). However, IPL was not associated with the age (r^2 = 0.0227, P = 0.0547), the body weight of patients (r^2 = 0.0217, P = 0.4431), BSA (r^2 < 0.0001, P = 0.8765), and the daily IM dose (r^2 = 0.1348, P = 0.1011), (Ishikawa *et al*, 2010).

In another study of Japanese CML patients by Takahasi *et al,* among all 314 evaluated patients, 166 (65.3 %) achieved MMR and 218 (85.8 %) achieved CCR. IPL were significantly higher in patients with MMR than in those without MMR; the mean values were 1107.4 ± 594.4 ng/mL (median, 986 ng/mL) and 872.7 ± 528.5 ng/mL (median 719.5 ng/mL), respectively (P = 0.002). It was also found that patients with IPL ≥ 1002 ng/mL had a higher probability of achieving MMR than those with IPL < 1002 ng/ml (P = 0.0120). In addition, IPL was significantly higher in patients with CCR (n = 219) than in those without CCR (n = 36), the mean values were 1057.8 ± 585.0 ng/mL (median 916 ng/mL) and 835.0 ± 524.3 ng/mL, respectively (P = 0.033), (Takahashi *et al*, 2010).
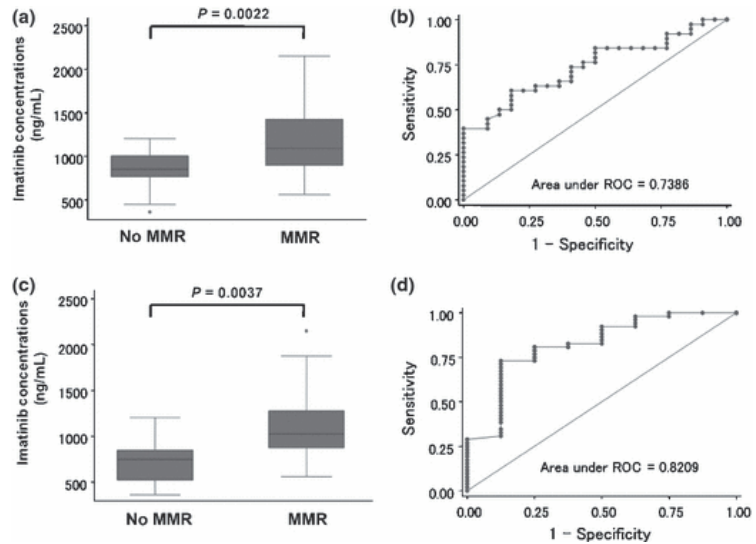
Figure 14: C$_{min}$ threshold for major molecular response (MMR). (a) Cmin levels of imatinib among patients who achieved MMR at the 12th month (n = 38) were significantly higher than in patients who did not achieve MMR (n = 22); the median values were 1092.5 ng/mL and 853 ng/mL, respectively (P = 0.0022, Mann–Whitney U-test). (b) Concentration-effect ROC analysis of the discrimination potential of imatinib Cmin levels for MMR at the 12th month. The area under the ROC (AUC) was 0.7386 with the best sensitivity (63.2%) and specificity (68.2%) at a Cmin threshold of 974 ng/mL. (c) Cmin levels of imatinib among patients who achieved MMR during treatment (n = 52) were higher than in patients who did not achieve MMR (n = 8); the median values were 1025 ng/mL and 748.5 ng/mL, respectively (P = 0.0037, Mann–Whitney U-test). (d) Concentration-effect ROC analysis of the discrimination potential of imatinib Cmin levels for MMR during treatment. The AUC was 0.8209 with the best sensitivity (80.8%) and specificity (75.0%) at a Cmin threshold of 817 ng/mL (adapted from Ishikawa Y *et al*, 2010).

In long-term JALSG CML202 study, 481 patients were evaluated. Estimated 7-year overall survival (OS) and event-free survival (EFS) at a medium follow-up of 65 months were 93 % and 87 %, respectively. Due to frequent adverse events, standard 400 mg dosage was reduced in several patients. There were no significant differences in OS and EFS between the 400- and 300- mg groups; however, there was a significant difference in achieving CCR or MMR between these groups (P = 0.018 and P = 0.017). There was a significant correlation between IPL and age only in the 400-mg group (P = 0.034), with weak correlations between IPL and BW or BSA.

50

Results of this study suggest that, although a daily dose of 400 mg imatinib is associated with better outcomes, 300 mg imatinib may be adequate for a considerable number of Japanese patients who are intolerant to 400 mg imatinib. Blood level monitoring would be useful to determine the optimal dose of imatinib (Ohnishi *et al*, 2012).

On the other hand, in a study of Forrest *et al,* no correlation of IPL and CCR at 1 year (CCR 1010 ng/ml vs no CCR 1175 ng/ml P = 0.29) or MMR (MMR 1067 ng/ml vs no MMR 1063 ng/ml P = 0.74) after a median of 1298 days of IM therapy was found. CCR and MMR correlated with Sokal risk scores with the odds of achieving CCR or MMR for a low risk vs high risk score of 10.8 (95% CI 2.2–53.5) and 6.4 (95% CI 1.4–29.4), respectively (Forrest *et al*, 2009).

Based on results of several independent studies from different study groups, association of IPL with CCR and MMR was demonstrated and theoretical IPL threshold for achieving optimal treatment response above ~1000 ng/mL was set. Monitoring of IPL could be important tool in CML clinical practice for dosage adjustment ant treatment individualization.

Due to IM intracellular mechanism of action, determination of its concentration in leukaemic cells could be better predictive marker for estimation of optimal dose adjustment for favourable treatment response. However, determination of IM intracellular levels is quite complex; IM is substrate of multidrug resistance protein (p-glycoprotein pump) and it is promptly effluxed out of the cell. A few methods for determination of IM and other TKIs in cultured cells (Roche *et al*, 2009; Klawitter *et al*, 2009; Mlejnek *et al*, 2011), leukocytes of patients (D'Avolio *et al*, 2012) and patient's bone marrow (Iqbal *et al*, 2011) were published.

2. Aims of the work

- The method development for therapeutic monitoring of imatinib and other tyrosine kinase inhibitors in human plasma using LC-MS/MS

2.1. Mičová K, Friedecký D, Faber E, Polýnková A, Adam T. Flow injection analysis vs. ultra high performance liquid chromatography coupled with tandem mass spectrometry for determination of imatinib in human plasma. Clin Chim Acta. 2010;411(23-24):1957-62.

2.2. Mičová K, Friedecký D, Faber E, Adam T. Isotope dilution direct injection mass spectrometry method for determination of four tyrosine kinase inhibitors in human plasma. Talanta. 2012;93:307-13.

- Following the relation between imatinib plasma level and treatment response in patients with chronic myeloid leukaemia

2.3. Faber E, Friedecký D, Mičová K, Divoká M, Katrincsáková B, Rožmanová S, Jarošová M, Indrák K, Adam T. Imatinib dose escalation in two patients with chronic myeloid leukaemia, with low trough imatinib plasma levels measured at various intervals from the beginning of therapy and with suboptimal treatment response, leads to the achievement of higher plasma levels and major molecular response. Int J Hematol. 2010;91(5):897-902.

2.4. Faber E, Friedecký D, Mičová K, Rožmanová S, Divoká M, Jarošová M, Indrák K, Adam T. Imatinib trough plasma levels do not correlate with the response to therapy in patients with chronic myeloid leukaemia in routine clinical setting. Ann Hematol. 2012;91(6):923-9.

- Imatinib metabolite profiling in plasma using high resolution mass spectrometry

2.5. Friedecký D, Mičová K, Faber E, Hrdá M, Adam T. Detailed study of imatinib metabolism using high-resolution mass spectrometer Orbitrap Elite. (Article in process)

2.1. Flow injection analysis vs. ultra high performance liquid chromatography coupled with tandem mass spectrometry for determination of imatinib in human plasma.

Kateřina Mičová[a], David Friedecký[a], Edgar Faber[b], Adriana Polýnková[a] and Tomáš Adam[a]

a) Laboratory of Inherited Metabolic Disorders, University Hospital and Palacký University in Olomouc, I.P. Pavlova 6, CZ 775 20 Olomouc, Czech Republic
b) Department of Hemato-Oncology, Faculty of Medicine and Dentistry, Palacký University in Olomouc, I.P. Pavlova 6, CZ 775 20 Olomouc, Czech Republic

Aims: In this work we present the development, validation, and comparison of two methods – flow injection analysis and ultra-performance liquid chromatography with tandem mass spectrometry to determine IM in plasma samples taken from patients with CML undergoing IM therapy.

2.1.1. Abstract

Background: The aim of this study was to develop, validate and compare flow injection analysis (FIA) and ultra-high-performance liquid chromatography (LC) / tandem mass spectrometry methods for the determination of imatinib in plasma from patients with chronic myeloid leukaemia.

Methods: The plasma for analysis by both methods was deproteinated by methanol containing d8-imatinib. Separation was achieved on a 1.7 µm C18 column with a linear gradient (4 mM ammonium formiate and acetonitrile, pH 3.2). FIA was performed at flow rate of 0.03 mL/min (0.1% formic acid in methanol). Multiple reaction monitoring mode on the tandem mass spectrometer (API 4000, AB Sciex) in positive ESI was used for detection.

Results: The total analysis times were 3.2 (LC) and 0.75 min (FIA). Both methods were successfully validated and applied to the plasma patients samples. The limits of quantification were 4.1 and 30.8 ng/mL; imprecisions were less than 5.7% and recovery ranged between 93 and 105%, for the LC and FIA, respectively. The methods revealed agreement with a mean difference of 1.46 ng/mL (SD 28.95 ng/mL).

Conclusions: The high-throughput methods that were developed are suitable for the therapeutic drug monitoring of imatinib in plasma. They can be used in routine clinical practice.

2.1.2. Matherials and methods

Chemicals

Imatinib mesylate, d8-imatinib mesylate (d8IM, deuterated internal standard), and desmethylated imatinib mesylate (desIM, major metabolite), (Fig. 15) were kindly provided by Novartis Pharmaceuticals (East Hanover, NJ, USA). Formic acid, ammonium hydroxide, and methanol were all LC/MS grade and purchased from Sigma (St. Louis, MO, USA). Deionized water was prepared with an EASY pure LF (Werner Reinstwassersysteme, Leverkusen, Germany).
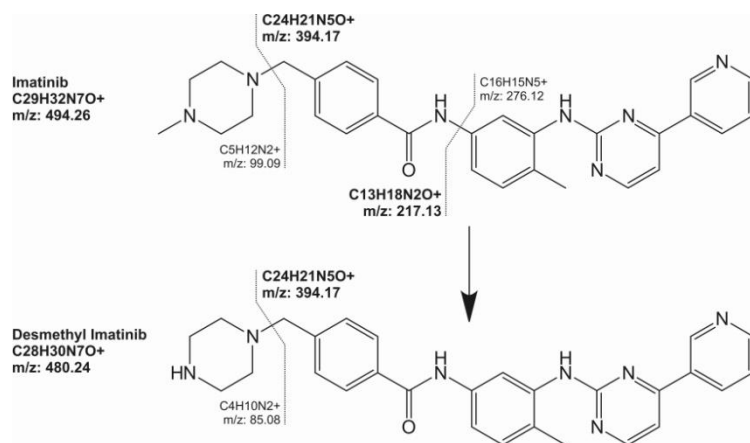
Fig. 15: Structures and fragmentation of IM and desIM.

Standard solutions

Stock solutions of IM, desIM, and d8IM were prepared by dissolving them in methanol to yield concentrations of 0.5 mg/mL expressed as free substances. IM was further diluted with methanol to obtain solutions at concentration levels in the range 100 – 50000 ng/mL. The internal standard was finally diluted to a working concentration of 30 ng/mL. The calibration

55

standards and controls were prepared by addition to drug-free plasma from healthy volunteers. All the solutions were stored at -20 °C.

Sample preparation

This study was conducted in accordance with the Declaration of Helsinki, and the protocol was reviewed by the hospital ethics committee. All examined patients gave their informed consent with participation in the study before blood sampling. The plasma samples were prepared from blood with an anticoagulation additive (EDTA) of control healthy volunteers and patients treated with IM by centrifuging (1200xg; 5 min). On the basis of previously published methods (De Francia *et al*, 2009; Velpandian *et al*, 2004), the plasma (0.02 mL) was deproteinated by methanol containing internal standard d8IM (0.18 mL). The sample was sonicated (1 min), shaken (5 min), frozen (30 min; -20 °C), and centrifuged (14500xg; 5 min). The prepared supernatant was displaced into vials and directly analysed or stored at - 20 °C prior to analysis.

Ultra high performance liquid chromatography / Flow injection analysis

For both methods IM plasma levels were measured using UltiMate 3000 RS (Dionex, Sunnyvale, CA). The processed samples were maintained at + 5 °C in a thermostated autosampler rack during analysis by LC and FIA methods.

Chromatographic separations were performed at 40 °C on a reverse phase analytical column Acquity®BEH C18 1.7 µm, 2.1 x 50 mm (Waters, Milford, MA). The mobile phase consisted of acetonitrile (B) and 4 mM ammonium formiate buffer; pH = 3.2 (A). During the first minute the mobile phase consisted of 17% B; 1.0 - 2.0 min linear gradient of acetonitrile from 17 to 50 % was applied; 2.0 - 2.1 mobile phase consisted of 50% B. Then the system was set to its initial conditions in 0.1 minute and was conditioned for 1 minute before the next injection. The analysis proceeded at a flow rate of 0.5 mL/min and 2 µL of the samples were injected into the system. The total run time of the analysis was 3.2 min, including the autosampler wash.

The mobile phase consisted of methanol and 0.1 % formic acid during FIA. The flow was set at 0.3 mL/min for 0.0 - 0.1 min and 0.4 - 0.5 min; in the measuring period between 0.1 and 0.4 min it was reduced to 0.03 mL/min. The volume of the injected sample was 0.5 µL and the total run time of the analysis, including the autosampler wash, was 45 s.

Tandem mass spectrometry

All the experiments were performed on an API 4000 tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA) with electrospray ionization in positive mode. The mass spectrometer was operated in multiple reaction monitoring mode (MRM). The dwell time was adjusted to a time of 200 ms for each mass transition between the precursor and product ion.

The ion source settings consisted of a temperature of 450 °C, capillary voltage of 5500 V, nebulizer gas 40/20 psi (LC/FIA method), auxiliary gas 40 psi, curtain gas 20/14 psi (LC/FIA method) and high-purity nitrogen as the collision gas 6 psi. Characteristic mass spectrometry parameters of declustering potential (70 V for desIM and 115 V for all other compounds), collision energy (35 V), and collision exit potential (10 V) were determined for all the analytes using standard solutions (1000 ng/mL) dissolved in methanol. The tandem mass spectrometer was set to "unit" and "low" resolution for the LC and FIA methods, respectively. Transitions m/z 494.0 → 394.4 (IM), m/z 480.3 → 394.1 (desIM) and m/z 502.4 → 394.0 (d8IM) were used for LC method. Transitions m/z 494.0 → 394.4 (IM) and m/z 502.4 → 394.0 (d8IM) were used for quantification in FIA method. In order to identify possible interferences of the FIA method, a ratio of the two m/z transitions 494→394 and 494→217 was set at 3.0 - 3.4. The quantification of the LC analysis was done with the Analyst 1.5 software (Applied Biosystems, USA) by the ratio of the corrected peak area of IM and d8IM. The FIA analysis were quantified by the ratio of average intensities of IM and d8IM (30 data points per analyse) in the Chemoview 2.0 software (Applied Biosystems, USA).

Method validation

The methods validation was based on the recommendations published by the Food and Drug Administration (FDA), (FDA, 2001), recommendations of the Conference Report of the Washington Conference on "Analytical methods validation: Bioavailability, Bioequivalence and Pharmacokinetic studies" (Shah *et al*, 2000) and Matuszewski's *et al* studies were also considered (Matuszewski *et al*, 2003).

The plasma of healthy volunteers, with the addition of 100, 300, 500, 1000, 3000, and 5000 ng/mL of IM in triplicates, was used for the construction of the calibration curve. The linear regression model (least-square method) with the inversion of concentration as the weighting factor ($1/x^2$) was chosen for the calculation of the dependence between the peak-area/intensity (for LC/FIA method) ratio of IM/d8IM and concentration. In order to determine the linearity slope, the difference, correlation coefficient, and standard deviations were calculated.

The sensitivity of the methods was determined on plasma samples with IM added with low concentration near to LOQ (50 ng/mL) and calculated as a signal-to-noise ratio of 3 and 10 for LOD and LOQ, respectively. For calculated LOQ bias and coafficient of variation were determined (n=6, spiked plasma).

Intra- and interday imprecisions and recoveries were measured in blank plasma and in plasma samples with the addition of 300, 1000, and 3000 ng/mL of IM in six replicates within one day and six consecutive days, respectively. The ANOVA method was used for the calculation of means and standard deviations. Recovery was expressed as a percentage of the target value.

The specificity of this method was investigated by analysing six different blank plasma samples from healthy volunteers without IM intake. Each blank sample was tested to check the lack of interferences with IM or d8IM using the presented method.

The stability of IM at different conditions has been previously reported in many articles (Titier *et al*, 2005; Haouala *et al*, 2009; Bakhtiar *et al*, 2002). Imatinib in plasma samples is stable at least 12 month in the freezer

(-20°C) with decomposition less than 3.3%. Stability of plasma and blood is better than 95% at room temperature for 96 hours (Widmer *et al*, 2004).

Ion suppression was explored by the post-column continuous infusion of IM into the tandem mass spectrometer during the chromatographic analysis of eight blank human plasma extracts. The concentration of the standard solution was 1000 ng/mL and it was infused at a flow rate of 10 µL/min. Subsequently, the quantitative determination of the matrix effects was also assessed for both methods. Blank plasma samples were prepared by a standard procedure and subsequently the supernatant was spiked with IM to three final concentrations of 300, 1000, and 3000 ng/mL. Ion suppression was calculated by an equation:

$$\text{Ion suppression \%} = \left[1 - \frac{I_{std\ in\ blank} - I_{blank}}{I_{std\ in\ MeOH}} \right] \times 100$$

comparing the signal intensities of the standard solutions in methanol and blank plasma extract with identical amount of IM added.

In order to compare the methods that were developed, 120 plasma samples from patients suffering from CML and treated with IM were analysed and the results were evaluated by Bland & Altman plot and regression analysis. The equation of linear regression, correlation coefficient, mean difference from zero, and standard deviation were calculated.

Validation characteristics were calculated and plotted with the QC Expert 3.0 software (Trilobyte, Czech Republic).

2.1.3. Results

Ultra high performance liquid chromatography

For the separation of IM mostly conventional LC columns filled with C18 stationary phase with particles with 5-µm dimensions are used, with a common retention time of IM about 4 minutes (De Francia *et al*, 2009; Titier *et al*, 2005; Parise *et al*, 2003; Widmer *et al*, 2004). In order to

achieve a short time of analysis we used a C18 column filled with 1.7-μm BEH particles (Waters), which provides higher resolution and faster separation. Therefore IM was separated in 1.61 min with a high separation efficiency of more than 250.000 theoretical plates per meter under a back-pressure of 320 - 400 bar. In addition, the column allows fast re-equilibration during 1.5 min.

Ion suppression by the post-column injection method was studied with eight plasma samples. We observed chromatograms with possible areas of expressive ion suppression and no significant reduction of the signal at the time of 1.61 min was found. Only the region of 0.25 - 0.40 min which corresponds to dead volume containing salts and other analytes without retention was significantly suppressed (Figure 16).
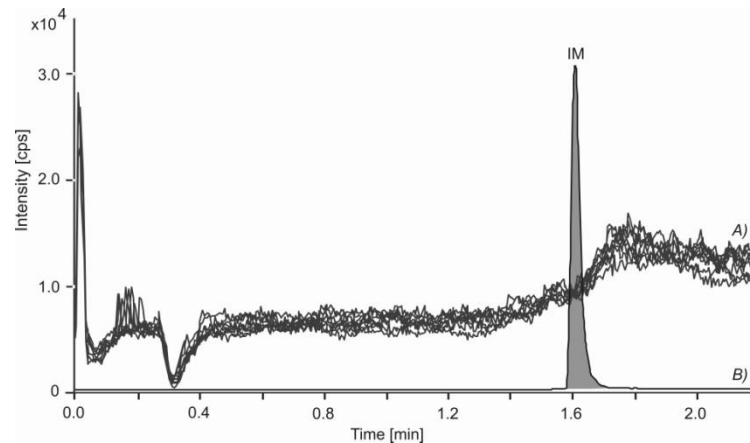
Figure 16: Ion suppression study - continuous infusion of IM to eight blank plasma analyses (A), separation of IM standard (B).

Representative chromatogram patient suffering from CML during IM therapy is shown in Figure 17. Apart from IM and d8IM, the transition of main metabolite desIM is also recorded.
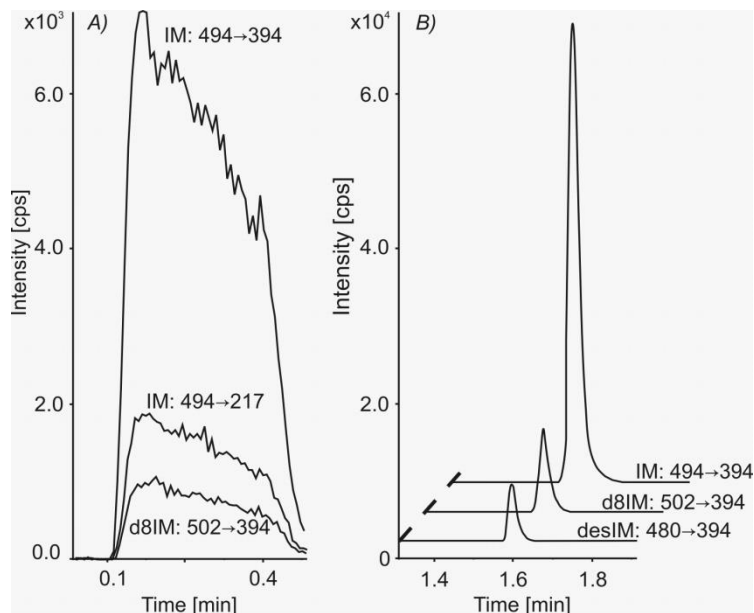
Fig. 17: Analysis of plasma samples from patient on long-term IM therapy (400 mg) by FIA (A, 895 ng/mL, 20 h after dose) and LC method (B, 1153 ng/mL, 25 h after dose).

Flow injection analysis

In order to minimize the analysis time and costs a method using the direct infusion of the sample to a mass spectrometer was developed. For the analysis the same preparation of samples, consisting of only plasma deproteination, is applied, without any additional cleaning steps. Signal intensities that were approximately five times lower were caused by ion suppression, but it has no influence on the precision and accuracy of the method as a result of the use of internal standard d8IM.

More accurate results were achieved by reducing the flow rate during the measurement of the signal from 0.30 to 0.03 mL/min, which offers a longer time and more points for the measurement of the signal. In addition, the "low" resolution of the first and the third quadrupoles offers intensities that are more than three times higher. For all patients samples analysed we found no data ratio of IM *m/z* transitions outside the set limits

61

and variation of intensity of d8IM was less than 6.6 %. Considering variation of sample injection of 5.5 % (for 0.5 µL) interferences in real samples are negligible.

Representative flow injection analysis of patient suffering from CML and treated with IM is shown in Figure 17. The total analysis time between two injections (45 s) consisted of the acquisition time of the mass spectrometer (30 s) and the washing of the autosampler (25 s), which begins during the twentieth second from the start of the analysis.

<u>Validation</u>

The linear correlation between the analyte and internal standard peak area or intensity and concentration was determined in the range 100 - 5000 ng/mL; each concentration was measured 3 times within 1 day. The equations of the calibration curves were: $y = 0.0054x - 0.0741$; ($R = 0.998$) and $y = 0.0067x - 0.1869$; ($R = 0.9975$); LOD was assessed at 1.24 and 9.2 ng/mL, and the LOQ values were 4.12 and 30.8 ng/mL (bias 7.96 and 15.21 %; CV 11.89 and 14.32 %) for LC and FIA methods, respectively. Inter-day and intra-day imprecision, ion suppression, and the analytical recoveries of the methods were determined using blank plasma added to low, medium, and high concentrations (300, 1000, and 3000 ng/mL). Both of the methods provided comparable results; recoveries varied between 93.05 and 105.50 % and 93.56 and 103.24 % and inter/intra-day imprecisions were less than 5.72 % for the LC and FIA methods, respectively (Table 2). The assay of six blank plasma samples did not show any interference in the retention time of all the analytes (data not shown). Ion suppressions were significantly higher in the FIA method (73.61 - 88.42 %) compared to the LC method (7.21 - 18.86 %) because, together with IM, a number of suppressing species were ionized in the ion source during the same analysis time. The matrix effects were fully eliminated by the use of d8IM.

Table 2: Mean values of imprecision, recovery and ion suppression of plasma samples with IM added (300, 1000 and 3000 ng/mL).

LC-MS/MS

Concentration (ng/mL)	Imprecision (CV, %)		Recovery (%), (CV, %)	Ion suppression (%)
	Interday	intraday		
3000	5.72	4.02	99.80 (5.71)	16.84
1000	5.05	3.51	99.92 (5.05)	13.02
300	3.02	3.73	98.85 (2.99)	10.63

FIA-MS/MS

Concentration (ng/mL)	Imprecision (CV, %)		Recovery (%), (CV, %)	Ion suppression (%)
	Interday	intraday		
3000	5.06	4.02	98.78 (5.00)	78.11
1000	4.48	3.67	99.11 (4.44)	87.09
300	2.64	4.88	97.04 (2.56)	85.79

Comparison of the methods

Both methods (LC vs. FIA) were compared by Bland-Altman plot and regression analysis. Plasma samples from 120 patients with CML on IM therapy were analysed by paired tests. Regression analysis provided a correlation equation of y = 0.995x + 3.295 and a correlation coefficient of 0.997. Comparison by Bland-Altman plot showed mean difference of -1.46 ng/mL and standard deviation of 28.95 ng/mL (Figure 18).
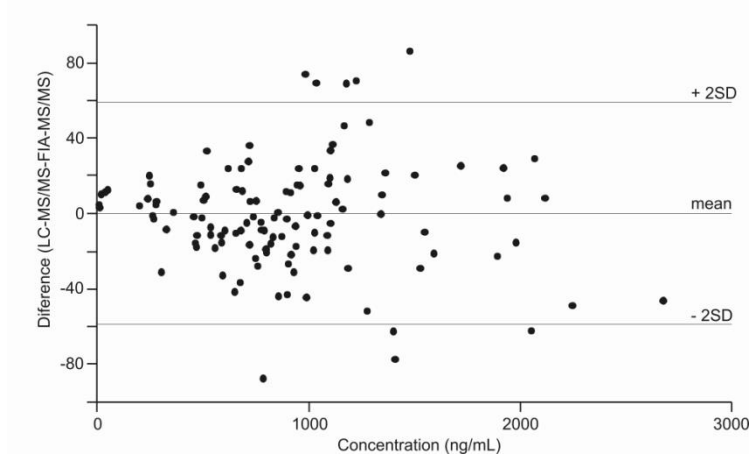
Fig. 18: Bland-Altman plot of IM difference between LC and FIA method.

2.1.4. Discussion

Over the last years, tandem mass spectrometry with liquid chromatography has been an expanding technique in clinical chemistry laboratories. In particular, methods based on isotope dilution are widespread. We developed, validated, and compared the FIA and LC methods for the determination of IM in plasma samples from patients suffering from CML. Our LC method utilizes the recently introduced columns filled with sub-two-micron particles, which allow fast separations at high efficiency. In the last year more than one thousand samples of plasma samples have been analysed on one column and no change in the retention time and only a slight decrease in separation efficiency resulted. An excellent sample throughput of up to 80 samples per hour was achieved with the use of the FIA method that was developed. The exclusion of the separation step speeds up the analysis and creates a lower requirement for consumable expenditures. In our laboratory the FIA method is currently in use due to higher throughput and also easier method switching. The methods are fully comparable and applicable for blood level testing in the range 100 – 5000 ng/mL of IM achieved at clinically used dosages. Although the clinical trials of CML patients with

64

high IM plasma levels has been reported (le Coutre *et al*, 2004; Widmer *et al*, 2006), in routine clinical practice levels close 5000 ng/mL are never reached even at maximum IM dose 800 mg/day. In more than one thousand samples analysed we found only one sample above 5000 ng/mL in a patient who has taken 1600 mg/day of IM by an error when switching from 100 mg to 400 mg tablets.

2.1.5. Acknowledgments

2.2. Isotope dilution direct injection mass spectrometry method for determination of four tyrosine kinase inhibitors in human plasma.

Kateřina Mičová[1*], David Friedecký[1*], Edgar Faber[2] and Tomáš Adam[1]

*authors contributed equally to the work

[1] _Laboratory for Inherited Metabolic Disorders, University Hospital and Palacký University in Olomouc, I.P. Pavlova 6, CZ 775 20 Olomouc, Czech Republic_
[2] _Department of Hemato-Oncology, Faculty of Medicine and Dentistry, Palacký University in Olomouc, I.P. Pavlova 6, CZ 775 20 Olomouc, Czech Republic_

Aims: In this work we developed and validated a fast and inexpensive isotope dilution direct injection method coupled with tandem mass spectrometry for the simultaneous determination of TKIs in human plasma without the need of chromatographic separation using common sample preparation.

2.2.1. Abstract

Background: Therapeutic drug monitoring is recommended for the optimal management of patients with several malignant diseases. The aim of this study was to develop and validate an isotope dilution direkt injection mass spectrometry method for the high throughput determination of tyrosine kinase inhibitors in plasma from leukaemic and cancer patients.

Methods: The plasma for analysis was deproteinated by methanol and the centrifuged supernatant was directly injected to mass spectrometer without separation step. Multiple reaction monitoring modes on a hybrid triple quadrupole – linear ion trap mass spectrometer (5500 QTRAP) were used for the detection and quantification of imatinib, nilotinib, lapatinib, and dasatinib.

Results: We developed a fast method with analysis time of 55 s and 19 s in multiple injection setting. The method was successfully validated and applied to the patient plasma samples. In order to overcome insufficient sensitivity of dasatinib, multiple reaction monitoring cube (MRM^3) mode in linear ion trap was successfully applied. The limits of quantification were in the range 1.0–5.5 ng/ml. Imprecisions were lower than 6.9 % and the accuracy of the quality control samples ranged between 99.0 and 107.9 %.

Conclusions: Isotope dilution direct injection mass spectrometry method allows high-throughput therapeutic drug monitoring of tyrosine kinase inhibitors in plasma. The method offers low-cost analyses as a result of its speed and the exclusion of separation step and can be advantageously used in routine clinical practice. The method can be applied on various drugs and biochemical markers with the use of triple quadrupole instruments.

2.2.2. Materials and Methods

Chemicals and reagents

Imatinib mesylate, nilotinib, and dasatinib were purchased from LC Laboratories (Woburn, MA, USA) and deuterated standards (D8-imatinib, D6-nilotinib, D8-dasatinib, D4-lapatinib-ditosylate) and lapatinib from TLC PharmaChem (Vaughan, Ontario, Canada). Formic acid, ammonium hydroxide, water, and methanol were all LC/MS grade and were purchased from Sigma (St. Louis, MO, USA); dimethylsulfoxide (DMSO) was obtained from Lachema (Brno, Czech Republic). Plasma samples of healthy volunteers and patients in tri-potassium salts of ethylenediaminetetraacetic acid (K_3EDTA) were obtained from the Department of Hemato-Oncology, University Hospital Olomouc (Czech Republic). The internal quality control (IQC) samples for imatinib, nilotinib, and dasatinib were obtained from Chromsystems (Munich, Germany), and the external quality control (EQC) samples for imatinib were obtained from Service de pharmacologie clinique (Centre Hospitalier Universitaire de Bordeaux, France).

Standard solutions, quality comtrol samples

Stock solutions of IM mesylate and D8-IM were prepared by dissolving them in methanol to a yield concentration of 1mg/mL expressed as free substances. IM was further diluted in methanol to obtain solutions with concentration levels in the range 0.1–100 µg/mL. The internal standard was finally diluted in methanol to a working concentration of 22.5 ng/mL. NIL, DAS, LAP, and their deuterated internal standards (D6-NIL, D8-DAS, D4-LAP-ditosylate) were dissolved in DMSO to a yield concentration of 1 mg/mL expressed as free substances. All the drugs were subsequently diluted in methanol to obtain a concentration of 0.01–10 µg/mL for DAS, 0.1–100 µg/mL for NIL, 0.1–200 µg/mL for LAP, 50 ng/mL for D8-DAS, 100 ng/mL for D6-NIL, and 75 ng/mL for D4-LAP. The calibration standards and controls were prepared from drug-free plasma from healthy volunteers enriched with particular analytes to the required concentration. All the solutions were stored at -20 °C. Lyophilized IQC samples were dissolved in

2 mL of pure water and the definite aliquot was processed by a standard procedure as described below. The EQC plasma sample aliquots were processed in the same way.

<u>Sample preparation</u>

This study was conducted in accordance with the Helsinki Declaration, and the protocol was reviewed by the hospital ethics committee. All the patients who were examined gave their informed consent to participation in the study before blood sampling. The blood from healthy control volunteers and patients treated with TKI was put into a test tube with the addition of an anticoagulant (EDTA), and subsequently centrifuged (1200 g; 5 min). A small volume of the plasma (20 µL) was precipitated by methanol enriched by an adequate internal standard (180 µL) in 1.5 mL Eppendorff tubes. Subsequently, the sample was put into a sonicator for 1 min, shaken for 5 min, cooled for 30 min at -20 °C, and centrifuged for 5 min at 14,300 g. The supernatant was placed into 350 µL glass vial (12 x 32 mm, fused insert) and directly injected into a mass spectrometer or stored in a freezer at -20 °C before analysis.

<u>Isotope dilution direct injection analysis</u>

TKI plasma levels were measured using UltiMate 3000 RS (Dionex, Sunnyvale, CA). The samples were maintained in a thermostated autosampler rack with the temperature set to +5 °C during analysis.

In order to prevent carryover of plasma samples and enhance ionisation organic solvents with addition of formic acid are commonly used for direct injection analyses (Chace *et al*, 2003). Based on the physical and chemical properties of TKIs and deproteination solvent the mobile phase consisting of methanol and 0.1% formic acid was chosen. The flow rate was set at 0.30 mL/min for 0.00-0.12 min and 0.40-0.60 min; in the measuring period between 0.12 and 0.40 min the flow rate was reduced to 0.03 mL/min. The samples (0.5 µL) were injected directly into a mass spectrometer without using chromatographic separation. In order to obtain maximum sample throughput with optimized multiple injection in

one analysis the flow rate was increased to 0.5 mL/min and injections of all samples were performed in sequence every 19 sec.

Tandem mass spectrometry

All the experiments were performed on a QTRAP 5500 triple quadrupole instrument (AB Sciex, Foster City, CA, USA). Mass spectrometric detection of the analytical run was monitored in positive MRM and MRM[3] modes. The dwell times of two MRM transitions between the precursors and product ions for each compound and each appropriate internal standard were set to 30 ms. The parameters of the ion source were optimized to the following settings: an ionization spray voltage of 5500 V, curtain gas of 30 psi, heater gas of 40 psi, turbo ion spray gas of 40 psi, a source temperature of 350 °C, and entrance potential of 10 V. High-purity nitrogen was used as the collision gas. The gas pressure for collision-activated dissociation was adjusted to "medium settings". Declustering potential, collision energy, and collision cell exit potential were optimized on standards of the analytes under study in a methanolic solution of 0.1% formic acid. All the parameters are detailed in Table 3. Both quadrupoles (Q1 and Q3) were set to unit resolution. The Analyst 1.5.1 software (AB Sciex, USA) was used for tuning the mass spectrometry parameters and data evaluation and quantification on the basis of the ratio of the corrected peak area of the compound and its deuterated internal standard. In order to exclude possible interferences the ratios of the two m/z transitions for each analyte and its deuterated standard were determined.

Due to low signal-to-noise ratio in MRM mode of DAS the MRM[3] mode was used to improve sensitivity and the selectivity of the method. In order to achieve secondary fragmentation of first product ion, the third quadrupole operated in the linear ion trap (LIT) mode. In this experiment, the protonated DAS (m/z 488.0) was isolated as a first precursor in the first quadrupole and fragmented in collision cell (the second quadrupole) to a first product using collision gas flow at the medium instrument setting, a collision energy of 41 V, and a declustering potential of 50 V. The first product was subsequently trapped in the third quadrupole (working in LIT mode) using a dynamic LIT fill time of up to 250 ms and an excitation time

of 25 ms and fragmented under excitation energy of 0.13 V, giving the most intensive second product ion at m/z 232.0. Finally, the MRM[3] ion transition of 488.0 → 401.0 → 232.0 was monitored for the quantification of DAS. LIT was set to perform a mass scan centered at m/z 232.0 with a mass window of 0.5 Da (231.75 – 232.25 Da).

<u>Method validation</u>

The method validation was based on the recommendations published by the Food and Drug Administration (FDA), (FDA, 2001), and European Medicine Agency (Guideline on bioanalytical method validation, 2011). Validation procedure was performed for all inhibitors in one method by the use of 0.3 and 0.03 mL/min flow rate. In order to obtain maximum speed, finally we tested multiinjection method with flow rate of 0.5 mL/min and compared it with LC-MS/MS method.

- Linearity, limit of detection, limit of quantification

The plasma of healthy volunteers, with the addition of 10, 30, 100, 300, 1000, 3000, and 10,000 ng/mL of IM and NIL, 3, 10, 30, 100, 300, and 1000 ng/mL of DAS, and 25, 50, 250, 500, 2500, 5000, and 10,000 of LAP in triplicates was used for the construction of the calibration curves. For the quantification and calculation of the linear regression model (least-square method) fitted by 1/x weighting, the concentration as dependence between the peak area ratio of the TKI/internal standard and concentration was used. The linearity slope, intercept, correlation coefficient, and standard deviation were calculated. The sensitivity of the method was determined on blank plasma with addition of low TKI standard concentration: 30 and 10 ng/mL for IM and NIL, 10 and 5 ng/mL for DAS, and 25 and 10 ng/mL for LAP to reflect the lowest clinically relevant concentrations based on previously published pharmacokinetics data (van Erp *et al*, 2009). The limit of detection (LOD) and limit of quantification (LOQ) were calculated as a signal-to-noise ratio of 3 and 10, respectively; the bias and coefficient of variation were determined on LOD/LOQ concentration levels (n=10, spiked plasma).

- Imprecision, recovery, accuracy

Intra- and inter-day imprecision and recoveries were measured using plasma samples added to 3000, 1000, and 300 ng/mL for IM and NIL, 300, 100 and 30 ng/mL for DAS, and 5000, 2500, and 500 ng/mL for LAP in six replicates within one day and consecutive six days, respectively. Means and standard deviations were calculated by the ANOVA method; recovery was expressed as a percentage of target value on IQC samples. Accuracy was measured using IQC for IM, NIL, DAS on two concentration levels, and EQC in 15 samples for IM.

- Ion suppression

Quantitative determination of matrix effects was performed on six different blank plasma samples with the addition of TKIs to the same final concentration as for imprecision evaluation. Ion suppression was calculated as the ratio of signal intensities of a plasma sample supplemented by TKIs to the final concentration equal to the standard solution and the standard solution of TKIs dissolved in methanol.

- Comparison of the direct injection method with liquid chromatography

Developed method was compared with our previously published LC-MS/MS method (Mičová *et al*, 2010a,b). Plasma samples (n = 28) were analysed and the results were evaluated by Bland-Altman plot and regression analysis. The equation of linear regression, correlation coefficient, mean difference from zero, and standard deviation were calculated.

2.2.3. Results and discussion

<u>Sample preparation</u>

On the basis of previously published methods (De Francia *et al*, 2009; Velpandian *et al*, 2004; Bakhtiar *et al*, 2002) we chose an easy, cheap, and effective way to prepare the plasma samples for direct injection into a

mass spectrometer with high efficiency. This simple sample preparation by protein precipitation using organic solvent without any additional cleaning steps is sufficient and usable for the high-throughput bioanalysis of TKIs in human plasma. In order to remove proteins before analysis deproteination by organic solvents compatible with mass spectrometry were tested. In comparison to acetonitrile tenfold amount of methanol followed by cooling offered homogenous precipitate with clear supernatant. Under these conditions we analysed more than 1000 plasma samples without any reduction in the mass spectrometer sensitivity.

Isotope dilution direct injection method

The developed method allows the simultaneous determination of the most commonly used tyrosine kinase inhibitors in chronic myeloid leukaemia and breast cancer treatment – IM, NIL, DAS, and LAP in human plasma – by flow injection analysis coupled with tandem mass spectrometry. A representative direct injection analysis data of the plasma samples of particular TKIs is shown in Figure 19. Transitions of IM, NIL, and LAP offer a high signal-to-noise ratio and MRM mode is fully suitable for determination in plasma samples from patients. The transition of DAS provides significantly lower ionization and higher noise, which results in insufficient sensitivity in MRM mode. In addition, plasma samples from patients on DAS treatment contain concentration levels that are ten times lower as a result of the lower dosage and faster pharmacokinetics. Therefore we applied MRM^3 mode and we obtained excellent sensitivity with high selectivity of DAS. On the other hand, for one transition an MRM^3 period consumes a cycle time of 2.0 s. Together with the MRM period, we obtained 2.6 s per one cycle and 14 data points in one analysis. As a result of time consumption, MRM^3 mode has limited use in fast multianalyte chromatographic analyses where it is necessary to achieve more than ten data points per compound. Mass spectrum and direct injection analysis data for DAS are shown in Figure 20.

73
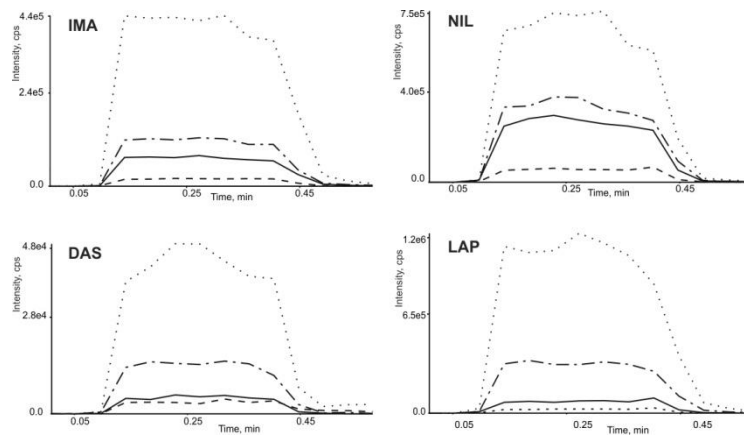
Figure 19: Representative direct injection analysis-data of IQC (IM, c = 1911 ng/mL; NIL, c = 1184 ng/mL; DAS, c = 252 ng/mL) and added plasma (LAP, c = 2500 ng/mL) samples measured in MRM mode. Dotted, dot-dashed, full and dashed lines correspond to first and second transitions of analytes and deuterated internal standards, respectively (see Table 3).
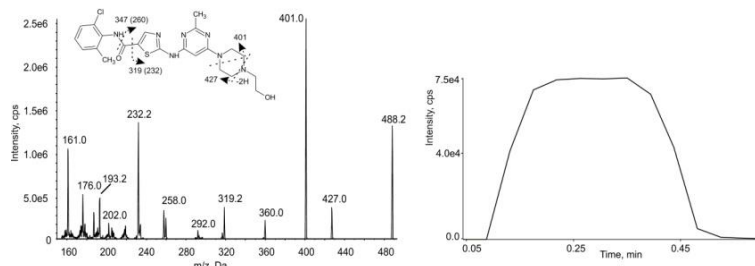
Figure 20: Mass spectrum and MRM[3] direct injection analysis-data of dasatinib (c = 252 ng/mL).

Table 3: Optimized mass spectrometry parameters for analysed compounds.

	MRM transition	DP (V)	CE (V)	CXP (V)
IM	494.0 → 394.1	16	39	34
IM	494.0 → 217.2	16	35	18
D8-IM	502.0 → 394.1	16	39	34
D8-IM	502.0 → 225.2	16	35	18
NIL	529.9 → 289.1	41	43	24
NIL	529.9 → 259.1	41	77	22
D6-NIL	536.3 → 295.1	41	39	8
D6-NIL	536.3 → 266.1	41	69	18
DAS	488.9 → 401.0	31	41	34
DAS	488.0 → 232.0	31	57	14
D8-DAS	497.0 → 406.0	31	41	34
D8-DAS	497.0 → 237.0	31	57	14
LAP	580.9 → 365.0	36	53	32
LAP	580.9 → 350.0	36	53	28
D4-LAP	586.1 → 366.0	36	47	26
D4-LAP	586.1 → 351.9	36	61	24

Whereas the analyses for the frequently used LC-MS/MS methods for simultaneous TKI determination take few minutes, the total analysis time of the isotope dilution direct injection method we developed is 55 s. This

includes injection of the sample (20 s) and measurement with a washing step (35 s). Signal intensities are one order lower in comparison with liquid chromatography methods as a result of the higher ion suppression. But this has negligible effect on the accuracy and precision of the method because of the utilization of a deuterated internal standard.

In order to obtain more sensitive and accurate results, the flow rate was reduced from 0.3 mL/min to 0.03 mL/min (0.12 - 0.40 min). This affords longer time and more points for the data acquisition and lower ion suppression. For compounds measured in MRM mode the acceptable ratios of peak areas of two transitions were set to 3.2-3.6, 3.4-4.0, 1.8-2.2, 4.3-4.9, 3.1-3.6, and 2.7-3.3 for IM, D8-IM, NIL, D6-NIL, LAP, and D4-LAP, respectively. We found no data ratio of m/z transitions outside the set limits for any of the measured samples. The variation in the peak area of internal standards was in the range 1.4 - 6.5% (intra-day).

Multiinjection analysis that we developed by the use of higher flow rate of 0.5 mL/min enables measuring of 32 samples during 10 minutes (Fig. 21). Calculated concentrations were compared with results from routine used UHPLC-MS/MS method by Bland-Altman graph and regression analysis. Calibrators, quality control samples and patient plasma samples on IM therapy were analysed by pair test. Comparison by Bland-Altman plot showed mean difference of -4.27 ng/mL and standard deviation of 46.89 ng/mL. Regression analysis provided correlation equation of $y = 1.0144 x - 13.003$ and a correlation coefficient of 0.991. High throughput of the method is caused by no delay of new analysis start and direct samples injection in sequence of each 19 seconds. Dwell time of 150 ms for IM measurement offers 15 points per peak which is reliable for accurate and precise quantitation.
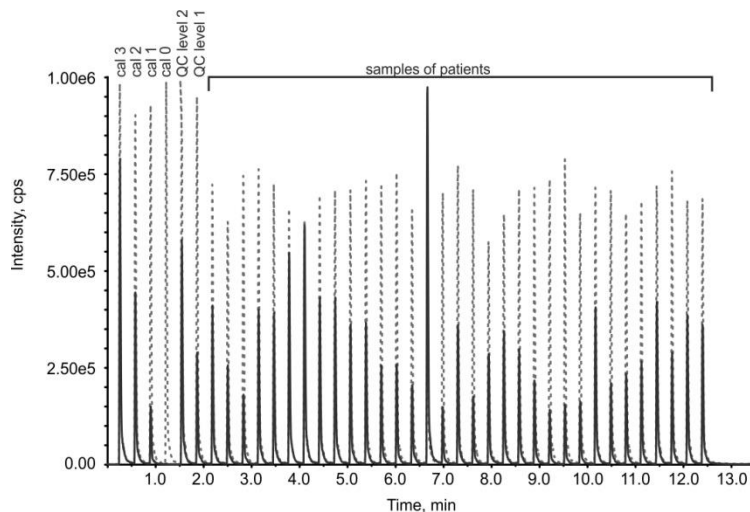
Figure 21: Flow multipleinjection analysis of IM plasma samples. Intenzities of dashed line of internal standard correspond to differences in ion suppressions in the plasma samples which is about 25 %.

Validation

In order to validate the linearity of the method, LOD, LOQ, imprecision, analytical recovery, and accuracy were determined. The method offers linear dependences over entire ranges with correlation coefficients r > 0.994. The limits of quantification better than 6 ng/ml for analytes measured in MRM mode (IM, NIL, LAP) are well below the clinically relevant range of concentration encountered in patients (typically > 100 ng/ml). In the case of DAS MRM[3] mode LOQ was five times lower, which corresponds to the requirement of higher sensitivity as a result of lower plasma levels (Tab. 4). Intra- and inter-day imprecisions and accuracies expressed as variation coefficient and bias are summarized in Table 5. Applied to IQC and EQC blood samples, the method offers excellent bias and standard deviation (Tab. 6). The stability of TKIs at different conditions was described in previously published studies. All of the analytes are stable in plasma at least five months stored at -20°C with maximum loss of 10 % of the nominal concentration (De Francia *et al*, 2009; Haouala *et al*, 2009).

77

Table 4: Validation – linearity (n=3), LOD (n=10) and LOQ (n=10) of TKIs.

Analyte	Linearity $y = a(SD) x + b(SD)$	LOD (ng/mL)	LOQ (ng/mL) (bias, CV, %)
IM	$y = 1.37e^{-4}(1.68e^{-6}) x + 8.45e^{-4}(1.64e^{-3})$	1.64	5.45
	(r = 0.9986)		(-12.58, 24.04)
NIL	$y = 3.39e^{-4}(5.91e^{-6}) x + 1.51e^{-3}(1.88e^{-3})$	1.30	4.35
	(r = 0.9976)		(-5.90, 12.22)
DAS	$y = 1.12e^{-2}(1.22e^{-4}) x + 6.75e^{-2}(3.54e^{-3})$	0.29	0.96
	(r = 0.9990)		(9.59, 12.22)
LAP	$y = 6.24e^{-4}(1.15e^{-5}) x + 1.86e^{-2}(3.04e^{-2})$	1.36	4.55
	(r = 0.9964)		(14.33, 10.67)

Table 5: Intra- and Inter-day imprecission and accuracy (n=6).

Analyte	Concentration (ng/mL)	Imprecision (CV, %)		Accuracy (bias, %)	
		Intra-day	Inter-day	Intra-day	Inter-day
IM	3000	3.18	2.47	-0.28	1.95
	1000	3.50	4.03	1.12	3.97
	300	3.87	4.93	1.07	-3.11
NIL	3000	4.16	2.48	-1.72	-0.67
	1000	4.16	1.89	5.54	5.67
	300	4.53	6.93	-1.73	-1.81
DAS	300	3.90	3.67	0.17	1.61
	100	4.31	3.33	2.53	1.85
	30	4.36	5.36	1.22	0.06
LAP	5000	3.27	4.01	-0.50	-0.29
	2500	4.17	3.24	1.13	0.69
	500	2.83	4.30	-0.67	2.93

		Intra-day	Inter-day	Intra-day	Inter-day
	(ng/mL)				

Table 6: Accuracy of IQC and EQC blood samples.

Analyte	level (ng/mL)	accuracy	
		(bias, %)	(CV, %)
IM-iqc	941	2.40	4.29
	1911	1.34	3.68
NIL-iqc	711	0.15	5.58
	1184	-1.00	4.13
DAS-iqc	116	0.93	5.78
	252	2.62	5.59
IM-eqc	80-6000	1.62	7.37

Ion suppression determined using plasma samples added to low, medium, and high concentrations of TKI in six replicates was significantly higher in comparison to liquid chromatography methods as a result of the ionization of many suppressing substances in the ion source during the same analysis time. Ion suppressions were 66.5 – 74.1 %, 77.8 – 86.9 %, 54.0 – 60.0 %, and 65.3 – 84.0 % for IM, NIL, DAS, and LAP, respectively. Thanks to the use of deuterated internal standards, the matrix effects were eliminated.

The direct injection analysis and LC methods were compared by Bland-Altman plots and regression analysis. Quality controls and samples from patients were analysed by paired test. Bland-Altman plot showed mean difference of -12.5, 0.02, -2.55, -20.00 ng/mL and standard deviation of 39.22, 21.73, 8.36 and 113.24 ng/mL for IM, NIL, DAS and LAP, respectively. Regression analyses provided a linear correlation with slope of 1.023, 1.005, 0.995, 1.029, intercept of -3.158, -4.395, 2.881, 5.773 and correlation coefficient of 0.997, 0.999, 0.994 and 0.998 for IM, NIL, DAS and LAP, respectively.

2.2.4. Acknowledgements

2.3. Imatinib dose escalation in two patients with chronic myeloid leukaemia, with low trough imatinib plasma levels measured at various intervals from the beginning of therapy and with suboptimal treatment response, leads to the achievement of higher plasma levels and major molecular response.

Edgar Faber[1], David Friedecký[2], <u>Kateřina Mičová[2]</u>, Martina Divoká[1], Beata Katrincsáková[1], Šárka Rožmanová[1], Marie Jarošová[1], Karel Indrák[1], Tomáš Adam[2]

[1]Department of Hemato-Oncology
[2]Department of Biochemistry and Immunogenetics, Laboratory of Inherited Metabolic Disorders
Faculty of Medicine and Dentistry, Palacky University in Olomouc
Faculty Hospital Olomouc
Olomouc, Czech Republic

Aims: Among other evidence lacking, there is no conclusive report about the success of imatinib dose escalation in patients with low IPL associated with suboptimal treatment response. We provide this evidence in two patients now.

2.3.1. Abstract

Despite the prognostic value of trough imatinib plasma levels (IPL) identified in some studies, no recommendations for the use of IPL results in routine management of CML patients have been issued. We report two patients in whom daily imatinib dose was increased from 400 mg to 600 or 800 mg because of low IPL found at various intervals from the beginning of treatment (7 measurements; mean IPL values=616.33 and 764.5 ng/mL, respectively). Both patients achieved suboptimal response according to the European LeukaemiaNet criteria (complete cytogenetic response was not achieved after one year of treatment in Patient 1 and major molecular response after 47 months of standard-dose imatinib therapy in Patient 2). In addition, we have demonstrated low hOCT-1 expression at diagnosis in both patients, retrospectively. Escalation of imatinib daily dose resulted in an significant increase of IPL (6 measurements; mean=1790 and 1416.66 ng/mL, respectively) and in achievement of complete cytogenetic response in Patient 1 after 3 months and major molecular response within 15 and 6 months in both patients. Our cases demonstrate that low IPL identified at various non-predefined intervals from the beginning of therapy may be used for deciding on dose escalation in selected CML patients in the routine clinical setting, especially in the cases with suboptimal treatment response.

2.3.2. Methods

Cytogenetics and FISH

Cytogenetic examinations were performed according to standard procedure from bone marrow cells at the time of starting imatinib therapy and then once a year. FISH with LSI BCR/ABL ES and/or LSI BCR/ABL Dual Fusion probes (Abbott-Vysis, Downers Grove, IL, USA) was applied at the beginning of imatinib treatment and than at least once in six months. In case of additional cytogenetic abnormalities, FISH with centromeric (Abbott-Vysis) and/or painting probes (Cambio Ltd., Cambridge, UK) was performed. Cytogenetic response was classified as complete (0 % Ph positive mitoses or interphase cells using FISH), major (1-34 %), minor (35-67 %) and minimal (68-99 %) as described previously (The Italian Cooperative Study Group on chronic myeloid leukaemia, 1994).

Quantitation of BCR/ABL mRNA

Quantitative reverse-transcriptase polymerase chain reactions (RQ-PCR) for BCR/ABL mRNA transcript levels were performed using the LightCycler Instrument (Roche Diagnostics, Mannheim, Germany). Total leukocyte RNA was extracted from peripheral blood and/or bone marrow aspirate after lysis of red blood cells according to standard protocol (Chomczynski *et al*, 1987). RNA was reverse transcribed to cDNA with Transcriptor Reverse Transcriptase (Roche Diagnostics, Mannheim, Germany) using random hexamers. Primers and TaqMan probe sequences published in the EAC network protocol were used for RQ-PCR (Gabert *et al*, 2003). Briefly, 100 ng cDNA was added to 2 µl Master Mix (LightCycler FastStart DNA Master Hybridization Probes; Roche Diagnostics, Mannheim, Germany) in 20 µl total reaction volume with final concentration of 4 mM MgCl$_2$, 300 nM primers and 200 nM TaqMan probe. The PCR program included initial denaturation step for 10 min at 95°C and 50 cycles (95°C, 10 sec, 20°C/s)/(60°C, 30 sec, 20°C/s). BCR/ABL and ABL assays were performed in duplicate. Mean crossing points (Cp) were used to interpolate standard curves and to calculate the transcript copy number. Normalized levels were calculated as the BCR-ABL/ABL ratio and expressed

as percentage. The standard curve was generated using serial dilutions of a linearized plasmid, containing BCR/ABL insert FGRS10 (Ipsogen, Marseille, France). The plasmid used to quantify the endogenous control gene ABL was CGRS01 (Ipsogen, Marseille, France).

Measurement of imatinib plasma levels

Imatinib plasma concentrations were measured by ultra-performance liquid chromatography-tandem mass spectrometry assay (Parise *et al*, 2003) using UHPLC UltiMate 3000 RS (Dionex, USA) coupled with the API 4000 tandem mass spectrometer (Applied Biosystems, USA). Samples were obtained at routine clinical check-ups at 18.25-24 hours after the latest dose of imatinib in both patients, with exception of two measurements in Patient 1 that were taken 14.75 hours after the latest imatinib dose.

Sequencing analysis of BCR/ABL kinase domain

RNA and cDNA were prepared as described [13, 14]. cDNA was amplified by nested PCR using primers located in the BCR (external 5´-GCTACGGAGAGGCTGAAGAAG-3´ and internal 5´-CAGATGCTGACCAACTCGTGT-3´) and ABL (external 5´-GCTCGCATGAGTTCATAGACC-3´and internal 5´-CTTCTCTAGCAGCTCATACACC-3´) regions of the BCR/ABL gene. The amplicons cover the region flanked by amino acids 200-448, according to GenBank accession no. M14752. PCR products were directly sequenced using forward (5´-AGAGATCAAACACCCTAACC-3´) and reverse (5´-GTTCTCCCCTACCAGGCAG-3´) primers and ABI PRISM Genetic Analyser 3100 (Applied Biosystems, Foster City, CA) using Chromas, Version 1.5 sequence analysis software (Technelysium Pty Ltd, Queensland, Australia).

Measurement of hOCT1 mRNA levels by real-time quantitative reverse transcriptase polymerase chain reaction

To assess the expression of *hOCT1* (SLC22A1) in CML patients total RNA was isolated and subjected to RNase-free DNase treatment performed according to the manufacturer's instructions (Ambion, Austin,TX). DNase-

free RNA was reverse transcribed into cDNA using SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) was performed using EXPRESS SYBR GreenER qPCR SuperMix (Invitrogen, Carlsbad, CA) on LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) using the following conditions: 95°C for 5 min followed by 40 cycles at 95°C for 10s, 60°C for 10s and 72°C at 20s. Primers described by White et al (White et al, 2007) were used to amplify the hOCT1 gene. The ABL1 gene was amplified as an internal control using the primers designed as follows: CCAACCTTTTCGTTGCACTGTA (ABL1_F) and CATTTTTGGTTTGGGCTTCAC (ABL1_R). The samples were analysed in triplicate, and data were assessed using the comparative Ct method (ΔΔCt) using expression of hOCT1 in healthy volunteers as a calibrator. Briefly, a total of eight normal peripheral blood samples were pooled, treated with DNase and reverse transcribed into cDNA as described above in order to be used as a calibrator to assess hOCT1 expression in CML patients. qRT-PCR data were analysed using LightCycler Software Version 1.5.0 (Roche Diagnostics, Mannheim, Germany). PCR conditions were optimized to obtain a single peak on melting curve analysis for both genes. PCR products were directly sequenced in order to ensure the 100 % match with the target gene. The *hOCT1* gene expression levels were normalized with the *ABL1* gene expression levels in the same sample. Next, *hOCT1* gene expression was normalized between samples by relating them to the respective *hOCT1* expression level in peripheral blood of healthy volunteers (calibrator), in which expression of *hOCT1* was arbitrarily scored as 1.0.

2.3.3. Results

Clinical features, history of CML and imatinib therapy in both patients are depicted in Table 9. Both patients were diagnosed on the basis of standard bone marrow cytogenetic examination that confirmed the presence of the Philadelphia chromosome without any additional chromosomal changes. Both patients had their liver transaminases, bilirubin, albumin, creatinine and glomerular filtration within normal limits.

After the introduction of *hOCT1* gene expression measurement in our laboratory in March 2010 we have assessed the patients´ samples taken at the diagnosis. In both cases low normalized *hOCT1* gene expression was demonstrated (0.1233 and 0.0506, respectively). Both patients signed informed consent with IPL blood sampling. In both patients, standard treatment with 400 mg of imatinib daily was initiated after cytoreduction with hydroxyurea. Patient 1 did not achieve CCR before imatinib dose escalation, but achieved it after 3 months of escalated dosage. Patient 2 achieved CCR after 12 months with a standard dose of imatinib 400 mg daily, but did not achieve MMR after 18 months. At the time of this suboptimal response we were not able to demonstrate any mutation in *BCR-ABL* tyrosine kinase domain using DNA sequencing in neither patient. Repeated IPL measurements revealed low IPL (7 examinations; mean values 616.33 and 764.5, range 507-728 and 649-898 ng/mL, respectively) and the patients did not achieve optimal response to standard imatinib treatment (see Table 10 and Figure 26). Therefore, it was decided to escalate the dose to 800 and 600 mg daily. Assessments of IPL were performed at routine check-ups at non-predefined time intervals from the beginning of the therapy. Repeated IPL measurements after imatinib dose escalation have confirmed achievement of higher IPL (mean values 1790 and 1416.66 ng/mL, respectively; see Table 10). After 3 months from the start of escalated dosage, CCR was achieved in Patient 1, while after 15 and 6 months of escalated dosage, MMR was achieved in both patients (see Table 9, Figure 26 for molecular, cytogenetic and IPL monitoring of the patients). Both patients had already experienced grade 3 hypophosphatemia according NCI toxicity scale after standard dosage of imatinib. Escalation of a daily imatinib dose to 800 mg was associated with makrocytosis, grade 1 hypokalemia, significant fluid retention and peripheral edema with the need of diuretic therapy in Patient 1 whereas in Patient 2, grade 1 elevation of ALT and grade 1 hypokalemia were observed after imatinib dose escalation to 600 mg daily, while there were no clinical side-effects. No haematologic toxicity was recorded. Both patients have a very good compliance to imatinib therapy irrespective of the dosage applied.

Table 9: Clinical and laboratory characteristics of patients and their course of treatment.

	Patient 1	Patient 2
Sex	male	male
Age at the time of diagnosis	53	46
Weight (kg)	86	120
Body surface area (m^2)	2.04	2.44
Sokal index	1.67	0.84
Hasford index	1737	504
Albumin (g/l; normal range: 35-50)	48	46.7
Glomerular filtration accoring MDRD (normal range: 1.15-2.0)	1.59	1.48
Time from diagnosis to start of imatinib therapy (400 mg daily) (days)	7	39
Time from diagnosis to CCR (months)	18	12
Time from diagnosis to imatinib dose escalation (months)	15	48
Dose after escalation (mg daily)	800	600
Time from imatinib dose escalation to MMoR (months)	15	6
Time from diagnosis to the latest follow-up (months)	30	60

Table 10: Imatinib dosage (absolute and calculated per kg of body weight and per m^2 of body surface area) and imatinib plasma levels before and after imatinib daily dose escalation from 400 mg to 600 or 800 mg. Samples were taken 23 to 24 hours after the dose in Patient 1 and 18.25 to 20 hours after the dose in Patient 2. * Samples were taken 14.75 hours after ingestion of the latest dose (dosage 400 mg twice daily).

	Patient 1	Patient 2
Initial imatinib daily dose (mg)	400	400
Daily dose per kg body weight (mg/kg)	4.65	3.33
Daily dose per m^2 (mg/m^2)	196.08	163.93
Trough imatinib plasma levels before dose escalation (ng/mL) [month of the sample from beginning of imatinib therapy]	507 [9], 728 [12], 614 [15] Mean=616.33	649 [30], 850 [33], 898 [36], 661 [42] Mean=764.5
Imatinib daily dose after escalation (mg)	800	600
Daily dose per kg body weight after escalation (mg/kg)	9.3	5
Daily dose per m^2 after escalation (mg/m^2)	392.16	245.9
Trough imatinib plasma levels after dose escalation (ng/mL) [month of the sample from beginning of imatinib therapy]	1010 [18], 2040 [21]*, 2320 [24]* Mean=1790.00	1390 [48], 1460 [51], 1400 [54] Mean=1416.66
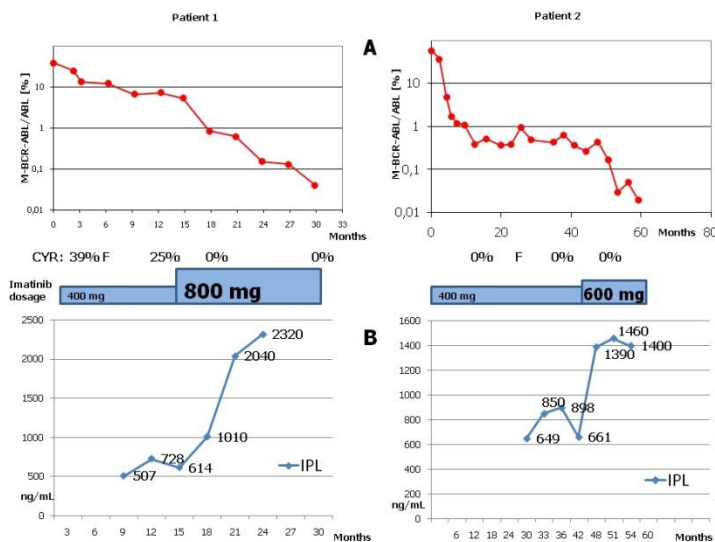
Figure 26: Results of molecular (Part A), cytogenetic (CYR) and trough imatinib plasma level (IPL; Part B) monitoring in both patients. Imatinib daily dosage is shown in the middle. (F=cultivation failure).

2.3.4. Discussion

Picard *et al* showed for the first time that IPL may have a prognostic value in patients with CML treated with first-line imatinib (Picard *et al*, 2007). They measured IPL in 68 patients in chronic or accelerated phase CML treated with 400 or 600 mg of imatinib daily at unspecified time intervals from the start of imatinib treatment and they found significantly higher IPL in patients achieving CCR and MMR (Picard *et al*, 2007). Their observation was confirmed by Larson *et al* who reported results of a pharmacokinetic part of the IRIS study using day 29 IPL measurements and found IPL to be associated not only with CCR and MMR but also with significantly different progression-free survival of patients divided into four groups according to IPL (Larson *et al*, 2008). Both studies confirmed high inter-patient variability of IPL that may be influenced by various factors including drug absorption, different binding to plasma proteins, variability in metabolizing liver enzymes and patients' demographic factors (Cortes *et*

90

al, 2009). Also our team was able to confirm the correlation of IPL with the probability of achieving MMR in a small group of patients (Faber *et al*, 2008). However, there are at least two other studies that found no correlation between IPL and patients' prognosis (Forrest *et al* 2009; Davies *et al*, 2009). Despite speculations that IPL could assist with decisions about whether to increase an imatinib dose, IPL measurement is recommended as helpful in situations when poor adherence to treatment is suspected, severe site-effects are observed or drug interactions should be ruled out (Cortes *et al*, 2009). Further studies are awaited to confirm prospectively the link between IPL and response to treatment and to define effective trough concentrations in different patient populations (Cortes *et al*, 2009). No clear recommendation has been issued regarding IPL estimations in CML patients in the recent statement paper of ELN experts (Baccarani *et al*, 2009). Among other evidence lacking, the usefulness of imatinib dose escalation due to the low IPL for the subsequent achieving of optimal treatment response has not been reported in a full article. In a recent abstract, Australian authors published the results of the TIDEL II prospective study that used IPL measured at day 22 of imatinib therapy in 74 CML patients for subsequent dosage adjustment (Osborn *et al,* 2009). In 11 (15 %) patients, IPL lower than 1000 ng/mL was found and 7 (9.5 %) of them were able to escalate the imatinib daily dose to 800 mg. These patients achieved comparable mean IPL with the rest of the group at 3 and 6 months and their molecular response also improved (Osborn *et al,* 2009). Our two patients were identified among 12 patients with suboptimal treatment response from 40 patients treated with first-line imatinib at our centre since 2004. Only these two patients (5 %) were shown to display significantly lower than recommended IPL (< 1000 ng/mL) assessed at various time points between months 9 and 42 of therapy. Both patients showed an increase of IPL after imatinib dose escalation and also achievement of optimal treatment response. Of interest in both our patients, low expression of *hOCT1* gene at the diagnosis as the other possible cause of suboptimal treatment reponse was retrospectively demonstrated. White et al have shown the association of low expression of *hOCT1* with suboptimal treatment response and suggested imatinib dose escalation as a suitable strategy for its´ management (White *et al*, 2007).

The population of patients suitable for imatinib dose management according to IPL may not be large as most patients respond well to imatinib first-line treatment and in non-responding patients several other causes of resistance may play a role. However, we believe that our report showing that IPL estimated at various time points of treatment may be used for dose adjustment in individual patients has important implications for optimalization of routine management of CML patients outside clinical trials.

2.3.5. Acknowledgement

2.4. Imatinib trough plasma levels do not correlate with the response to therapy in patients with chronic myeloid leukaemia in routine clinical setting.

Edgar Faber[1*], David Friedecký[2*], Kateřina Mičová[2], Šárka Rožmanová[1], Martina Divoká[1], Marie Jarošová[1], Karel Indrák[1], Tomáš Adam[2]

* authors contributed equally to the work

[1]Department of Hemato-Oncology
[2]Department of Biochemistry and Immunogenetics, Laboratory of Inherited Metabolic Disorders
Faculty of Medicine and Dentistry, Palacký University Olomouc
University Hospital Olomouc
Olomouc, Czech Republic

Aims: In this study, the prognostic value of IPL for achievement of molecular response in patients in chronic phase CML treated with imatinib for more than 18 months in a single center was analysed.

2.4.1. Abstract

Association of trough imatinib plasma levels (IPL) with cytogenetic or molecular response to treatment in patients with chronic myeloid leukaemia (CML) was repeatidly reported. We analysed their value in the routine clinical setting in 131 patients with chronic phase CML in whom imatinib was applied as first- or second-line treatment. A total of 1118 measurements were obtained by ultra-performance liquid chromatography-tandem mass spectrometry assay in patients treated with daily dose of imatinib ranging from 100 mg to 800 mg. Samples were obtained from 1 to 96 hours after drug ingestion. High inter- (36%) and intra-individual variability (9-33%) of IPL was observed. For analysis of correlation of IPL with treatment response according European LeukaemiaNet (ELN) criteria two sets of samples were selected. The first set consisted of 241 samples taken 24±2 hours after dosing in 54 patients and the second one of 329 samples taken 24±4 hours after imatinib ingestion in 84 patients. In both sets only patients treated with 400 mg imatinib once daily for at least 18 months were included. From multiple measurements in individual patients, mean IPL were used. In both sets we were not able to demonstrate correlation between IPL and response to treatment according ELN. We believe that this was due to the differences in patients' compliance, leukaemia biology and other variables that are difficult to eliminate in the routine clinical practice. The use of IPL for prognostic estimation in CML treatment outside the clinical trials is probably limited.

2.4.2. Patients and methods

<u>Patients</u>

The study was conducted in accordance with the ethical standards based upon 1964 Declaration of Helsinki. All patients consecutively treated at the Department who gave informed consent approved by the local Ethics Committee were included into this retrospective study. Diagnosis of CML was established in each case from the findings in blood count and bone marrow and confirmed by the demonstration of Philadelphia chromosome by standard cytogenetics and/or BCR-ABL fusion gene by fluorescence in situ hybridization (FISH) or reverse-transcriptase polymerase chain reaction. The phase of disease at diagnosis was identified according to the ELN criteria (Baccarani *et al*, 2006). Sokal score was calculated according to the formula in the original publication (Socal *et al*, 1984). At the beginning of the treatment, standard dosage of 400 mg daily in one dose was given to all patients. Due to hematologic or non-hematologic toxicity, the dose was individually reduced to 300, 200 or 100 mg daily and in some patients, intermittent dosage was used in addition (Faber *et al*, 2006). In cases of suboptimal response, the dose of imatinib was escalated to 500, 600 or 800 mg daily. Median duration of imatinib treatment was 82 months (range; 25-121) in the second-line treatment after interferon and 59 months (range; 21-99) when imatinib was given as the front-line therapy. IPL results in all 131 patients were analysed for the impact of absolute given dose of imatinib and interval from the ingestion of imatinib to blood sampling on IPL. However, only samples taken in two groups of 54 and 84 patients treated with 400 mg of imatinib in one daily dose in whom sampling of the blood was performed 24±2 and 24±4 hours after ingestion of the drug, respectively, were included into the analysis of IPL and gender, age, body weight, body surface area and response to treatment correlation. Hematologic, cytogenetic and molecular responses to treatment were assessed according to the ELN criteria (Baccarani *et al*, 2006).

Cytogenetics and FISH

Cytogenetic examinations were performed according to the standard procedure from bone marrow cells at the time of starting imatinib therapy and then once a year or at the time of progression. FISH with LSI BCR-ABL ES and/or LSI BCR-ABL Dual Fusion probes (Abbott-Vysis, Downers Grove, IL, USA) was applied at the beginning of imatinib treatment and than at least once in six months. In case of additional cytogenetic abnormalities, FISH with centromeric (Abbott-Vysis) and/or painting probes (Cambio Ltd., Cambridge, UK) was performed. Cytogenetic response was classified as complete (0 % Ph-positive mitoses), major (1-34 %), minor (35-67 %) and minimal (68-99 %) as described previously (The Italian Cooperative Study Group on chronic myeloid leukaemia; 1994). Interphase FISH was used for assessment of the response in cases of culture failure. In that case, finding up to 1 % of positive cells represented complete cytogenetic response in the study.

RQ-PCR for BCR-ABL

Quantitative reverse-transcriptase polymerase chain reactions (RQ-PCR) for BCR-ABL mRNA transcript levels were performed using the LightCycler Instrument (Roche Diagnostics, Mannheim, Germany). Total leukocyte RNA was extracted from peripheral blood and/or bone marrow aspirate after lysis of red blood cells according to the standard protocol (Chromczynski *et* Sacchi, 1987). RNA was reverse transcribed to cDNA with Transcriptor Reverse Transcriptase (Roche Diagnostics, Mannheim, Germany) using random hexamers. Primers and TaqMan probe sequences published in the EAC network protocol were used for RQ-PCR (Gabert *et al*, 2003). Briefly, 100 ng cDNA was added to 2 μl Master Mix (LightCycler FastStart DNA Master Hybridization Probes; Roche Diagnostics, Mannheim, Germany) in 20 μl total reaction volume with final concentration of 4 mM $MgCl_2$, 300 nM primers and 200 nM TaqMan probe. The PCR program included initial denaturation step for 10 min at 95°C and 50 cycles (95°C, 10 sec, 20°C/s)/(60°C, 30 sec, 20°C/s). BCR-ABL and ABL assays were performed in duplicate. Mean crossing points (Cp) were used to interpolate standard curves and to calculate the transcript copy number.

Normalized levels were calculated as the BCR-ABL/ABL ratio and expressed as percentage. The standard curve was generated using serial dilutions of a linearized plasmid, containing BCR-ABL insert FGRS10 (Ipsogen, Marseille, France). The plasmid used to quantify the endogenous control gene ABL was CGRS01 (Ipsogen, Marseille, France). Results of RQ-PCR were set on International Scale (IS) with conversion factor 0.8261 estimated during European LeukaemiaNet external quality control. Major molecular response (MMR) was defined as BCR-ABL ≤ 0.1% IS (at least 1000 copies of ABL), corresponding to a 3 log reduction (Baccarani *et al*, 2009).

Measurement of imatinib plasma levels

Imatinib plasma concentrations were measured by ultra-performance liquid chromatography-tandem mass spectrometry assay (Mičová *et al*, 2010b) using the UHPLC UltiMate 3000 RS (Dionex, USA) coupled with the API 4000 tandem mass spectrometer (Applied Biosystems, USA). Samples were obtained from 1 to 96 hours after drug ingestion. In order to assess correlation between IPL and treatment response two sets of samples were analysed. First set consisted of 241 samples taken 24±2 hours after 400 mg given in one daily dose in 54 patients. Second set consisted of 329 samples taken 24±4 hours after imatinib ingestion in 84 patients. Both groups have been treated with imatinib for at least 18 months. From multiple measurements in individual patients, mean IPL were used for statistical assessment. Patients with BCR-ABL kinase domain mutations that caused the resistance to treatment were excluded from the first set. In order to perform statistical evaluation of measured data regression analysis, t-statistics and description statistics were applied.

2.4.3. Results

Characteristics of our cohorts of CML patients are depicted in Table 7. In both groups of patients used for evaluation of IPL and treatment response only 2 and 7 patients, respectively, did not achieve complete cytogenetic response and therefore the impact of IPL on probability of achievement of cytogenetic response could not have been estimated. 28 (52 %) and 46 (55 %) patients, respectively, achieved optimal cytogenetic

response according to the ELN criteria. Optimal molecular response according to the ELN recommedations was achieved in 20 (37 %) and 31 (37 %) patients from both sets, respectively.

A total of 1118 measurements were obtained by ultra-performance liquid chromatography-tandem mass spectrometry assay in 131 patients (79 males, 52 females) treated with various regimens ranging from 100 mg to 800 mg daily, interval between last dose administration and blood taking varied from 1 to 96 h and IPL reached 2 – 10800 ng/mL (Table 8).

In most patients IPL were measured at several ocassions: in 4 (3 %) patients only once; in 5 (4 %) patients twice, in 19 (14 %) patients 3 to 5 times, and in 103 (79 %) patients more that 5 times. Assessments of IPL were performed at routine check-ups at non-predefined time intervals from the beginning of the therapy. Selected 241 samples were taken 24±2 hours after 400-mg given in one daily dose from 54 patients and they were used for following analyses: relation between IPL of males (mean 954.13 ng/mL; SD = 342.81 ng/mL) and females (mean 941.95 ng/mL; SD = 334.68 ng/mL; P=0.898), relation between IPL and body weight (R^2 = 0.001), body surface area (BSA), (R^2 = 5.371E-05), dose per kilogram (R^2 = 0.002) and dose per square meter (BSA), (R^2 = 9.754E-05). No significant tendencies were observed.

Associations of IPL with age was also estimated and weak correlation (R^2 = 0.030) was found. We observed a high inter-individual variability (36 %) but in several cases also intra-individual variability (means between 9-33 %, n >5) of IPL. We confirmed a correlation of IPL with absolute dose (R^2 = 0.092; Fig. 22) and interval between ingestion and sampling (R^2 = 0.226, exponential correlation; Fig. 23). However, we were not able to demonstrate a clear correlation between IPL and MMR (P=0.673), IPL in patients with (n=40; 960.75±314.52, mean±SD) and without (n=14; 916.07±403.71) MMoR were almost equal each other.

98

Table 7: Baseline characteristics of patients' cohorts at sampling time of 24±2 h (A), 24±4 h (B) and 0–96 h (C)

	A	B	C
Number of patients – no. (%)	54 (100)	84 (100)	131 (100)
Male	32 (59)	50 (60)	79 (60)
Female	22 (41)	34 (40)	52 (40)
Age			
Median (yr)	56	57	57
Range (yr)	30 – 75	30 – 89	21 – 89
Body weight			
Median (kg)	74	75	75
Range (kg)	54 – 118	50 – 120	29 – 120
Body surface area			
Median (m^2)	1.87	1.90	1.89
Range (m^2)	1.55 – 2.39	1.5 – 2.44	1.02 – 2.44
Phase of disease at diagnosis			
Chronic phase	54 (100)	83 (99)	126 (96)
Accelerated phase	0 (0)	1 (1)	4 (3)
Blastic phase	0 (0)	0 (0)	1 (1)
Sokal score – no. (%)			
Low risk (< 0.8)	22 (41)	31 (37)	51 (39)
Medium risk (0.8-1.2)	19 (35)	34 (40)	47 (36)
High risk (> 1.2)	10 (18)	16 (19)	29 (22)
Unknown	3 (6)	3 (4)	4 (3)
Presence of BCR-ABL mutations	0 (0)	1 (1)	5 (4)
Presence of additional cytogenetic abnormalities	2 (4)	5 (6)	12 (9)
Imatinib dose			
400mg once daily	54 (100)	84 (100)	103 (78.5)
lower than 400mg daily	0 (0)	0 (0)	10 (7.5)
higher than 400mg daily	0 (0)	0 (0)	18 (14)
Patients pretreated with interferon-α – no. (%)	32 (59)	41 (49)	58 (44)

We also were not able to confirm any statistically significant difference between IPL in patients with optimal (888.4±344.4, mean±SD; n=25) and suboptimal (1083.2±400.0; n=13) treatment response (P=0.1261) and IPL in patients with treatment failure (935.2±245.3; n=16), (P=0.6400) according to the ELN when both criteria for cytogenetic and molecular response were applied simultaneously (Fig. 24). A separate comparison between IPL in

patients pretreated with interferon (n=32) who achieved MMoR (n=23) and those who did not achieve MMR (n=9) gained also statistically insignificant results despite certain difference in IPL (1022±83.42 and 836±336.74, median±medSD; P=0.960).

Table 8: Response to the treatment and IPL in the subgroup of patients at sampling time of 24±2 h (A), 24±4 h (B) and 0–96 h (C)

Response to the treatment	A		B		C	
	Number of patients – no. (%)	IPL ng/mL (min-max; median)	Number of patients – no. (%)	IPL ng/mL (min-max; median)	Number of patients – no. (%)	IPL ng/mL (min-max; median)
Group of the patients analyzed	54 (100)	396-2012; 874	84 (100)	139-3460; 967	131 (100)	2-10800; 1140
Cytogenetic response – no. (%)						
Complete (irrespective of time of achievement)	52 (96)	396-2012; 893	77 (92)	139-2957; 924	116 (89)	17-10800; 1935
Complete achieved within 12 months	28 (52)	396-2012; 894	46 (55)	139-2012; 967	64 (49)	2-5650; 1145
Not achieved	2 (4)	607-836; 721.5	6 (7)	607-2407; 1481	14 (11)	27-3732; 953
Molecular response – no. (%)						
Major (irrespective of time of achievement)	40 (74)	396-1710; 903	60 (71)	139-3460; 998	92 (70)	2-6770; 1100
Major achieved within 18 months	20 (37)	396-1608; 860	31 (37)	139-3460; 896	45 (34)	2-6770; 1070
Not achieved	14 (26)	503-2012; 808	24 (29)	473-2407; 900	39 (30)	27-10800; 1190
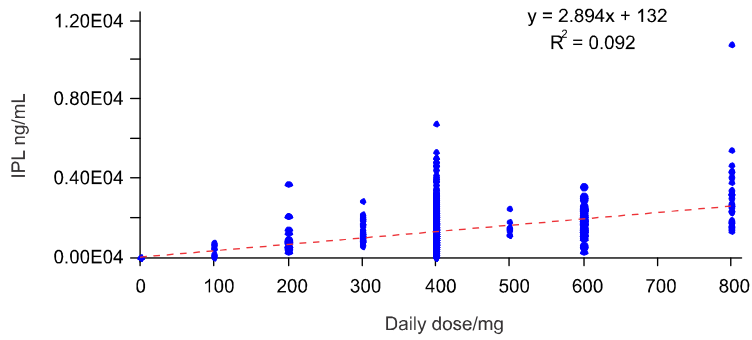
Figure 22: Relation between IPL and dose of imatinib (R^2 = 0.114).
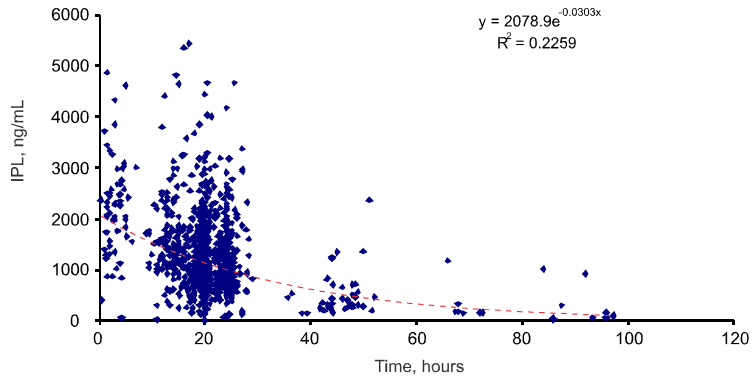
Figure 23: Correlation between IPL and interval between drug administration and blood sampling (n=1118 samples).

Finally, we performed analysis in the second set of samples (n=329) collected from 84 patients 24±4 hours after imatinib dose administration. Into this analysis also samples of patients with mutations in BCR-ABL kinase domain were included but other conditions for inclusion were not changed (a single dose of 400 mg per day and duration of the treatment at least 18 month). No correlation between IPL and body weight (R^2=0.0002), BSA (R^2=0.006), dose per kilogram (R^2=0.0002) and dose per square meter (R^2=0.005) was found. IPL in men and women did not differ significantly (P

= 0.456). A weak correlation was observed between IPL and age (P=0.131). lso in this extendend group of patients and samples we did not find any relation between IPL and MMR (P=0.761). Finally, we did not find any association between IPL and the treatment response according to the ELN (Fig. 25).

Figure 24: Comparison between IPL in patients with optimal (A; 888.4±344.4; n=25) and suboptimal (B; 1083.2±400.0; n=13) treatment response and al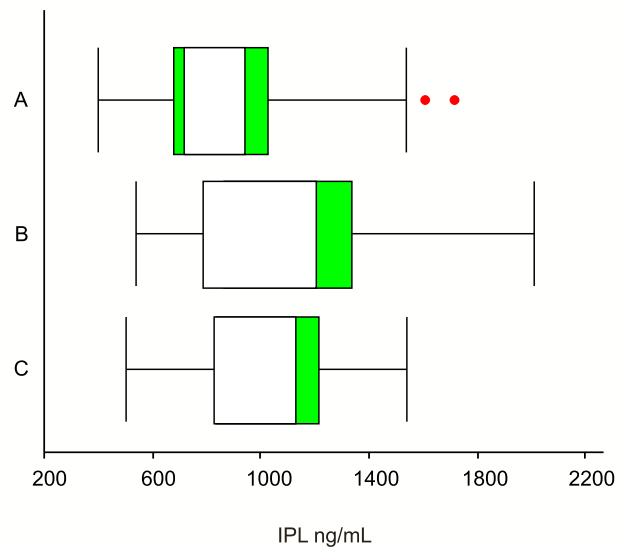so patients with treatment failure (C; 935.2±245.3; n=16) according to the ELN (54 patients; A vs B: P=0.126; A vs C: P=0.640).

102
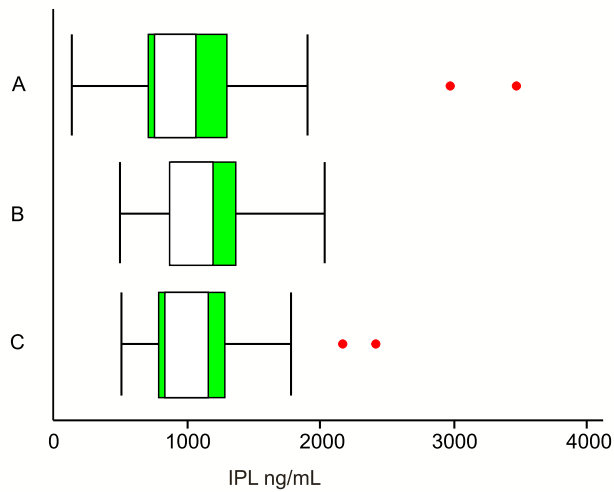
Figure 25: Comparison between IPL in patients with optimal (A; 1096.6±651.4; n=38) and suboptimal (B; 1098.4±404.6; n=22) treatment response and also patients with treatment failure (C; 1086.8±479.2; n=24) according to the ELN (84 patients; A vs B: P=0.990; A vs C: P=0.949).

2.4.4. Discussion

Picard *et al* showed for the first time that IPL may have a prognostic value in patients with CML treated with first-line imatinib (Picard *et al*, 2007). They measured IPL in 68 patients in chronic or accelerated phase CML treated with 400 or 600 mg of imatinib daily at unspecified time intervals from the start of imatinib treatment and they found significantly higher IPL in patients achieving CCR and MMR (Picard *et al*, 2007). Their observation was confirmed by Larson et al who reported results of a pharmacokinetic part of the IRIS study using day 29 IPL measurements and found IPL to be associated not only with CCR and MMR but also with significantly different progression-free survival of patients divided into four groups according to IPL (Larson *et al*, 2008). Both studies confirmed high inter-patient variability of IPL that may be influenced by various factors including drug absorption, different binding to plasma proteins, variability in metabolizing liver enzymes and patients' demographic factors (Cortes *et*

103

al, 2009). Also our team was able to confirm the correlation of IPL with the probability of achieving MMoR in a small group of patients (Faber *et al*, 2008). However, there are at least two other studies that found no correlation between IPL and patients' prognosis (Forrest *et al*, 2009; Davies *et al*, 2009). Despite speculations that IPL could assist with decisions about whether to increase an imatinib dose, IPL measurement is recommended as helpful in situations when poor adherence to treatment is suspected, severe site-effects are observed or drug interactions should be ruled out (Cortes *et al*, 2009). Further studies are awaited to confirm prospectively the link between IPL and response to treatment and to define effective trough concentrations in different patient populations (Cortes *et al*, 2009). No clear recommendation has been issued regarding IPL estimations in CML patients in the recent statement paper of ELN experts (Baccarani *et al*, 2009). Major overlap of IPL between patients with optimal and suboptimal response observed in our study but even in the studies that found significant difference in IPL between responding and failure patients represents a significant obstacle to the use of IPL for clear prognostic discrimination and for dosage adjustments in the routine clinical setting.

To our best knowledge, there is only one prospective study that uses low IPL for routine escalation of the dosage. Osborn and coworkers published the interim results of the TIDEL II prospective study that used IPL measured at day 22 of imatinib therapy in 74 CML patients for subsequent dosage adjustment (Osborn *et al*, 2009). In 11 (15%) patients IPL lower than 1000 ng/mL was found and 7 (9.5%) of them were able to escalate the imatinib daily dose to 800 mg. These patients achieved mean IPL comparable with the rest of the group at 3 and 6 months and their molecular response also improved (Osborn *et al*, 2009). We were able to identify 7 patients with IPL lower than recommended concentration (<1000 ng/mL) among 13 patients with suboptimal treatment response according to ELN from 54 patients treated with first-line imatinib at our center since 2004. Furthermore, only 8 patients in a group with optimal treatment response (n=25) achieved IPL higher than 1000 ng/mL. Only in the case of two patients we escalated therapeutic dose of imatinib and obtained an increase of IPL and also optimal treatment response was achieved (Faber *et al*, 2010a).

Our results might have been influenced by the number of patients analysed including lower proportion of suboptimal or failure patients. Irrespective to that limitation, we believe that our failure to demonstrate the correlation between IPL and molecular response to imatinib is caused by difference in patients' compliance, leukaemia biology and other variables that are difficult to eliminate in routine clinical practice. IPL may be used for identification of patients with poor compliance or in cases of excluding prominent toxicity but their use for prognostic estimate outside the clinical trials might be limited.

Our observed data confirmed correlation between IPL and absolute dose as also reported in several studies (Picard *et al*, 2007; Ishikawa *et al*, 2010; Sakai *et al*, 2009). These data do not provide any additional information useful for management of CML treatment of patients with suboptimal response.

2.4.5. Acknowledgements

2.5. Detailed study of imatinib metabolism using high-resolution mass spectrometer Orbitrap Elite.

David Friedecký[1,2], Kateřina Mičová[1], Edgar Faber[3], Marcela Hrdá[1], Tomáš Adam[1,2,4]

[1] *Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacký University in Olomouc*
[2] *Laboratory of Inherited Metabolic Disorders, Faculty of Medicine and Dentistry, Palacký University in Olomouc, University Hospital Olomouc*
[3] *Department of Hemato-Oncology, Faculty of Medicine and Dentistry, Palacký University in Olomouc, University Hospital Olomouc*
[4] *Department of Clinical Chemistry, University Hospital Olomouc*

Aims: We focused on imatinib metabolite profiling in plasma and leukocytes of patients with chronic myeloid leukaemia. This study demonstrates the capability of HRMS for finding and identification of metabolites in plasma. Our study was performed on LTQ Orbitrap Elite instrument with resolution power > 240,000 FWHM. This hybrid MS combine high-field Orbitrap analyser with the dual-pressure linear ion trap, therefore parallel MS and MSn analysis using multiple fragmentation techniques (CID, HCD and optional ETD) are allowed.

2.5.1. Abstract

Therapeutic drug monitoring is widely applied useful tool for treatment individualization in order to achieve optimal clinical response and avoid toxicity. Due to drug metabolizing enzymes several bioactive or toxic metabolites are produced. Therefore determination of parent drug and also of metabolites may have clinical relevance and could be helpful for treatment adjustment. In this work imatinib metabolite profiling in plasma of patients with chronic myeloid leukaemia was performed. Separation proceeded on Phenomenex Kinetex C18 column (100 x 2.1 mm; 1.7 μm) using UltiMate 3000 RS (Thermo Scientific) liquid chromatography with mass spectrometry detection using Orbitrap Elite instrument (Thermo Scientific) based on exact mass measurement. Scan range of *m/z* 350 – 1200 was chosen and the resolution was set at 60,000 FWHM. For confirmation of metabolite identities characterized by *m/z* values, MS^2 exact mass fragmentation of phase I metabolites and MS^3 fragmentation of phase II metabolites (conjugates) in orbitrap mass analyser were performed. All measurements were performed with mass accuracy < 5 ppm. Data were evaluated using Excalibur 2.2 SP1, MetWorks 1.3 SP3 and Mass Frontier 7.0 software. In plasma samples 90 metabolites in concentration range of 0.1 nmol/L - 1 μmol/L were found.

2.5.2. Material

Imatinib mesylate was purchased from LC Laboratories (Woburn, MA, USA) and deuterated standard (D8-imatinib) from TLC PharmaChem (Vaughan, Ontario, Canada). Formic acid, ammonium hydroxide, water, dimethylsulfoxide (DMSO), acetonitrile and methanol were all LC/MS grade and were purchased from Sigma (St. Louis, MO, USA). Plasma samples of patients were collected into Vacuette® Blood Collection Tube, K_3EDTA, pull cap, 9 ml in Hemato-Oncology Clinic, University Hospital Olomouc (Czech Republic).

2.5.3. Sample preparation

This study was conducted in accordance with the Helsinki Declaration, and the protocol was reviewed by the hospital ethics committee. All the patients who were examined gave their informed consent to participation in the study before the blood sampling. The blood from patients treated with IM was transfered into a test tube containing an anticoagulant (EDTA). Plasma was separated from the whole blood by centrifugation at 3,000 g for 5 minutes. Plasma (50 µL) was precipitated by methanol (150 µL) with addition of an internal standard D8-Imatinib (100 ng/mL). Subsequently, samples were put into a sonicator for 1 min, shaken for 1 min, freezed for 30 min at -20 °C, and centrifuged for 15 min at 14,300 g. The supernatant was placed into glass vial and directly analysed or stored in a freezer at -20 °C before analysis. As a blank sample pooled plasma from healthy adults was prepared identically.

2.5.4. Methods

<u>Samples of patients</u>

Overall 37 plasma samples from patients with different imatinib daily dosage and sampling time from last dose were analysed. For further experiments and data evaluation eight patients with dose of 400 mg/day and sampling time of 24±4 hours after last dose (steady state) were chosen.

<u>LC-MS system</u>

LC-MS system consisted of an UltiMate 3000 RS (ThermoFisher Scientific, San Jose, CA, USA) coupled with LTQ Orbitrap Elite mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA). Mass spectrometer was controlled by Xcalibur 2.2 SP1 software. Chromatographic system was controlled by Chromeleon Xpress and DCMSLink 2.12 software.

<u>Chromatographic conditions</u>

Separation was performed on a Phenomenex Kinetex C18 column (100 x 2.1 mm; 1.7 µm; Torrance, CA, USA) at 40°C. 10 uL of sample was injected on the column. The temperature of autosampler was set at 10°C. For imatinib metabolites separation three mobile phases were examined: 4 mM ammonium formate pH 3.2; 10 mM ammonium formate pH 4.0 and 20 mM ammonium acetate pH 9.0 as mobile phase A and acetonitrile as mobile phase B. Separation efficiency and signal intensity depending on the degree of ionization were studied. The best results were obtained using 10 mM ammonium formate pH 4.0 which was chosen for the further experiments (data not shown). Gradient elution was applied at the flow rate 0.4 mL/min. The column was maintained at initial conditions of 5 % B for 3 min, followed by a linear gradient to 40 % over 10 minutes, then from 40 to 95 % over 0.5 min, held at 95 % B till 18 minute and then decreased during 0.5 minute to 5 % B and equilibrated at initial conditions till the end of the analysis. Total analysis time was 20 minutes.

Mass spectrometry conditions

   LTQ Orbitrap Elite operated in positive heating electrospray ionization mode. The mass spectrometer ion source parameters were adjusted as follows: electrospray voltage of + 1.5 kV, ion source heater temperature of 360°C, capillary temperature of 350°C, flow rates of 35, 10 and 0 arbitrary units for sheath, auxiliary and sweep gas, respectively; S-lens RF level was set to 60%. Three different settings were applied. Search for molecular ions of metabolites was performed in full scan mode ranging from m/z 350 – 1200 at the resolution of 60,000 FWHM, data frequency of 2 Hz and the maximum injection time of 100 ms. For confirmation of metabolite identities MS^2 exact mass fragmentation of phase I metabolites and MS^3 fragmentation of phase II metabolites (conjugates) in orbitrap mass analyser were performed. Energy for collision induced dissociation (CID) was set at 35 eV with the isolation width of m/z 1. Fragmentation spectra were collected within m/z 135 – 800 at resolution 30,000 FWHM. The mass spectrometer was externally calibrated from 100–2000 Da using a Pierce® LTQ Velos ESI Positive Ion Calibration Solution (ThermoFisher Scientific). The ion m/z 391.2843 (diisooctylphtalate) was used as an internal lock mass to provide exact mass accuracy bellow 5 ppm for all analyses. List of potential metabolites was designed by MetWorks 1.3 SP3 software (ThermoFisher Scientific, Table 11). Identification of fragments and structure elucidation was processed in Mass Frontier 7.0 software (ThermoFisher Scientific).

Table 11: Listof metabolites found in plasma of patients treated by IM. Each biotransformation product is described by molecular formula [M+H]⁺ and exact *m/z* modification formula with corresponding mass shift and number of metabolite observed in chromatographic profile (see Fig. 28, 29)

Modification	Modification	Formula	m/z	m/z shift	Metabolite
Imatinib		C29H32N7O+	494.2663		
Imatinib + glucuronidation	+C6H8O6	C35H40N7O7+	670.2984	+176.0321	1-3
Demethylation	-CH2	C28H30N7O+	480.2506	-14.01565	4
Demethylation + glucuronidation	-CH2+C6H8O6	C34H38N7O7+	656.2827	+162.0164	5
Oxidation	+O	C29H32N7O2+	510.2612	+15.9949	6-16
Oxidation + glucuronidation	+O+C6H8O6	C35H40N7O8+	686.2933	+192.0270	17-21
Demethylation + oxidation	-CH2+O	C28H30N7O2+	496.2455	+1.9793	22-33
Demethylation + oxidation + glucuronidation	-CH2+O+C6H8O6	C34H38N7O8+	672.2776	+178.0114	34-36
Dioxidation	+2O	C29H32N7O3+	526.2561	+31.9898	37-49
Dioxidation + glucuronidation	+2O+C6H8O6	C35H40N7O9+	702.2882	+208.0219	50-57
Desaturation	-H2	C29H30N7O+	492.2506	-2.0157	-
Demethylation + desaturation	-CH2-H2	C28H28N7O+	478.2350	-16.0313	58
Desaturation + oxidation	+O-H2	C29H30N7O2+	508.2455	+13.9793	59-61
Desaturation + oxidation + glucuronidation	+O-H2+C6H8O6	C35H38N7O8+	684.2776	+190.0114	62-44
Dioxidation + desaturation	+2O-2H	C29H32N7O3+	524.2405	+29.9742	65-74
Dioxidation + desaturation + glucuronidation	+2O-2H+C6H8O6	C35H38N7O9+	700.2726	+206.0063	75
Oxidation + hydrolysis	+H2O2	C29H34N7O3+	528.2718	+34.0055	76-77
Oxidation + hydrolysis + glucuronidation	+H2O2+C6H8O6	C35H42N7O9+	704.3039	+210.0376	78
Methylation	+CH2	C30H34N7O+	508.2819	+14.0157	79
Oxidation + methylation	+CH2O	C30H34N7O2+	524.2768	+30.0106	80
Demethylation + desaturation + oxidation	+O-CH2H2	C28H28N7O2+	494.2299	-0.0364	81-84
Demethylation + desaturation + oxidation + glucuronidation	+O-CH2H2+C6H8O6	C34H36N7O8+	670.2620	+175.9957	85-87
Glycine conjugation	-OH+C2H4NO2	C31H35N2O2+	551.2877	+57.0215	88
Glucuronidation + decarboxylation	+C5H8O5	C34H40N7O6+	642.3035	+148.0372	89-90

2.5.5. Results and discussion

<u>Analysis of Glivec tablet and standard powder</u>

In a first part of experiment a Glivec tablet and standard powder of imatinib and were analysed. In order to determine metabolites and minor impurities from synthesis the samples were dissolved by methanol to final concentration of 10 μg/mL that is one order more compared to concentration in plasma from CML patients (sample taking 24h after dose of 400mg/day). Using Metworks 1.3 software several peaks corresponding to oxidation (+O), desaturation (–2H), desaturation+oxidation (+O-2H), methylation (+CH2) and demethylation (-CH2) of imatinib were observed (Fig. 27).
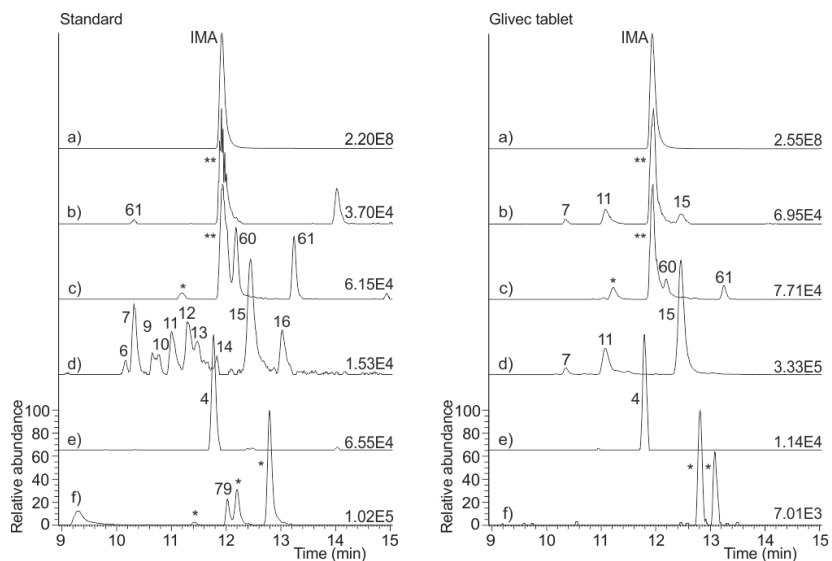
Figure 27: Metabolite profiles of IM standard and Glivec tablet. Analyses represent extracted ion chromatograms of selected metabolisations a) parent drug with *m/z* 494.2663, b) desaturation with *m/z* 492.2506, c) desaturation+oxidation with *m/z* 508.2455, d) oxidation with *m/z* 510.2612, e) demethylation with *m/z* 480.2506 and f) methylation with *m/z* 508.2819. * - metabolites not present in plasma sample from patients with CML; ** - metabolites generated in ion source from IM.

In Figure 27 (Line I) extracted ion chromatograms of the parent drug three orders more abundant compared to impurities are shown. Thus, the content of observed impurities from synthesis is less than 0.1 %.

Three peaks (No. 7, 11 and 15) representing oxidative impurities were present in Glivec tablet compared to ten peaks with one order less abundance in standard powder (Figure 27, Line IV). Based on retention times and fragmentation spectra these impurities were identical with metabolites detected in plasma.

Line II and III in Figure 27 represent extracted ion chromatograms of desaturation and desaturation+oxidation. The major peaks in RT 11.87 (marked **) were found in all analysed samples (including plasma from patients) with consistent intensity ratio to IM. The ion source fragmentation is unlikely to apply because the exact masses of metabolite molecular ions are not the part of IM fragmentation spectra. This suggests a theory of electrochemical process of desaturation and desaturation+oxidation of IM in ion source. This phenomenon was verified by analysis under different separation conditions (pH of mobile phase adjusted to 3, 4 and 5; data not shown) where IM, desaturated (m/z 492.2506) and desaturated+oxidized (m/z 508.2455) compounds eluted in the same retention time with the same intensity ratios. Three peaks No. 7, 11 and 15 (Line b, figure 27) found in Glivec tablet are result of ion source fragmentation of oxidative metabolites (Line d, figure 27). The fragmentation spectra of these metabolites contain m/z of 492.2506 as one of the main fragments and they were verified by experiments under different pH of mobile phase (data no shown).

In addition, in Glivec tablet two desaturated+oxidized (No. 60 and 61), one demethylated (No. 4) and two unidentified methylated (marked by asterisk) impurities were present. Standard powder differs from Glivec tablet especially in profiles and intensities of oxidative, demethylated and methylated metabolites (Lines IV, V and VI, Figure 27). All of these compounds were confirmed by fragmentation spectra measured in orbital ion trap with high resolution and accuracy and fragments were identified by Mass Frontier 7.0 software.

Metabolite profile of plasma samples from patients with CML

Eight plasma samples from patients on standard therapy by 400 mg per day were taken into analysis. In extracted ion chromatograms generated by software Metworks 1.3 more than 180 unique chromatographic peaks in the range 8.14 and 13.24 min with specific *m/z* values (Table 11) were found. For subsequent evaluation peaks as products of ion source fragmentation and electrochemical conversion (see chapter 2.5.5) and peaks with very low intensity observed in less than a half of plasma samples were excluded. Finally ninety detected peaks corresponding to first and second phase metabolites were taken for subsequent confirmation of identity and calculations (Table 11). The intensity ratios of the metabolites compared to IM (100 %) in plasma samples were in the range of 0.0008 - 6.6300 % with average metabolite intensities of $2x10^2$ - $1.5x10^6$ and $2.3x10^7$ of IM (Table 12). Based on these results scheme of metabolisation was designed (Figure 28). The calculated concentrations for all metabolites derived from concentration of IM in plasma were in range 0.1 nmol/L - 1 µmol/L.

Table 12: Fragmentation profile of the metabolites compared to IM (100 %) in plasma samples.*calculated *m/z* deduced from the structure but not found by Mass Frontier 7.0 software; ** *m/z* without structure proposed by Mass Frontier 7.0 software

Met	tr	Fragmentation (intensity, %)	Reative abundance	Ratio (%)
IM	11.90	394.1662 (100); 217.1335 (5); 476.2557 (2); 189.1386 (0.6); 396.1819 (0.4); 174.0913 (0.1)	2,32E+07	100,0000
1	8.49	394.1662 (100); 476.2557 (7); 217.1335 (5)	2,23E+03	0,0096
2	8.62	394.1662 (100); 217.0974* (6); 217.1335 (5); 476.2185* (2); 476.2557 (1); 396.1819 (1); 189.1386 (0.7);	2,48E+05	1,0686
3	11.15	394.1662 (100); 217.1335 (4)	1,63E+04	0,0704
4	11.72	394.1662 (100); 203.1179 (7); 462.2401 (2); 396.1819 (0.6); 175.123 (0.4)	1,54E+06	6,6303
5	8.45	394.1662 (100); 203.1179 (6); 462.2401 (1)	1,74E+04	0,0751
6	10.12	410.1612 (100); 492.2506 (35); 392.1509 (5); 395.1741 (4); 394.1662 (3); 217.1335 (2); 393.1584 (1); 423.1928 (0.9); 450.2163 (0.7); 277.1084 (0.6); 277.1322 (0.6); 189.1386 (0.5)	1,92E+05	0,8282
7	10.26	492.2506 (100); 410.1612 (7); 480.2506 (0.4); 392.1506 (0.4); 395.1741 (0.2); 217.1335 (0.2);	9,64E+04	0,4155

		276.1244 (0.1); 394.1662 (0.1); 393.1584 (0.1)		
8	10.33	410.1612 (100); 492.2506 (13); 217.1335 (5); 189.1386 (0.5); 412.1768 (0.5); 412.4606** (0.1); 394.1662 (0.1); 493.2357** (0.1); 467.2557 (0.1); 407.8825** (0.1); 392. 1506 (0.1); 174.0925** (0.1); 453.2034 (0.1); 479.219 (0.1)	6,38E+04	0,2749
9	10.62	410.1612 (100); 217.1335 (5); 412.1768 (5); 453.2034 (3); 492.2506 (2); 424.1766** (1); 479.219 (0.9); 413.1846 (0.9); 393.1345 (0.8); 222.0915** (0.8); 189.1386 (0.7); 392.1506 (0.3)	1,53E+05	0,6587
10	10.75	410.1612 (100); 217.1335 (35); 492.2506 (25); 413.1846 (13); 189.1386 (5); 412.1768 (4); 321.2074 (3); 215.1183* (3); 174.0913 (1); 453.2034 (1); 424.1774** (1); 187.1232 (0.7); 294.1349 (0.5); 493.2363** (0.4); 398.1612 (0.3)	1,06E+05	0,4572
11	10.91	394.1662 (100); 395.1741 (96); 492.2876** (3); 392.1506 (0.4); 396.1819 (0.3)	1,78E+04	0,0766
12	11.22	410.1612 (100); 395.1741 (21); 394.1662 (19); 492.3321** (9); 492.2506 (8); 217.1335 (7); 424.1769** (2); 492.2119** (2); 412.1768 (2)	5,41E+03	0,0233
13	11.44	410.1612 (100); 492.2754** (22); 392.1506 (15); 217.1335 (9); 501.1716** (2); 423.2354** (1); 394.1662 (1); 189.1386 (0.9)	6,40E+03	0,0276
14	11.79	410.1612 (100); 492.3325** (7); 392.1506 (5); 394.1662 (5); 395.1741 (4); 492.2506 (3); 217.1335 (2); 492.2995** (2); 466.2864** (2); 353.1847** (2)	1,56E+03	0,0067
15	12.31	450.2163 (100); 423.1928 (85); 492.2506 (70); 393.1584 (68); 410.1612 (68); 395.1741 (52); 409.1911** (42); 394.1662 (29); 436.2006 (19); 437.2084 (13); 392.1506 (9); 451.2241 (8); 449.2084 (7); 463.2241 (6); 338.1863 (5); 464.2334** (5); 410.1846** (5); 421.1935** (4); 408.1852** (4); 217.1335 (4); 493.2585 (4); 478.235 (4); 435.1928 (3); 189.1386 (3)	2,33E+05	1,0054
16	12.95	278.1400 (100); 410.1612 (63); 233.1285 (30); 492.3327 (22); 492.2506 (15); 450.2163 (12); 423.1928 (10); 393.1584 (9); 395.1741 (9); 409.1897** (6); 236.1498** (5); 394.1662 (5); 423.2157** (4); 350.2304** (3); 451.2082** (2); 436.2006 (2); 492.3009 (1)**; 217.1335 (1)	3,55E+03	0,0153
17	8.52	410.1612 (100); 217.1335 (76); 492.2506 (33); 189.1386 (10)	2,53E+03	0,0109
18	8.77	492.2506 (100); 410.1612 (76); 450.2163 (53); 423.1928 (51); 393.1584 (39); 395.1741 (33)	4,17E+03	0,0180
19	9.24	410.1612 (100); 217.1335 (6); 492.2506 (3)	1,58E+04	0,0682
20	9.41	410.1612 (100); 217.1335 (6); 492.2506 (2)	2,26E+05	0,9744
21	9.74	410.1612 (100); 217.1335 (7); 492.2506 (5); 412.1768 (5); 453.2034 (4); 392.2486** (3); 424.1766** (2); 189.1386 (1); 413.2083** (1);	3,03E+03	0,0130

115

		479.2190 (1)		
22	9.97	410.1612 (100); 478.2350 (40); 392.1506 (12); 395.1741 (5)**; 479.2778** (3); 203.1179 (3); 479.2428(2); 394.1662 (2)	1,20E+04	0,0517
23	10.09	478.2350 (100); 410.1612 (62); 392.1506 (11); 214.1453** (2)	3,35E+03	0,0144
24	10.16	410.1612 (100); 478.2350 (4); 203.1179 (3); 409.1630** (2); 392.1516 (12); 479.2776** (1); 487.7917** (1)	2,41E+03	0,0104
25	10.35	395.1741 (100); 410.1612 (87); 394.1662 (70); 409.1632** (39); 437.1575** (14); 412.1768 (8); 203.1179 (8); 184.0741** (7); 453.2034 (6)	7,32E+03	0,0315
26	10.45	410.1612 (100); 395.1741 (16); 394.1662 (10); 412.1768 (8), 203.1179 (7); 453.2034 (4); 478.2350 (3); 214.1446** (3); 396.1455 (2); 479.2843** (2); 487.7916** (2); 479.219 (2)	1,74E+04	0,0751
27	10.61	410.1612 (100); 203.1179 (35); 478.2350 (28); 214.1442** (18); 413.1846 (7); 303.1803** (6); 479.2858** (3); 412.1768 (3); 396.1441** (2); 347.2074** (2); 392.1506 (9); 440.2067** (2)	6,98E+03	0,0301
28	10.67	410.1612 (100); 347.1244** (38); 478.7863** (33); 409.1631** (24); 479.2792 (14); 184.0746** (13); 203.1179 (11); 349.1407** (10); 392.1506 (8)	5,22E+02	0,0022
29	11.08	478.2350 (100); 410.1612 (25); 394.1662 (4); 396.1725** (3); 478.3244** (1); 409.1631** (1)	1,64E+03	0,0071
30	11.15	478.2350 (100); 410.1612 (4); 394.1662 (2); 396.1725** (1)	2,81E+03	0,0121
31	11.27	478.2350 (100); 410.1612 (16); 437.1342** (7); 478.1986 (3); 392.1506 (2); 203.1179 (2); 450.2736** (2); 301.1194** (2); 409.1643** (2); 440.2058** (1)	2,85E+03	0,0123
32	11.40	478.2350 (100); 218.1011** (20); 217.0974 (15); 219.1040** (8); 410.1612 (8); 396.1732** (7); 395.1698** (3); 394.1612** (3); 184.0740** (2); 478.1986 (1)	1,34E+03	0,0058
33	11.60	no fragmentation	2,81E+02	0,0012
34	9.11	410.1612 (100); 203.1179 (8)	5,99E+02	0,0026
35	9.27	410.1612 (100)	1,44E+04	0,0619
36	9.62	no fragmentation	3,04E+02	0,0013
37	8.85	508.2455 (100); 526.2913** (25); 426.1561 (18); 292.1193 (13); 516.7818** (intenzita)	5,14E+02	0,0022
38	9.15	508.2455 (100); 426.1561 (2); 526.2920** (2); 482.1669** (1)	1,30E+03	0,0056
39	9.27	508.2455 (100); 426.1561 (7); 411.169 (3); 439.2306** (1); 526.2909** (1); 217.1335 (1); 426.2402** (1)	3,20E+03	0,0138
40	9.41	508.2455 (100); 426.1561 (13); 411.169 (8); 526.2902** (6); 392.1506 (6); 508.3254** (5); 217.1335 (2); 439.2286 **(2)	1,28E+03	0,0055

41	9.79	411.169 (100); 439.1877 (66); 466.2112 (40); 508.3271** (36); 508.2455 (34); 452.1955 (14); 236.1501** (9); 410.1612 (7); 526.2913** (7); 508.2091 (6); 426.1561 (6); 479.219 (5); 453.2034 (5); 482.2299 (2); 451.1877 (2); 506.3148** (2); 438.1799 (1)	5,52E+02	0,0024
42	10.15	426.1561 (100); 508.2455 (41); 508.3274** (38); 392.1506 (24); 408.1460** (17); 217.1335 (15); 236.1500** (9); 380.2084** (5); 509.2278** (3); 526.2908** (3); 439.1877 (6)	1,52E+03	0,0066
43	10.44	492.2506 (100); 508.3274** (46); 508.2455 (29); 439.1877 (27); 394.1662 (25); 410.1612 (24); 411.169 (24); 494.2299 (22); 426.1561 (21); 491.2428 (15); 449.2084 (11); 408.1454** (10); 464.2193 (9); 236.1493** (8); 526.2932** (7); 466.2112 (5)	2,62E+03	0,0113
44	10.73	508.2455 (100); 426.1561 (32); 508.3270** (26); 439.1877 (20); 509.3060** (17); 466.2112 (12); 410.1612 (11); 490.235 (9); 409.1534** (7); 501.7536** (7); 236.1503** (7); 425.1857** (5); 383.1504** (5); 411.169 (5); 498.2616** (3); 217.1335 (3); 494.2324** (2); 398.1612 (2); 526.2918** (2)	2,40E+03	0,0104
45	10.92	439.1877 (100); 508.2455 (67); 466.2112 (55); 411.169 (33); 508.3262 (23); 452.1955 (21); 426.1561 (12); 479.219 (7); 453.2034 (9); 451.1877 (8); 517.3743** (5); 236.1496** (5); 409.1523** (4); 410.1612 (4); 526.2899** (4); 438.1799 (3); 465.2019** (3); 494.2299 (2)	3,17E+03	0,0137
46	11.09	508.2455 (100); 439.1877 (41); 466.2112 (29); 426.1561 (24); 508.3260** (18); 411.169 (18); 217.1335 (8); 479.219 (7); 429.1784** (6); 452.1955 (5); 424.1768** (5); 410.1612 (4); 188.1308 (3); 453.2034 (9); 412.1679** (3); 467.2242** (2); 236.1493 (2); 526.2905** (2)	1,85E+03	0,0080
47	11.26	508.2455 (100); 428.2811** (68); 508.3300** (61); 426.1561 (21); 526.2926** (17)°; 439.1877 (12); 353.2473** (11); 451.2155** (10); 517.3699** (10); 517.8688** (8)	3,39E+02	0,0015
48	12.67	508.2455 (100); 394.1662 (48); 494.2299 (31); 509.2302** (11); 508.3252** (9); 409.1900** (6); 468.2506 (4); 410.1980** (4); 184.0739** (4); 480.2421** (3); 483.2152** (3); 482.2673** (2); 236.1506 (2)	6,74E+03	0,0291
49	12.91	508.2355** (100)	3,27E+03	0,0141
50	8.25	426.1561 (100); 508.2455 (98)	3,82E+03	0,0165
51	8.30	426.1561 (100); 217.1335 (8); 508.2455 (4)	5,61E+03	0,0242
52	8.59	no fragmentation	4,28E+02	0,0018
53	8.80	no fragmentation	2,68E+02	0,0012
54	9.04	426.1561 (100); 392.2333** (30)	6,02E+02	0,0026

55	9.22	no fragmentation	6,39E+02	0,0028
56	9.57	no fragmentation	5,88E+03	0,0253
57	9.91	no fragmentation	2,40E+02	0,0010
58	11.28	394.1662 (100), 461.2850** (5)	9,76E+02	0,0042
59	10.72	394.1662 (100); 408.1463** (95); 490.235 (27); 249.1238** (12), 374.1482** (10)	2,12E+03	0,0091
60	12.03	231.1130 (100); 394.1662 (20); 490.235 (17); 480.2506 (4)**; 203.1179 (4)**; 249.1239* (3), 411.2060** (3)	1,40E+05	0,6045
61	13.12	394.1662 (100); 231.1133 (10); 249.1239* (9); 396.1819 (8); 490.235 (6); 395.1741 (3)**; 480.2506 (3)**; 203.1179 (1)**	4,58E+05	1,9753
62	8.29	no fragmentation	2,77E+02	0,0012
63	8.90	231.1129 (100); 490.235 (18); 394.1662 (15); 203.1179 (5);** 175.123 (4); 480.2506 (4)**	1,81E+04	0,0779
64	9.21	394.1662 (100); 231.1133 (10); 249.1239* (8); 396.1819 (8); 490.235 (6); 395.1742 (3); 480.2506 (2); 203.1179 (1)	3,01E+04	0,1298
65	10.17	no fragmentation	9,63E+02	0,0041
66	10.84	no fragmentation	5,07E+02	0,0022
67	11.03	506.2311* (100); 424.1414* (6); 480.2506 (2); 410.1612 (2)	3,71E+03	0,0160
68	11.11	506.2314* (100); 410.1612 (10); 392.1506 (6); 424.1417* (5); 395.1741 (3)**; 480.2506 (2)	3,87E+03	0,0167
69	11.44	506.2314* (100); 424.1795** (55); 365.2224** (6); 506.2770** (4); 337.1908** (4); 367.2032** (2); 353.1865** (2); 350.2320** (2); 524.3521** (2); 351.2352** (1); 410.1612 (1)	1,81E+03	0,0078
70	11.59	no fragmentation	4,86E+02	0,0021
71	11.85	no fragmentation	7,49E+02	0,0032
72	12.61	478.235 (100); 265.1200** (59); 394.1662 (20); 506.3619** (20); 506.2288** (18); 396.1750** (13); 246.0975** (10); 245.0934** (8); 337.2780** (7); 247.0978 (7); 395.1719** (6)	4,49E+03	0,0194
73	12.70	478.235 (100); 265.1201** (55); 394.1662 (17); 506.2314 (16)*; 506.3627** (10); 350.2326** (7); 496.2487** (7); 336.2749** (6); 337.2779** (6); 247.1100** (4); 321.2828** (4)	2,18E+03	0,0094
74	12.83	506.2314* (100); 478.235 (94); 506.3626** (47); 265.1184** (42); 336.2732** (31); 394.1662 (19); 350.2282** (18); 337.2742** (16); 365.2243** (16); 350.2344 **(13); 337.2804** (11)	3,64E+03	0,0157
75	9.21	no fragmentation	1,97E+02	0,0008
76	8.33	no fragmentation	2,85E+02	0,0012
77	10.57	510.2612 (100); 278.1504** (5); 296.1592** (2); 392.1506 (2); 484.2577** (2); 354.2826** (1)	8,40E+02	0,0036
78	9.60	no fragmentation	3,34E+02	0,0014
79	12.00	231.1130 (100); 394.1662 (28); 490.2350 (19); 480.2506 (5); 203.1179 (4); 175.1230 (4); 411.2065** (3); 396.1819 (3); 400.1754** (2);	2,43E+04	0,1046

|				
		231.0779** (2); 275.0935** (1); 408.1819 (1)		
80	11.47	506.2314* (100); 424.1780 (56); 410.1612 (2); 337.1906** (2); 506.3600 (2); 350.2320** (2); 506.3227** (1); 426.1931** (1); 506.2699** (1); 492.1359** (1); 467.219 (1); 506.2799** (1)	1,28E+03	0,0055
81	10.88	305.1498** (100); 394.1612 (40); 273.1241** (28); 450.2369** (18); 476.2193 (15)	3,71E+03	0,0160
82	11.37	217.0971 (100); 476.2193 (14); 189.1021 (2); 218.1005** (1); 323.1721** (1); 161.1084** (1); 423.1928 (1); 475.2305** (1)	1,55E+04	0,0667
83	12.47	394.1662 (100); 217.1335 (25); 235.1082 (20), 396.1819 (8); 395.1741 (6); 476.2390** (6); 466.2347 (4)	1,20E+05	0,5190
84	12.84	423.1928 (100); 466.235 (96);	4,00E+03	0,0172
85	8.19	217.0974 (100)	3,69E+02	0,0016
86	8.57	394.1662 (100); 217.0973 (75); 476.2193 (15)	6,04E+03	0,0260
87	8.74	394.1662 (100); 396.1819 (8)**; 217.0973 (8); 476.2193 (5); 395.1741 (5)**; 235.1079 (4); 466.235 (3); 217.1335 (2)	1,16E+04	0,0502
88	12.11	508.2455 (100)**; 480.2506 (6); 490.2356** (2); 478.2350 (1); 396.1819 (0.7); 394.1662 (0.7); 157.1221** (0.3); 451.0915** (0.2); 203.1179 (0.2); 353.1835** (0.1)	4,24E+04	0,1828
89	9.36	510.2612 (100); 391.9699 (53)	2,22E+03	0,0096
90	10.62	no fragmentation	2,58E+03	0,0111

119
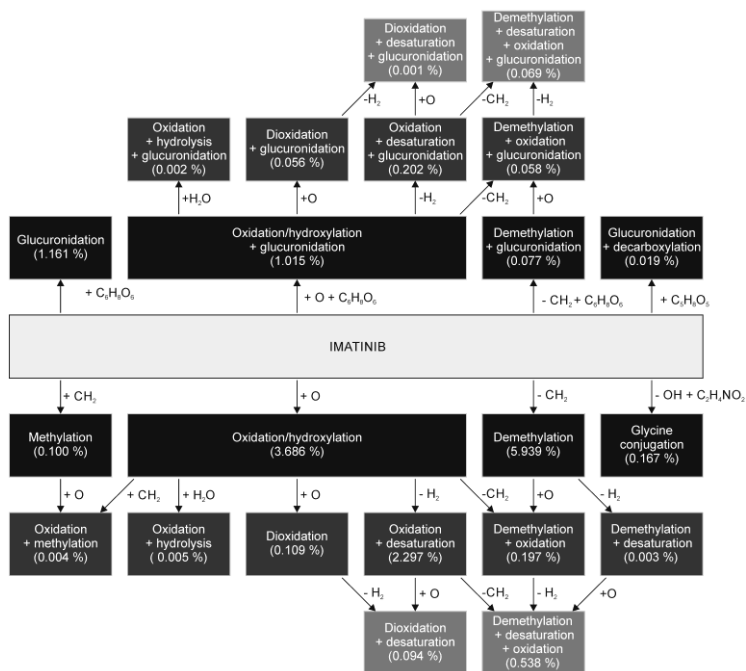
Figure 28: Designed scheme of IM metabolisation.

Majority of polar metabolites elute earlier than IM as expected in reverse phase liquid chromatography system (Figure 29 and 30) however four oxidative metabolites (no. 15, 16, 48 and 49) were more retained compared to IM. These metabolites offer significantly different fragmentation spectra in comparison with other oxidative metabolites no. 6-14 and 37-47 and are in according to previously published results (Marull *et* Rochat, 2006). Glucuronides as representatives of second phase metabolites offer weaker retention due to higher polarity of glucuronide part. Retention time shift correlations between metabolites and corresponding glucuronides for IM (no. IM/1-3), demethylation (no. 4/5), oxidation (no. 6-16/17-21), demethylation+oxidation (no. 22-33/34-36) and dioxidation (no. 37-49/50-57) were observed (Figure 29). Metabolite profiles of selected biotransformations were surprisingly very similar, e.g. oxidation vs. demethylation+oxidation (Figure 29e and 29g).
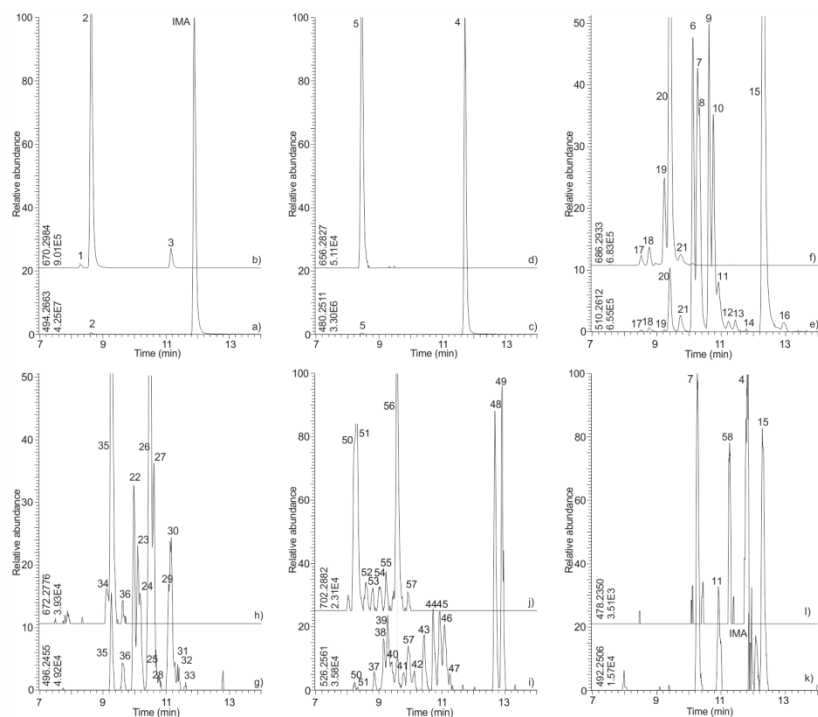
Figure 29: First part of complete metabolite profile of patient on Glivec dose of 400 mg/day (blood taken 24h after dose). Analyses represent extracted ion chromatograms of selected metabolisations a) IM with m/z 494.2663, b) glucuronidation with m/z 670.2984, c) demethylation with m/z 480.2506, d) demethylation+glucuronidation with m/z 656.2827, e) oxidation with m/z 510.2612, f) oxidation+glucuronidation with m/z 686.2933, g) oxidation+demethylation with m/z 496.2455, h) oxidation+demethylation+glucuronidation with m/z 672.2776, i) dioxidation with m/z 526.2561, j) dioxidation+glucuronidation with m/z 702.2882, k) desaturation with m/z 492.2506, l) desaturation+demethylation with m/z 478.2350.
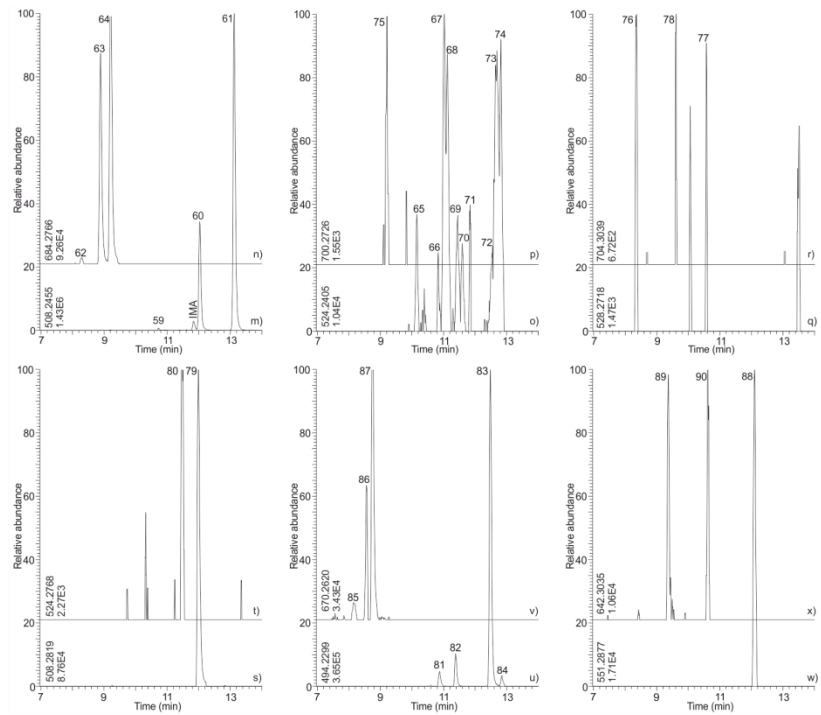
121

Figure 30: Second part of complete metabolite profile of patient on Glivec dose of 400 mg/day (blood taken 24h after dose). Analyses represent extracted ion chromatograms of selected metabolisations m) oxidation+desaturation with *m/z* 508.2455, n) oxidation+desaturation+glucuronidation with *m/z* 684.2766, o) dioxidation+desaturation with *m/z* 524.2405, p) dioxidation+desaturation+glucuronidation with *m/z* 700.2726, q) oxidation+hydrolysis with *m/z* 528.2718, r) oxidation+hydrolysis+glucuronidation with *m/z* 704.3039, s) methylation with *m/z* 508.2819, t) oxidation+methylation with *m/z* 524.2768, u) oxidation+demethylation with *m/z* 494.2299, v) oxidation+demethylation+glucuronidation with *m/z* 670.2620, w) glycine conjugation with *m/z* 551.2877, x) glucuronidation+decarboxylation with *m/z* 642.3035.

Differences in metabolic profiles between plasma samples were observed. Figure 31 shows the extracted ion chromatograms of selected metabolisations from two patients. Compared to IM there are differences in relative intensities of demethylation (Line II, metabolite no. 4), oxidation (Line II, metabolite no. 6 and 16), demethylation+oxidation (Line V, metabolite no. 22) and dioxidation (Line VI, metabolite no. 48 and 49). Further, samples are different in overall intensities of all metabolites of selected metabolisations.
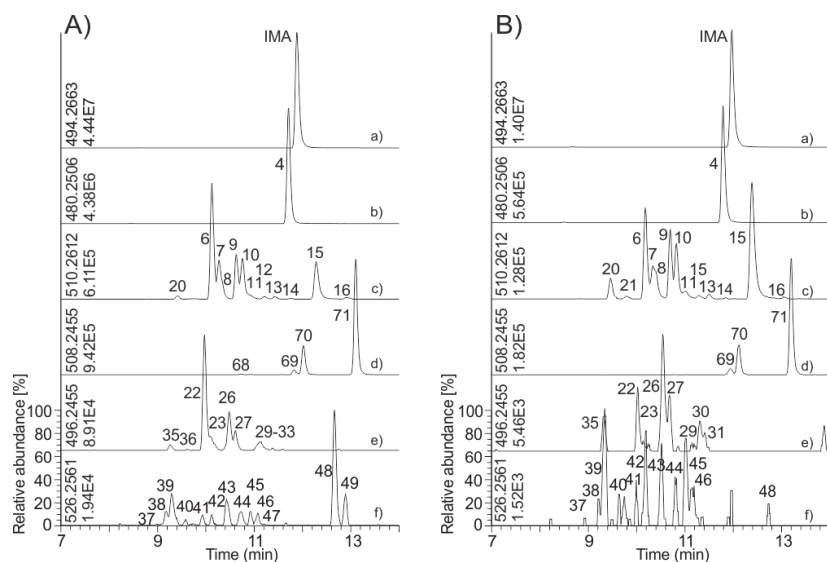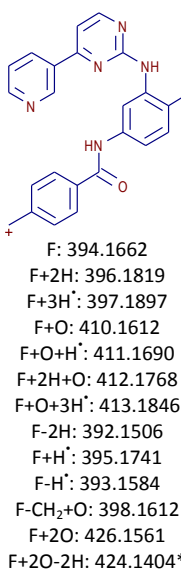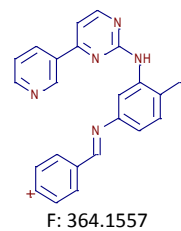

Figure 31: Metabolite profiles of two different patients (A, B) on Glivec dose of 400 mg/day (blood taken 24h after dose). Analyses represent extracted ion chromatograms of selected metabolisations a) parent drug with m/z 494.2663, b) demethylation with m/z 480.2506, c) oxidation with m/z 510.2612, d) desaturation+oxidation with m/z 508.2455, e) demethylation+oxidation with m/z 496.2455 and f) dioxidation with m/z 526.2561.

Confirmation of metabolite identity

In order to confirm identity, MS^2 fragmentation of all metabolites of phase I including IM was performed. Metabolites of phase II were identified by MS^3 fragmentation of molecular ions through the unconjugated ions corresponding to metabolites of phase I. Fragments from MS^2 and MS^3 experiments were measured at high resolution in Orbital ion trap and exact *m/z* values were processed and compared with theoretical predicted fragments generated by Mass Frontier 7.0 software (Figure 32, Table 12 and Figure 33). In order to achieve standard reproducible conditions of fragmentation collision induced dissociation was applied. Therefore the spectra are less complex since containing only MS^2 fragments.

Fragmentation of IM is well described in previously published articles (Marull *et* Rochat, 2006; Klawitter *et al*, 2009; Rochat *et al*, 2008; Titier *et al*, 2005). In our experiment three main fragments with abundance higher than 1% were observed: *m/z* 394.1662, 217.1335 and 476.2557. Other three low abundant fragments are present in spectra: *m/z* 189.1386; 396.1819; 174.0913. Structures of all six fragments were designed by Mass Frontier (Figure 33). For many metabolites parallel fragments with *m/z* value corresponding to *m/z* shift of appropriate metabolisation were detected, easily identified and confirmed by assigned structure (Table 12 and Figure 33).

Transformation of IM to demethylated product ($-CH_2$) is the main metabolic conversion. In fragmentation spectra of demethylated metabolite fragments that correspond to IM parent molecule fragments with mass shift of *m/z* -14.0157: 217.1335 → 203.1179; 476.2764 → 462.2401 were observed.

F: 174.0913

F: 175.1230

F: 187.1230

F: 189.1386
F-CH₂-2H+O: 189.1022*
F-H˙: 188.1308

F: 217.1335
F-2H: 215.1179*
F-CH₂: 203.1179
F+O: 233.1285
F+O-2H: 231.1129*
F-CH₂-2H+O: 217.0971*
F+2O: 249.1235*

F+O: 235.1441
F-CH₂-2H+2O: 235.1077*

F: 247.0978

F: 276.1244
F+O: 292.1193
F+O+2H: 294.1349
F+2H: 278.1400

F: 261.1135
F+O: 277.1084
F+O-2H: 275.0928*

F: 321.2074

F+O: 338.1863

F: 364.1557

F: 394.1662
F+2H: 396.1819
F+3H˙: 397.1897
F+O: 410.1612
F+O+H˙: 411.1690
F+2H+O: 412.1768
F+O+3H˙: 413.1846
F-2H: 392.1506
F+H˙: 395.1741
F-H˙: 393.1584
F-CH₂+O: 398.1612
F+2O: 426.1561
F+2O-2H: 424.1404*

125

F: 423.1928
F+O: 439.1877

F: 437.2084
F+O: 453.2034

F: 450.2163
F+O: 466.2112

F: 435.1928
F+O: 451.1877

F+O: 438.1799

F: 451.2241

F: 436.2006
F+O: 452.1955

F: 449.2084

F: 463.2241
F+O: 479.2190

126

F: 464.2193

F: 478.2350
F-CH$_2$-2H+O: 478.1986

F: 491.2428

F: 468.2506
F+O-2H: 482.2299

F: 479.2428

F: 492.2506
F+O: 508.2455
F-CH$_2$-2H+O: 492.2142*
F-CH$_2$-2H+2O: 508.2091*

F: 476.2557
F-CH2: 462.2401
F-CH$_2$-2H+O: 476.2194*

F: 490.2350
F+O: 506.2300*

F: 493.2585

Figure 33: Structures of theoretical predicted fragments generated by Mass Frontier 7.0 software. * calculated *m/z* deduced from the structure but not found by Mass Frontier 7.0

MS^2 spectra for oxidation/hydroxylation (+O) include characteristics fragments with mass shift m/z = 15.9949: 394.1662 → 410.1612; 217.1335 → 233.1285; 476.2557 → 492.2506. The main fragment of m/z = 410.1612 arises by loss of 100.0995 from parent molecule 510.2612 like the IM 494.2663 → 394.1662. For selected oxidative metabolites characteristic uncommon radical ions corresponding to cleavage on piperazine ring were found (m/z = 423.1928, 436.2010 and 450.2160, Figure 34).

Dioxidation (+2O) with mass shift m/z = 31.9898 offers similar fragmentation behavior: 394.1662 → 426.1561 (loss of 100.0995 from parent molecule 526.2561).

Other observed biotransformations include demethylation+hydroxylation (-CH$_2$+O; mas shift m/z = 1.9793), desaturation+hydroxylation (-2H+O; mas shift m/z = 13.9793), dioxidation+desaturation (+2O-2H; mass shift m/z = 29.9742), hydroxylation/oxidation+hydrolysis (+2H+2O; mas shift m/z = 34.0055), demethylation+desaturation+oxidation/hydroxylation (-CH$_2$-2H+O; mas shift m/z = -0.0364). All of these biotransformations including IM itself undergo glucuronidation (+C$_6$H$_8$O$_6$) with subsequent mass shift m/z = 176.0321. Methylation (+CH$_2$; mass shift m/z = 14.0157), methylation+oxidation/hydroxylation (+CH$_2$+O; mass shift m/z = 30.0106) and glycine conjugation (-OH+C$_2$H$_4$NO$_2$; mass shift m/z = 57.0215) are other significant metabolic reactions. Last reaction comprised decarboxylation after IM glucuronidation (+C$_5$H$_8$O$_5$; mass shift m/z = 148.0372) can be mentioned but decarboxylation is proceeded on glucuronide part. Structures of fragments proposed by Mass Frontier 7.0 software are showed in Figure 34.
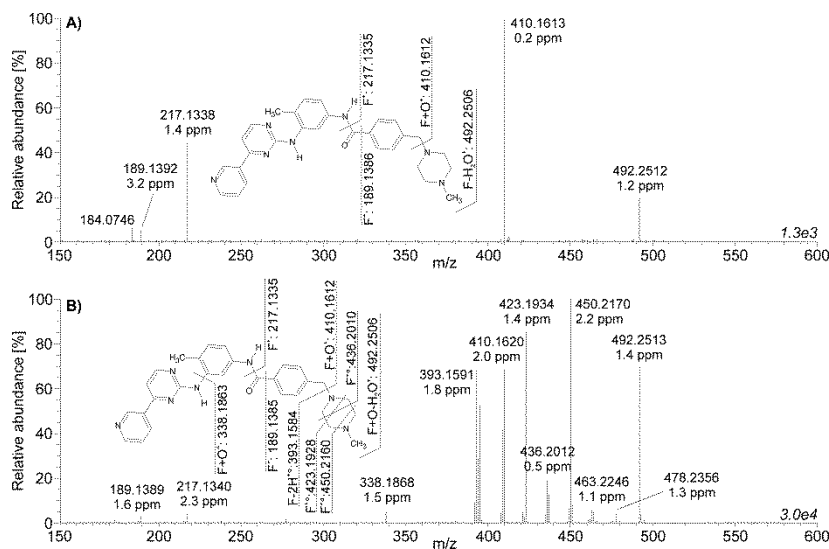
Figure 34: Fragmentation of oxidative metabolites A) No. 9 (t_r=10.33) a) and B) No. 15 (t_r=12.31).

Chromatographic peaks corresponding to desaturation (2H loss) were identified and characterized by *m/z* shift -1.9793 from parent molecule IM. Similarly, in MS^2 spectra characteristic fragments were observed: 217.1335 → 215.1542; 189.1386 → 187.1953 and 476.2557 → 474.2764. Since these compounds arise from electrospray ion source fragmentation or electrochemical conversion of the main oxidative metabolites, therefore were not included in the list of metabolites and scheme of metabolisation.

2.5.6. Acknowledgements

129

3. Conlusions

Targeted therapy using IM, NIL, DAS, and LAP, based on the inhibition of protein tyrosine kinases, represents an evolving concept in therapeutic strategies. The monitoring of TKI plasma levels has become an essential tool for the evaluation of adherence to the treatment and for the therapy management of CML, GIST or breast cancer patients. LC-MS/MS has spread as a major method for determination of a wide range of drugs, some of their metabolites, but also for several biomarkers in biological fluids, with better sensitivity, selectivity and enhanced throughput capability than previously used methods such as LC-UV/DAD or immunoassays.

Bioanalytical high-throughput methods (LC and FIA coupled to MS/MS) for determination of IM in plasma samples from patients with CML undergoing Glivec therapy were developed, validated and compared. Both methods use a simple sample preparation using deproteination with an organic solvent. LC separation was achieved on short C18 column with gradient elution programme. FIA was performed with total analysis time 0.75 min. Detection was based on MRM mode on the tandem mass spectrometer API 4000 (AB Sciex) in positive ESI. Methods fulfill FDA validation criteria (FDA, 2001) and provide comparable results based on Bland-Altman test. The methods allow high-throughput, sensitive, and specific quantification useful for the therapeutic drug monitoring of patients treated with IM. Currently, the developed LC-MS/MS method has been put into routine clinical practice in University hospital in Olomouc.

Subsequently, FIA-MS/MS method for IM determination was extended for other three TKIs nilotinib, dasatinib and lapatinib. In comparison with previously published methods using HPLC-MS/MS, our method has a short analysis time of 55 or 19 s in multiple injection settings as a result of the exclusion of the separation step, and sample preparation based on deproteination with an organic solvent is relatively simple. Therefore, the simultaneous determination of four tyrosine kinase inhibitors IM, NIL, DAS, and LAP in plasma with the isotope dilution direct injection mass spectrometry method is rapid, sensitive, selective, and high-throughput, requires a small amount of plasma sample, and has a markedly reduced requirement for consumable expenditures. In addition, we applied MRM^3

mode on the 5500 QTRAP in order to achieve the significantly better selectivity and sensitivity that is necessary for the correct quantification of low-level DAS. MRM^3 mode can be a new approach to the determination of drugs with a low concentration level, where MRM mode detection is not sufficient as a result of the poor ionization or the presence of interferences. In laboratories this method can be used instead of conventional HPLC-MS/MS because of easier method switching. The criteria for linearity, precision, accuracy, and recovery have been proven to be within the recommendations of the FDA and EMEA guidelines for the validation of bioanalytical methods (Chace *et al*, 2003; FDA, 2001). Consequently, this method is suitable for the use in routine clinical practice and it can be useful for the therapeutic drug monitoring of patients treated with IM, NIL, DAS, or LAP, especially for the evaluation of patient adherence to daily oral therapy, the efficacy of treatment, severe drug-related adverse events, drug-drug interaction, or the relationship between pharmacokinetics and pharmacodynamics.

Despite speculations that IPL could assist with decisions about whether to increase an imatinib dose, IPL measurement is recommended as helpful in situations when poor adherence to treatment is suspected, severe side-effects are observed or drug interactions should be ruled out (Cortes *et al*, 2009). We reported two patients, who were identified among 12 patients with suboptimal treatment response according to the European Leukaemia Net criteria from 40 patients treated with first-line imatinib at our centre since 2004. Their daily dose was escalated from 400 to 600 or 800 mg because low IPLs were found at various intervals since the beginning of treatment. Both patients showed an increase of IPL after imatinib dose escalation (mean values; 616 → 1790 ng/mL (400 → 800 mg/day) and 765 → 1417 ng/mL (400 → 600 mg/day)) and also achievement of optimal treatment response. Of interest in both our patients, low expression of *hOCT1* gene at the diagnosis as the other possible cause of suboptimal treatment reponse was retrospectively demonstrated. The population of patients suitable for imatinib dose management according to IPL may not be large as most patients respond well to imatinib first-line treatment and in non-responding patients several other causes of resistance may play a role. However, we believe that our report showing that IPL estimated at

131

various time points of treatment may be used for dose adjustment in individual patients has important implications for optimalization of routine management of CML patients outside clinical trials.

Association of trough IPL with cytogenetic or molecular response to treatment in patients with CML was several times reported (Picard *et al*, 2007; Larson *et al*, 2008; Singn *et al*, 2009; Awidi *et al*, 2010; Zhong *et al*, 2012; Ishikawa *et al*, 2010; Takahashi *et al*, 2010; Ohnishi *et al*, 2012; Forrest *et al*, 2009) but further studies are awaited to confirm prospectively the link between IPL and response to treatment and to define effective trough concentrations in different patient populations (Cortes *et al*, 2009). We analysed IPL in the routine clinical setting in 54 and 84 patients with chronic phase CML with IM 400 mg/day as first- or second-line treatment in whom blood sampling was performed 24±2 and 24±4 hours after last dose administration, respectively. In both sets, we were not able to demonstrate a statistically significant correlation between IPL and response to treatment according to the ELN. We suppose that low number of patients with minor proportion of suboptimal or failure patients, difference in patients' compliance, leukaemia biology, and other variables that are difficult to eliminate in routine clinical practice could affected our results. IPL may be used for identification of patients with poor compliance or in cases of excluding prominent toxicity but their use for prognostic estimate outside the clinical trials might be limited. Our observed data confirmed correlation between IPL and absolute dose as also reported in several studies (Picard *et al*, 2007; Ishikawa *et al*, 2010; Sakai *et al*, 2009). These data do not provide any additional information useful for management of CML treatment of patients with suboptimal response.

Chromatographic method using common sub-2-micron C18 stationaty phase was developed for high separation efficiency of IM ant its metabolites in human plasma. High-resolution mass spectrometry and MS^2 exact mass fragmentation for detailed study of IM metabolite profile in plasma was performed. By using of HRMS with high sensitivity and highly sophisticated software we were able to find and identified overall 90 metabolites and 75 of them fully characterized by MS^2 or MS^3 exact mass fragmentation and comparison of *m/z* of parental compound and its

fragments with structures characterized by exact *m/z* designed by Mass Frontier 7.0 software (mass accuracy bellow 5 ppm). High resolution offers advantage of precise assignment of *m/z* values to supposed structure of whole metabolites as well as fragments. Neverthless, the rest of 16 metabolites were not possible to identify and confirm their structure due to their low abundance in patients' plasma and instrument sensitivity limitation. As we showed all metabolisation studies should cover analysis of tablet which can contain many metabolites or impurities arising as minor products at synthesis of the drug. Other phenomenons have to be also taken into account when analysing minor metabolites – ion source fragmentation and electrochemical conversion which can play important role. But with respect to sensitivity of actual mass spectrometers it is important to exclude these false metabolites and final incorrect interpretation of the results. The theoretical importance of this study is in showing how complex can be metabolisation of drugs and how the software helps us with identification and confirmation of structures of metabolites. The practical potential of this study lies in subsequent studies focused on large cohort analysis of metabolites and finding relationships between levels of metabolites and clinical signs of patients like a good/poor response on treatment, symptoms, adverse reactions, failure of treatment, total cure of patients and many others.

4. Summary

New drugs based on targeted therapy are designed thanks to the advanced findings in the cancer molecular biology. Imatinib (IM) was the primarily developed compound, tyrosine kinase inhibitor that represented radical breakthrough in the treatment of chronic myeloid leukaemia with extensive prolonging event-free and progression-free survival. Despite of successful treatment results several inconveniences such as serious adverse events, treatment non-responders, food or drug interactions and others occur frequently. To avoid these problems and improve the life quality of patients as much as possible, monitoring of the drugs in body fluids, especially with narrow therapeutic index, became a useful tool to individualize treatment in terms of choose convenient medicament with appropriate adjusted dosage.

The mass spectrometry in the combination with an appropriate separation technique is recently the most frequent and suitable technique for studying the drug metabolism and therapeutic drug monitoring. Advantages of this technique are based on the extremely high sensitivity, selectivity, very low sample consumption and providing a complex information about sample. Methods for determination of IM in human plasma by LC-MS/MS and FIA-MS/MS and for simultaneous determination of four TKIs by direct injection isotope dilution MS method were developed and validated in this work. The methods are suitable for therapeutic drug monitoring in clinical laboratories.

Several studies demonstrated the correlation between IM plasma levels (IPL) and treatment response, therefore therapeutic monitoring of IM is currently used in clinical practice as a prognostic indicator for effective and safe individualized treatment. Interindividual variability in plasma exposure for a given dose of the drug and also treatment response is often caused by divergences in adherence to IM therapy, patient demographic factor, interindividual variability in the activity of metabolizing enzymes, food- or drug-drug interactions, differences in plasma protein binding of IM and variability in the activity of cellular uptake and efflux transporters. Two patients with suboptimal treatment response under standard IM daily dosage and low IPL in whom dose escalation led to optimal treatment

134

response and IPL elevation were reported. Another study aimed at relationship between IPL and treatment response according to the ELN was not able to demonstrate a statistically significant correlation.

The metabolite profiling could be a useful tool for finding relationships between levels of metabolites and clinical signs of patients like a good/poor response on the treatment, symptoms, adverse reactions, failure of treatment, total cure of patients and many others. Differences in the activity of particular drug metabolizing enzymes such as cytochrome P450 are responsible for changes in the metabolite amount and type production. The determination of drugs in body fluids became a subject of studies and it is widely applied in many experimental and clinical laboratories all over the world. In the consequence of drug metabolizing enzyme activity bioactive or toxic metabolites that should have clinical relevance may be produced. Our study presented method for identification and characterization of 90 IM metabolites by high-resolution mass spectrometry technique based on MS_2 and MS_3 exact mass fragmentation.

Mass spectrometry is constantly and increasingly leading technology in clinical and experimental laboratories and became an important tool for diagnosing and monitoring of diseases.

5. Souhrn

Díky novým poznatkům v molekulární biologii nádorových onemocnění jsou navrhovány nové léky založené na principu cílené terapie. Jako první vyvinutá sloučenina byl imatinib (IM), tyrosinkinasový inhibitor (TKI), který představoval radikální zlom v léčbě chronické myeloidné leukémie se značným prodloužením přežití bez příznaků a progrese. Navzdory úspěšným léčebným výsledkům se často objevovaly překážky jako vážné nežádoucí účinky, pacienti neodpovídající na léčbu, lékové či potravinové interakce a další.

Pro vyvarování se těmto problémům a co největšímu zlepšení kvality života pacientů se stalo monitorování léčiv, zejména s úzkým terapeutickým indexem, užitečným nástrojem k individualizaci léčby v podobě volby vyhovujícího léčebného přípravku s vhodně přizpůsobenou dávkou.

Hmotnostní spektrometrie v kombinaci s vhodnou separační technikou je v současnosti nejčastější používanou technologií pro studium metabolismu léčiv a terapeutického monitorování léčiv. Výhody této techniky jsou založeny na extrémně vysoké citlivosti, selektivitě, velmi nízké spotřebě vzorku a koplexním informacím získaných o vzorku. V této práci byly vyvinuty a validovány metody pro stanovení imatinibu v lidské plasmě pomocí LC-MS/MS a FIA-MS/MS a pro současné stanovení čtyř TKIs pomocí MS metody přímého nástřiku s izotopovým zřeďováním. Metody jsou vyhovující pro terapeutické monitorování léčiv v klinických laboratořích.

Několik studií demonstruje korelaci mezi plasmatickými hladinami IM (IPL) a léčebnou odpovědí, tudíž je terapeutické monitorování IM v klinické praxi v současnosti používáno jako prognostický ukazatel pro účinnou a bezpečnou individualizovanou léčbu. Interindividuální variabilita v plasmatické hladině pro danou dávku léčiva a také v léčebné odpovědi je často způsobena odlišným dodržováním léčby IM, demografickými factory pacientů, interindividuální variabilitě v aktivitě metabolizujících enzymů, lékových či potravinových interakcí, rozdílech ve vazbě IM na plasmatické

proteiny a variabilitě v aktivitě transportérů pro buněčnou absorpci a vylučování. V naší studii jsou uvedeni dva pacienti s nedostatečnou léčebnou odpovědí při standardním denním dávkování IM a s nízkou IPL, jejichž zvýšení denní dávky vedlo k optimální léčebné odpovědi a zvýšení IPL. Další studie zaměřená na vztah mezi IPL a léčebnou odpovědí podle ELN nebyla schopna prokázat statisticky významnou korelaci.

Metabolitové pofilování může být užitečným nástrojem pro nalezení vztahu mezi hladinami metabolitů a klinickými znaky pacientů jako dobrou/nízkou léčebnou odpovědí, příznaky onemocnění, nežádoucími účinky, selháním léčby, celkovým vyléčením pacienta a mnoha dalších. Rozdíly v aktivitě jednotlivých enzymů metabolizujících léky jako cytochrom P450 jsou zodpovědné za změny v množství a druhu produkovaných metabolitů. Stanovení léků v tělních telutinách se stalo předmětem studií a je široce aplikováno v mnoha výzkumných a klinických laboratořích po celém světě. V důsledku aktivity enzymů metabolizujících léky mohou být produkovány bioaktivní nebo toxické metabolity, které mohou být klinicky významné. Naše studie představuje metodu pro identifikaci a charakterizaci 90 metabolitů IM pomocí techniky hmotnostní spektrometrie s vysokým rozlišením založené na MS^2 a MS^3 fragmentaci na přesné hmotě.

Hmotnostní spektrometrie je neustále stoupající základní technologie v klinických a výzkumných laboratořích a stává se důležitým nástrojem pro diagnostiku a monitorování onemocnění.

6. List of abbreviations

ABCB1	ATP-binding cassette, sub-family B
ABL	Abelson murine leukaemia viral oncogene
ADP	adenosine diphosphate
AGP	α1-acid glycoprotein
ALL	acute lymphoblastic leukaemia
ALT	alanine transaminase
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
ATP	adenosine triphosphate
AUC	area under curve
BCR	breakpoint cluster region
BINGO	blood level testing of imatinib in the Nagoya study
BMT	bone marrow transplantation
BSA	body surface area
BW	body weight
CCR	complete cytogenetic response
cDNA	complementary DNA
CE	colission energy
CHR	complete hematologic response
CML	chronic myeloid leukaemia
CMR	complete molecular response
CV	coefficient of variation
CXP	collision exit potential
CYP	cytochrome P450
DAS	dasatinib
desIM	desmethyl imatinib
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EFS	event-free survival
ELN	European Leukaemia Net
EQC	external quality control

ESI	electrospray ionization
FDA	Food and Drug Administration
FIA	flow injection analysis
FISH	fluorescence *in situ* hybridization
FTICR	Fourier transform ion cyclotron resonance
GSH	glutathione
HILIC	hydrophilic interaction liquid chromatography
HLA	human leukocyte antigen
hOCT1	human organic cation transporter
HPLC	high-performance liquid chromatography
HR	hematologic response/high resolution
HSCT	human stem cell transplantation
IFN	interferon
IM	imatinib
IPL	imatinib plasma level
IQC	internal quality control
IRIS	International Randomized Study of Interferon vs STI571
IRK	insulin receptor kinase
IS	ion source/internal standard
JALSG	Japan adult leukaemia study group
LAP	lapatinib
LC	liquid chromatography
LIT	linear ion trap
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
MAPK/ERK	mitogen-activated protein kinase
MAOIs	monoamine oxidase inhibitors
MCR	major cytogenetic response
MDR1	multi drug resistence gene
MMR	major molecular response
MRM	multiple reaction monitoring
mRNA	messenger RNA
MS	mass spectromery

Mtor	mammalian targets of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NCI	National cancer institute
NIL	nilotinib
NNRTIs	non-nucleoside reverse transcriptase inhibitors
NP	normal phase
NRTIs	nucleoside reverse transcriptase inhibitors
OS	overal survival
PCR	polymerase chain reaction
PCyR	partial cytogenetic response
PDGFR	Platelet-derived growth factor receptor
PFS	progression-free survival
Ph	Philadelphia chromosome
PH	pleckstrin homology
PI	phosphoinositide
Pis	protease inhibitors
PP	protein precipitation
qRT-PCR	quantitative real-time polymerase chain reaction
Q-TOF	quadrupole time-of-flight
RNA	ribonucleic acid
ROC	receiver operating characteristic
RP	reverse phase
RT-PCR	real-time polymerase chain reaction
SD	standard deviation
SFKs	Src family kinases
SH	Src-homology
SIM	selected ion monitoring
SPE	solid phase extraction
STI571	signal transduction inhibitor 571
STIM	multicentre stop imatinib trial
SULT	sulfotransferase
TCAs	tricyclic anridepressants
TDM	therapeutic drug monitoring
TIDEL	trial of initial intensified imatinib therapy and sequential dose escalation

TKI	tyrosine kinase inhibitor
TMS	tandem mass spectrometry
TOPS	Tyrosine Kinase Inhibitor Optimization and Selectivity Study
UGT	uridine diphosphate glucuronyltransferase
UHPLC	ultra high-performance liquid chromatography

141

7. References

Adaway JE, Keevil BG. Therapeutic drug monitoring and LC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci. 2012;883-884:33-49.

Advani AS, Pendergast AM. Bcr-Abl variants: biological and clinical aspects. Leuk Res. 2002;26(8):713-20.

Apperley JF, Cortes JE, Kim DW, Roy L, Roboz GJ, Rosti G, Bullorsky EO, Abruzzese E, Hochhaus A, Heim D, de Souza CA, Larson RA, Lipton JH, Khoury HJ, Kim HJ, Sillaber C, Hughes TP, Erben P, Van Tornout J, Stone RM. Dasatinib in the treatment of chronic myeloid leukemia in accelerated phase after imatinib failure: the START a trial. J Clin Oncol. 2009;27(21):3472-9.

Awidi A, Ayed AO, Bsoul N, Magablah A, Mefleh R, Dweiri M, Ramahi M, Arafat E, Bishtawi M, Marie L. Relationship of serum imatinib trough level and response in CML patients: long term follow-up. Leuk Res. 2010;34(12):1573-5.

Baccarani M, Cortes J, Pane F, Niederwieser D, Saglio G, Apperley J, Cervantes F, Deininger M, Gratwohl A, Guilhot F, Hochhaus A, Horowitz M, Hughes T, Kantarjian H, Larson R, Radich J, Simonsson B, Silver RT, Goldman J, Hehlmann R; European LeukemiaNet. Chronic myeloid leukemia: an update of concepts and management recommendations of European LeukemiaNet. J Clin Oncol. 2009;27(35):6041-51.

Baccarani M, Druker BJ, Branford S, Kim DW, Pane F, Mongay L, Mone M, Ortmann CE, Kantarjian HM, Radich JP, Hughes TP, Cortes JE, Guilhot F; On behalf of the TOPS investigators. Long-term response to imatinib is not affected by the initial dose in patients with Philadelphia chromosome-positive chronic myeloid leukemia in chronic phase: final update from the Tyrosine Kinase Inhibitor Optimization and Selectivity (TOPS) study. Int J Hematol 2014;99(5):616-24.

Baccarani M, Rosti G, Castagnetti F, Haznedaroglu I, Porkka K, Abruzzese E, Alimena G, Ehrencrona H, Hjorth-Hansen H, Kairisto V, Levato L, Martinelli G, Nagler A, Lanng JL, Ozbek u, Palandri F, Palmieri F, Pane F, Rege-Cambrin G, Russo D, Specchia G, Testoni N, Weiss-Bjerrum O, Saglio G, Simonsson B. Comparison of imatinib 400 mg and 800 mg daily in the front-line treatment of high-risk, Philadelphia-positive chronic myeloid leukemia: a European LeukemiaNet Study. Blood. 2009;113:4497-504.

Baccarani M, Saglio G, Goldman J, Hochhaus A, Simmonsson B, Appelbaum F, Apperley J, Cervantes F, Cortes J, Deininger M, Gratwohl A, Guilhot F, Horowitz M, Hughes T, Kantarjian H, Larson R, Niederwieser D, Silver R, Hehlmann R; European LeukemiaNet. Evolving concepts in the management of chronic myeloid leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. Blood. 2006;108(6):1809-1820.

Baietto L, D'Avolio A, Ventimiglia G, De Rosa FG, Siccardi M, Simiele M, Sciandra M, Di Perri G. Development, validation, and routine application of a high-performance liquid chromatography method coupled with a single mass detector for quantification of itraconazole, voriconazole, and posaconazole in human plasma. Antimicrob Agents Chemother. 2010;54 (8):3408-13.

Bakhtiar R, Lohne J, Ramos L, Khemani L, Hayes F, Tse F. High-throughput quantification of the anti-leukemia drug STI-571 (Gleevec[TM]) and its main metabolite (CGP 74588) in human plasma using liquid chromatogramy-tandem mass spektrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2002;768:325-340.

Bharathi DV, Hotha KK, Sagar PV, Kumar SS, Reddy PR, Naidu A, Mullangi R. Development and validation of a highly sensitive and robust LC-MS/MS with electrospray ionization method for simultaneous quantitation of itraconazole and hydroxyitraconazole in human plasma: application to a bioequivalence study. J Chromatogr B Analyt Technol Biomed Life Sci. 2008;868(1-2):70-6.

Bhatt J, Subbaiah G, Singh S. Liquid chromatography/tandem mass spectrometry method for simultaneous determination of risperidone and its active metabolite 9-hydroxyrisperidone in human plasma. Rapid Commun Mass Spectrom. 2006;20(14):2109-14.

Bogusz MJ, Enazi EA, Hassan H, Abdel-Jawaad J, Ruwaily JA, Tufail MA. Simultaneous LC-MS-MS determination of cyclosporine A, tacrolimus, and sirolimus in whole blood as well as mycophenolic acid in plasma using common pretreatment procedure. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;850(1-2):471-80.

Brincker H. Population-based age- and sex-specific incidence rates in the 4 main types of leukaemia. Scand J Haematol. 1982;29(3):241-9.

Britzi M, Soback S, Isoherranen N, Levy RH, Perucca E, Doose DR, Maryanoff BE, Bialer M. Analysis of topiramate and its metabolites in plasma and urine of healthy subjects and patients with epilepsy by use of a novel liquid chromatography-mass spectrometry assay. Ther Drug Monit. 2003;25(3):314-22.

Buchdunger E, Zimmermann J, Mett H, Meyer T, Muller M, Druker BJ, Lydon NB. Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative. Cancer Res. 1996;56:100–4.

Bunch DR, Heideloff C, Ritchie JC, Wang S. A fast and simple assay for busulfan in serum or plasma by liquid chromatography-tandem mass spectrometry using turbulent flow online extraction technology. J Chromatogr B Analyt Technol Biomed Life Sci. 2010;878(31):3255-8.

Catena E, Perez G, Sadaba B, Azanza JR, Campanero MA. A fast reverse-phase high performance liquid chromatographic tandem mass spectrometry assay for the quantification of clindamycin in plasma and saliva using a rapid resolution package. J Pharm Biomed Anal. 2009;50(4):649-54.

144

Chace DH, Kalas TA, Naylor EW. Use of tandem mass spectrometry for multianalyte screening of dried blood specimens from newborns Clin Chem. 2003;49(11):1797-1817.

Chahbouni A, den Burger JC, Vos RM, Sinjewel A, Wilhelm AJ. Simultaneous quantification of erlotinib, gefitinib and imatinib in human plasma by liquid chromatography tandem mass spectrometry. Ther Drug Monit. 2009;31:683-7.

Chahbouni A, Wilhelm AJ, den Burger JC, Sinjewel A, Vos RM. Validated liquid chromatography-tandem mass spectroscopy method for the simultaneous quantification of four antimycotic agents in human serum. Ther Drug Monit. 2010;32(4):453-7.

Chomczynski P, Sacchi N. Single-step method of RNA isolation by quanidium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987;162:156-9.

Cortes JE, Egorin MJ, Guilhot F, Molimard M, Mahon F-X. Pharmacokinetic / pharmacodynamic correlation and blood-level testing in imatinib therapy for chronic myeloid leukemia. Leukemia. 2009;23:1537-44.

Cortes J, Giles F, O'Brien S, Thomas D, Garcia-Manero G, Rios MB, Faderl S, Verstovsek S, Ferrajoli A, Freireich EJ, Talpaz M, Kantarjian H. Result of high-dose imatinib mesylate in patients with Philadelphia chromosome-positive chronic myeloid leukemia after failure of interferon-alpha. Blood. 2003;102:83-6.

Cortes JE, Jones D, O'Brien S, Jabbour E, Konopleva M, Ferrajoli A, Kadia T, Borthakur G, Stigliano D, Shan J, Kantarjian H. Nilotinib as front-line treatment for patients with chronic myeloid leukemia in early chronic phase. J Clin Oncol. 2010;28(3):392-7.

Cortes JE, Jones D, O'Brien S, Jabbour E, Ravandi F, Koller C, Borthakur G, Walker B, Zhao W, Shan J, Kantarjian H. Results of dasatinib therapy in patients with early chronic-phase chronic myeloid leukemia. J Clin Oncol. 2010;28(3):398-404.

145

Danhauser-Riedl S, Warmuth M, Druker BJ, Emmerich B, Hallek M. Activation of Src kinases p53/56lyn and p59hck by p210bcr/abl in myeloid cells. Cancer Res. 1996;56:3589-96.

Davies A, Hayes AK, Giannoudis A, Lucas CM, Knight K, Watmough SJ, et al. A study of plasma levels of imatinib and its bioactive metabolite CGP-74588 reveals no correlation with subsequent clinical outcome in imatinib-treated chronic myeloid leukemia. Haematologica. 2009;94(Suppl. 2):259; Abstract 0637.

Davies A, Hayes AK, Knight K, Watmough SJ, Pirmohamed M, Clark RE. Simultaneous determination of nilotinib, imatinib and its main metabolite (CGP-74588) in human plasma by ultra-violet high performance liquid chromatography. Leuk Res. 2010;34:702-7.

D'Avolio A, Simiele M, De Francia S, Ariaudo A, Baietto L, Cusato J, Fava C, Saglio G, Di Carlo F, Di Perri G. HPLC-MS method for the simultaneous quantification of the antileukemia drugs imatinib, dasatinib and nilotinib in human peripheral blood mononuclear cell (PBMC). J Pharm Biomed Anal. 2012;59:109-16.

Dayer P, Desmeules J, Leemann T, Striberni R. Bioactivation of the narcotic drug codeine in human liver is mediated by the polymorphic monooxygenase catalyzing debrisoquine 4- hydroxylation (cytochrome P-450 dbl/bufl). Biochem Biophys Res Commun. 1988;152(1):411-6.

De Francia S, D'Avolio A, De Martino F, Pirro E, Baietto L, Siccardi M, Simiele M, Racca S, Saglio G, Di Carlo F, Di Perri G. New HPLC-MS method for the simultaneous quantification of the antileukemia drugs imatinib, dasatinib, and nilotinib in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2009;877:1721-1726.

de Sousa Maia MB, do Nascimento DF, Martins IL, Cunha AN, de Lima FE, Bezerra FA, de Moraes MO, de Moraes ME. Simultaneous quantitative analysis of oxcarbazepine and 10,11-dihydro-10-hydroxycarbamazepine in human plasma by liquid chromatography-electrospray tandem mass spectrometry. J Pharm Biomed Anal. 2007;45(2):304-11.

de Velde F, Alffenaar JW, Wessels AM, Greijdanus B, Uges DR. Simultaneous determination of clarithromycin, rifampicin and their main metabolites in human plasma by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2009;877(18-19):1771-7.

Decosterd LA, Rochat B, Pesse B, Mercier T, Tissot F, Widmer N, Bille J, Calandra T, Zanolari B, Marchetti O. Multiplex ultra-performance liquid chromatography-tandem mass spectrometry method for simultaneous quantification in human plasma of fluconazole, itraconazole, hydroxyitraconazole, posaconazole, voriconazole, voriconazole-N-oxide, anidulafungin, and caspofungin. Antimicrob Agents Chemother. 2010;54 (12):5303-15.

Deininger MWN, Druker BJ. Specific targeted therapy of chronic myelogenous leukemia with imatinib. Pharmacol Rev. 2003;55(3):401-23.

Deininger MW, Kopecky KJ, Radich JP, Kamel-Reid S, Stock W, Paietta E, Emanuel PD, Tallman M, Wadleigh M, Larson RA, Lipton JH, Slovak ML, Appelbaum FR, Druker BJ. Imatinib 800 mg daily induces deeper molecular responses than imatinib 400 mg daily: results of SWOG S0325, an intergroup randomized PHASE II trial in newly diagnosed chronic phase chronic myeloid leukaemia. Brit J Haematol. 2014;164:223–32.

Deng Y, Zhang H, Wu JT, Olah TV. Tandem mass spectrometry with online high-flow reversed-phase extraction and normal-phase chromatography on silica columns with aqueous-organic mobile phase for quantitation of polar compounds in biological fluids. Rapid Commun Mass Spectrom. 2005;19(20):2929-34.

Dickinson L, Robinson L, Tjia J, Khoo S, Back D. Simultaneous determination of HIV protease inhibitors amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir in human plasma by high-performance liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2005;829(1-2):82-90.

Dohse M, Scharenberg C, Shukla S, Robey RW, Volkmann T, Deeken JF, Brendel C, Ambudkar SV, Neubauer A, Bates SE. Comparison of ATP-binding cassette transporter interactions with the tyrosine kinase inhibitors imatinib, nilotinib, and dasatinib. Drug Metab Dispos. 2010;38:1371-80.

Druker BJ, Guilhot F, O'Brien SG, Gathmann I, Kantarjian H, Gattermann N, Deininger MW, Silver RT, Goldman JM, Stone RM, Cervantes F, Hochhaus A, Powell BL, Gabrilove JL, Rousselot P, Reiffers J, Cornelissen JJ, Hughes T, Agis H, Fischer T, Verhoef G, Shepherd J, Saglio G, Gratwohl A, Nielsen JL, Radich JP, Simonsson B, Taylor K, Baccarani M, So C, Letvak L, Larson RA; IRIS Investigators. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia, N Engl J Med. 2006;355:2408-17.

Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM, Capdeville R, Talpaz M. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. N Engl J Med. 2001;344:1038-42 b.

Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S, Sawyers CL. Efficacy and safety of a specific inhibitor of the Bcr-Abl tyrosine kinase in chronic myeloid leukemia. New Engl. J. Med. 2001;344:1031–37 a.

Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, Zimmermann J, Lydon NB. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med. 1996;2:561-6.

Else L, Watson V, Tjia J, Hughes A, Siccardi M, Khoo S, Back D. Validation of a rapid and sensitive high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) assay for the simultaneous determination of existing and new antiretroviral compounds. J Chromatogr B Analyt Technol Biomed Life Sci. 2010;878(19):1455-65.

Faber E. Imatinib. Farmakoterapie. 2005;3:233-38.

Faber E, Friedecký D, Mičová K, Divoká M, Katrincsáková B, Rožmanová S, Jarošová M, Indrák K, Adam T. Imatinib dose escalation in two patients with chronic myeloid leukemia, with low trough imatinib plasma levels measured at various intervals from the beginning of therapy and with suboptimal treatment response, leads to the achievement of higher plasma levels and major molecular response. Int J Hematol. 2010;91(5):897-902 a.

Faber E, Friedecký D, Mičová K, Rožmanová S, Divoká M, Jarošová M, Indrák K, Adam T. Imatinib trough plasma levels do not correlate with the response to therapy in patients with chronic myeloid leukemia in routine clinical setting. Ann Hematol. 2012;91(6):923-9.

Faber E, Indrák K et al. Chronická myeloidní leukemia, Galén 2010; 1st edition, ISBN 978-80-7262-680-9 b.

Faber E, Naušová J, Jarošová M, Egorin MJ, Holzerová M, Rožmanová Š, Marešová I, Divoký V, Indrák K. Intermittent dosage of imatinib mesylate in CML patients with history of significant hematologic toxicity after standard dosing. Leuk Lymphoma. 2006;47(6):1082-1090.

Fabbro D, Ruetz S, Buchdunger E, Cowan-Jacob SW, Fendrich G, Liebetanz J, Mestan J, O'Reilly T, Traxler P, Chaudhuri B, Fretz H, Zimmermann J, Meyer T, Caravatti G, Furet P, Manley PW. Protein kinases as targets for anticancer agents: from inhibitors to useful drugs. Pharmacol Ther. 2002;93:79–98.

Faderl S, Talpaz M, Estrov Z, Kantarjian HM. Chronic myelogenous leukemia: Biology and therapy. Ann Intern Med. 1999;131:207-19.

FDA (2001) Guidance for Industry: Bioanalytical Method Validation. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf (8.8.2014)

Forrest DL, Trainor S, Brinkman RR, Barnett MJ, Hogge DE, Nevill TJ, Shepherd JD, Nantel SH, Toze CL, Sutherland HJ, Song KW, Lavoie JC, Power MM, Abou-Mourad Y, Smith CA. Cytogenetic and molecular responses to

standard-dose imatinib in chronic myeloid leukemia are correlated with Sokal risk scores and duration of therapy but not trough imatinib plasma levels. Leuk Res. 2009;33(2):271-5.

Frank DA, Varticovski L. BCR/abl leads to the constitutive activation of Stat proteins, and shares an epitope with tyrosine phosphorylated Stats. Leukemia. 1996;10(11):1724-30.

Frerichs VA, DiFrancesco R, Morse GD. Determination of protease inhibitors using liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2003;787(2):393-403.

Gabert J, Beillard E, van der Velden VHJ, Bi W, Grimwade D, Pallisgaard N, Barbany G, Cazzaniga G, Cayuela JM, Cavé H, Pane F, Aerts JL, De Micheli D, Thirion X, Pradel V, González M, Viehmann S, Malec M, Saglio G, van Dongen JJ. Standardization and quality control studies of "real-time" quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - A Europe Against Cancer Program. Leukemia. 2003;17(12):2318-57.

Gao F, Zhang M, Cui X, Wang Z, Sun Y, Gu J.. Simultaneous quantitation of hydrochlorothiazide and metoprolol in human plasma by liquid chromatography–tandem mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis 2010;52(1):149-54.

Ghaffari S, Daley GQ, Lodish HF. Growth factor independence and BCR/ABL transformation: promise and pitfalls of murine model systems and assays. Leukemia. 1999;13(8):1200-6.

Gjerde J, Kisanga ER, Hauglid M, Holm PI, Mellgren G, Lien EA. Identification and quantification of tamoxifen and four metabolites in serum by liquid chromatography-tandem mass spectrometry. J Chromatogr A. 2005;1082(1):6-14.

Graham SM, Jørgensen HG, Allan E, Pearson C, Alcorn MJ, Richmond L, Holyoake TL. Primitive, quiescent, Philadelphia-positive stem cells from

patients with chronic myeloid leukemia are insensitive to STI571 in vitro. Blood. 2002;99(1):319-25.

Gratwohl A, Hermans J, Goldman JM, Arcese W, Carreras E, Devergie A, Frassoni F, Gahrton G, Kolb HJ, Niederwieser D, Ruutu T, Vernant JP, de Witte T, Apperley J. Risk assessment for patients with chronic myeloid leukaemia before allogeneic blood or marrow transplantation. Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation. Lancet. 1998;352(9134):1087-92.

Goldman JM, Szydlo R, Horowitz MM, Gale RP, Ash RC, Atkinson K, et al. Choice of pretransplant treatment and timing of transplants for chronic myelogenous leukemia in chronic phase. Blood. 1993;82:2235-8.

Gonzalez O, Alonso RM, Ferreirós N, Weinmann W, Zimmermann R, Dresen S. Development of an LC-MS/MS method for the quantitation of 55 compounds prescribed in combined cardiovascular therapy. J Chromatogr B Analyt Technol Biomed Life Sci. 2011;879(3-4):243-52.

Griffin JD, Todd RF 3d, Ritz J, Nadler LM, Canellos GP, Rosenthal D, Gallivan M, Beveridge RP, Weinstein H, Karp D, Schlossman SF. Differentiation patterns in the blastic phase of chronic myeloid leukemia. Blood. 1983;61:85-91.

Gschwind HP, Pfaar U, Waldmeier F, Zollinger M, Sayer C, Zbinden P, Hayes M, Pokorny R, Seiberling M, Ben-Am M, Peng B, Gross G. Metabolism and disposition of imatinib mesylate in healthy volunteers. Drug Metab. Dispos. 2005;33(10):1503-12.

Gu J, Soldin SJ. Modification of tandem mass spectrometric method to permit simultaneous quantification of 17 anti-HIV drugs which include atazanavir and tipranavir. Clin Chim Acta. 2007;378(1-2):222-4.

Guideline on bioanalytical method validation. European Medicines Agency, Committee for Medicinal Products for Human Use 2011. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_gui deline/2011/08/WC500109686.pdf (8.8.2014)

Guo T, Oswald LM, Mendu DR, Soldin SJ. Determination of levetiracetam in human plasma/serum/saliva by liquid chromatography-electrospray tandem mass spectrometry. Clin Chim Acta. 2007;375(1-2):115-8.

Guo JQ, Wang JY, Arlinghaus RB. Detection of BCR-ABL proteins in blood cells of benign phase chronic myelogenous leukemia patients. Cancer Res. 1991;51:3048-51.

Haouala A, Zanolari B, Rochat B, Montemurro M, Zaman K, Duchosal MA, Ris HB, Leyvraz S, Widmer N, Decosterd LA. Therapeutic drug monitoring of the new targeted anticancer agents imatinib, nilotinib, dasatinib, sunitinib, sorafenib and lapatinib by LC tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2009;877:1982-96.

Harmon DE. Leukemia: Current and Emerging Trends in Detection and Treatment. The Rosen Publishing Group, 2011. 1st edition, ISBN: 978-1-44881-311-7, p. 7-9

Hashimoto Y, Shibakawa K, Nakade S, Miyata Y. Validation and application of a 96-well format solid-phase extraction and liquid chromatography-tandem mass spectrometry method for the quantitation of digoxin in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2008;869 (1-2):126-32.

Hehlmann R, Heimpel H, Hasford J, Kolb HJ, Pralle H, Hossfeld DK, Queisser W, Löffler H, Hochhaus A, Heinze B. Randomized comparison of interferon-alpha with busulfan and hydroxyurea in chronic myelogenous leukemia. The German CML Study Group. Blood. 1994;84:4064–77.

Heisterkamp N, Groffen J. Philadelphia-positive leukemia: a personal perspective. Oncogene. 2002;21(56):8536-40.

Hochhaus A, Baccarani M, Deininger M, Apperley JF, Lipton JH, Goldberg SL, Corm S, Shah NP, Cervantes F, Silver RT, Niederwieser D, Stone RM, Dombret H, Larson RA, Roy L, Hughes T, Müller MC, Ezzeddine R, Countouriotis AM, Kantarjian HM. Dasatinib induces durable cytogenetic

responses in patients with chronic myelogenous leukemia in chronic phase with resistance or intolerance to imatinib. Leukemia. 2008;22(6):1200-6.

Hochhaus A, Berger U, Ansari H, Hasford J, Hehlmann R. Prospective comparative analysis of adverse effects during the treatment of patients with chronic myelogenous leukemia in chronic phase with interferon alpha, busulfan and hydroxyurea. Blood 1997;90(Suppl 1):278b

Hochhaus A, Kantarjian HM, Baccarani M, Lipton JH, Apperley JF, Druker BJ, Facon T, Goldberg SL, Cervantes F, Niederwieser D, Silver RT, Stone RM, Hughes TP, Muller MC, Ezzeddine R, Countouriotis AM, Shah NP. Dasatinib induces notable hematologic and cytogenetic responses in chronic-phase chronic myeloid leukemia after failure of imatinib therapy. Blood. 2007;109(6):2303-9.

Hochhaus A, Lin F, Reiter A, Skladny H, Vanrhee F, Shephers PCA, Allan NC, Hehlman R, Goldman JM, Cross NCP. Variable numbers of BRC-ABL transcripts persist in CML patients who achieve complete cytogenetic remission with interferon-alpha. Brit J Haematol. 1995;91(1):126-31.

Hochhaus A, Reiter A, Skladny H, Reichert A, Saussele S, Hehlmann R. Molecular monitoring of residual disease in chronic myelogenous leukemia patients after therapy. Recent Results Cancer Res. 1998;144:36-45.

Holcapek M, Kolárová L, Nobilis M. Anal. High-performance liquid chromatography-tandem mass spectrometry in the identification and determination of phase I and phase II drug metabolites. Bioanal. Chem. 2008;391(1):59-78.

Honeywell R, Yarzadah K, Giovannetti E, Losekoot N, Smit EF, Walraven M, Lind JSW, Tibaldi C, Verheul HM, Peters GJ. Simple and selective method for the determination of various tyrosine kinase inhibitors used in the clinical setting by liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2010;878:1059–1068.

Hori Y, Fujisawa M, Shimada K, Hirose Y, Yoshioka T. Method for screening and quantitative determination of serum levels of salicylic Acid,

acetaminophen, theophylline, phenobarbital, bromvalerylurea, pentobarbital, and amobarbital using liquid chromatography/electrospray mass spectrometry. Biol Pharm Bull. 2006;29(1):7-13.

Horowitz MM, Rowlings PA, Passweg JR. Allogeneic bone marrow transplantation for CML: a report from the International Bone Marrow Transplant Registry. Bone Marrow Transplant. 1996;17(Suppl 3):S5-6.

Hu Q, Noll RJ, Li H, Makarov A, Hardman M, Graham Cooks R. The Orbitrap: a new mass spectrometer. J Mass Spectrom. 2005;40(4):430-43.

Hunter T. The Croonian Lecture 1997. The phosphorylation of proteins on tyrosine: its role in cell growth and disease. Philos Trans R Soc Lond B Biol Sci. 1998;353:583–605.

Iqbal Z, Elliott M, Watson DG, Holyoake T, Jørgensen H. Analysis of imatinib in bone marrow and plasma samples of chronic myeloid leukaemia patients using solid phase extraction LC-ESI-MS. Pak J Pharm Sci. 2011;24(3):285-91.

Ishikawa Y, Kiyoi H, Watanabe K, Miyamura K, Nakano Y, Kitamura K, Kohno A, Sugiura I, Yokozawa T, Hanamura A, Yamamoto K, Iida H, Emi N, Suzuki R, Ohnishi K, Naoe T. Trough plasma concentration of imatinib reflects BCR-ABL kinase inhibitory activity and clinical response in chronic-phase chronic myeloid leukemia: a report from the BINGO study. Cancer Sci. 2010;101(10):2186-92.

Jiang T, Rong Z, Peng L, Chen B, Xie Y, Chen C, Sun J, Xu Y, Lu Y, Chen H. Simultaneous determination of citalopram and its metabolite in human plasma by LC-MS/MS applied to pharmacokinetic study. J Chromatogr B Analyt Technol Biomed Life Sci. 2010;878(5-6):615-9.

Kang JS, Lee MH. Overview of therapeutic drug monitoring. Korean J Intern Med. 2009;24:1-10.

Kantarjian HM, Deisseroth A, Kurzrock R, Estrov Z, Talpaz M. Chronic myelogenous leukemia: A concise update. Blood. 1993;82:691–703.

154

Kantarjian HM, Dixon D, Keating MJ, Talpaz M, Walters RS, McCredie KB, Freireich EJ. Characteristics of accelerated disease in chronic myelogenous leukemia. Cancer. 1988;61:1441-6.

Kantarjian HM, Giles F, Gattermann N, Bhalla K, Alimena G, Palandri F, Ossenkoppele GJ, Nicolini FE, O'Brien SG, Litzow M, Bhatia R, Cervantes F, Haque A, Shou Y, Resta DJ, Weitzman A, Hochhaus A, le Coutre P. Nilotinib (formerly AMN107), a highly selective BCR-ABL tyrosine kinase inhibitor, is effective in patients with Philadelphia chromosome-positive chronic myelogenous leukemia in chronic phase following imatinib resistance and intolerance. Blood. 2007;110(10):3540-6.

Kantarjian HM, Giles FJ, O'Brien SM, Talpaz M. Clinical course and therapy of chronic myelogenous leukemia with interferon-alpha and chemotherapy. Hematol Oncol Clin North Am. 1998;12:31-80.

Kantarjian H, Giles F, Wunderle L, Bhalla K, O'Brien S, Wassmann B, Tanaka C, Manley P, Rae P, Mietlowski W, Bochinski K, Hochhaus A, Griffin JD, Hoelzer D, Albitar M, Dugan M, Cortes J, Alland L, Ottmann OG. Nilotinib in imatinib-resistant CML and Philadelphia chromosome-positive ALL. N Engl J Med. 2006;354(24):2542-51.

Kantarjian H, Pasquini R, Lévy V, Jootar S, Holowiecki J, Hamerschlak N, Hughes T, Bleickardt E, Dejardin D, Cortes J, Shah NP. Dasatinib or high-dose imatinib for chronic-phase chronic myeloid leukemia resistant to imatinib at a dose of 400 to 600 milligrams daily: two-year follow-up of a randomized phase 2 study (START-R). Cancer. 2009;115(18):4136-47.

Kantarjian H, Sawyers C, Hochhaus A, Guilhot F, Schiffer C, Gambacorti-Passerini C, Niederwieser D, Resta D, Capdeville R, Zoellner U, Talpaz M, Druker B, Goldman J, O'Brien SG, Russell N, Fischer T, Ottmann O, Cony-Makhoul P, Facon T, Stone R, Miller C, Tallman M, Brown R, Schuster M, Loughran T, Gratwohl A, Mandelli F, Saglio G, Lazzarino M, Russo D, Baccarani M, Morra E; International STI571 CML Study Group. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. N Engl J Med. 2002;346: 45-52.

Kantarjian HM, Smith TL, O'Brien S, Beran M, Pierce S, Talpaz M. Prolonged survival in chronic myelogenous leukemia after cytogenetic response to interferon-alpha therapy. The Leukemia Service. Ann Intern Med 1995;122:254–61.

Kantarjian HM, Talpaz M, O'Brien S, Giles F, Garcia-Manero G, Faderl S, Thomas D, Shan J, Rios MB, Cortes J. Dose escalation if imatinib mesylate can overcome resistance to standard-dose therapy in patients with chronic myelogenous leukemia. Blood. 2003;101:473-5.

Kantarjian HM, Talpaz M, Giles F, O'Brien S, Cortes J. New insights into the pathopsysiology of chronic myeloid leukemia and imatinib resistance. Ann Intern Med 2006;145:913-23.

Kantarjian H, Talpaz M, O'Brien S, Garcia-Manero G, Verstovsek S, Giles F, Rios MB, Shan J, Letvak L, Thomas D, Faderl S, Ferrajoli A, Cortes J. High-dose imatinib mesylate therapy in newly diagnosed Philadelphia chromosome-positive chronic phase chronic myeloid leukemia. Blood. 2004;103(8):2873-8.

Keevil BG, Newman S, Lockhart S, Howard SJ, Moore CB, Denning DW. Validation of an assay for voriconazole in serum samples using liquid chromatography-tandem mass spectrometry. Ther Drug Monit. 2004;26(6):650-7.

Klawitter J, Zhang YL, Klawitter J, Anderson N, Serkova NJ, Christians U. Developement and validation of a sensitive assay for the quantification of imatinib using LC/LC-MS/MS in human whole blood and cell culture. Biomed Chromatogr. 2009;23:1251-58.

Koster RA, Dijkers EC, Uges DR. Robust, high-throughput LC-MS/MS method for therapeutic drug monitoring of cyclosporine, tacrolimus, everolimus, and sirolimus in whole blood. Ther Drug Monit. 2009;31(1):116-25.

Kutsev S, Kravchenko E, Turkina A. Imatinib blood level test in Russian federation. EUTOS Blood Level Testing Summit, November 16–17, 2009, Bordeaux, France (lecture).

Larson RA, Druker BJ, Guilhot F, O'Brien SG, Riviere GJ, Krahnke T, Gathmann I, Wang Y, IRIS (International Randomized Interferon vs STI571) Study Group. Imatinib pharmacokinetics and its correlation with response and safety in chronic-phase chronic myeloid leukemia: a subanalysis of the IRIS study. Blood. 2008;111:4022-8.

le Coutre P, Kreuzer KA, Pursche S, et al. Pharmacokinetics and cellular uptake of imatinib and its main metabolite CGP74588. Cancer Chemother Pharmacol 2004;53:313-323.

le Coutre P, Ottmann OG, Giles F, Kim DW, Cortes J, Gattermann N, Apperley JF, Larson RA, Abruzzese E, O'Brien SG, Kuliczkowski K, Hochhaus A, Mahon FX, Saglio G, Gobbi M, Kwong YL, Baccarani M, Hughes T, Martinelli G, Radich JP, Zheng M, Shou Y, Kantarjian H. Nilotinib (formerly AMN107), a highly selective BCR-ABL tyrosine kinase inhibitor, is active in patients with imatinib-resistant or -intolerant accelerated-phase chronic myelogenous leukemia. Blood. 2008;111(4):1834-9.

Lugo TG, Pendergast AM, Muller AJ, Witte ON. Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. Science. 1990;247:1079-82.

Ma S, Xu Y, Shou M. Characterization of imatinib metabolites in rat and human liver microsomes: differentiation of hydroxylation from N-oxidation by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. Rapid Commun. Mass Spectrom. 2009; 23 (10): 1446-50.

Madhusudan S, Ganesan TS. Tyrosine kinase inhibitors in cancer therapy. Clin Biochem. 2004;37(7):618-35.

Mahon FX. Pharmacologic monitoring and determinants of intracytoplasmic drug levels. Best Pract Res Clin Haematol. 2009;22:381-6.

Mahon FX, Réa D, Guilhot J, Guilhot F, Huguet F, Nicolini F, Legros L, Charbonnier A, Guerci A, Varet B, Etienne G, Reiffers J, Rousselot P; Intergroupe Français des Leucémies Myéloïdes Chroniques. Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. Lancet Oncol. 2010;11(11):1029-35.

Makarov A. Electrostatic axially harmonic orbital trapping: a high-performance technique of mass analysis. Anal Chem. 2000;72(6):1156-62.

Manley PW, Cowan-Jacob SW, Buchdunger E, Fabbro D, Fendrich G, Furet P, Meyer T, Zimmermann J. Imatinib: a selective tyrosine kinase inhibitor. Eur J Cancer. 2002;38(Suppl 5):S19–27.

Martinelli G, Soverini S, Rosti G, Cilloni D, Baccarani M. New tyrosine kinase inhibitors in chronic myeloid leukemia. Haematologica 2005;90:534-41.

Marull M, Rochat B. Fragmentation study of imatinib and characterization of new imatinib metabolites by liquid chromatography-triple-quadrupole and linear ion trap mass spectrometers. J. Mass Spectrom. 2006;41(3):390-404.

Matsuura K, Ohmori T, Nakamura M, Itoh Y, Hirano K. A simple and rapid determination of valproic acid in human plasma using a non-porous silica column and liquid chromatography with tandem mass spectrometric detection. Biomed Chromatogr. 2008;22(4):387-93.

Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem 2003;75:3019-3030.

McFarland KL, Wetzstein GA. Chronic myeloid leukemia therapy: Focus on second-generation tyrosine kinase inhibitors. Cancer Control. 2009;16:132-40.

Meinitzer A, Gartner G, Pilz S, Stettin M. Ultra fast liquid chromatography-tandem mass spectrometry routine method for simultaneous determination of cyclosporin A, tacrolimus, sirolimus, and everolimus in whole blood using deuterated internal standards for cyclosporin A and everolimus. Ther Drug Monit. 2010;32(1):61-6.

Melo JV. The diversity of BCR-ABL fusion proteins and their relationship to leukemia phenotype. Blood. 1996;88(7):2375-84.

Miyamura K, Kiyoi H, Ohtake S, Naoe T; Japan Adult Leukemia Study Group. Long-term outcome following imatinib therapy for chronic myelogenous leukemia, with assessment of dosage and blood levels: the JALSG CML202 study. Cancer Sci. 2012;103(6):1071-8.

Mičová K, Friedecký D, Faber E, Adam T. Determination of imatinib levels by capillary electrophoresis at patients with chronic myeloid leukemia. Chem Papers 2009;103:s185-s188.

Mičová K, Friedecký D, Faber E, Polýnková A, Adam T. Flow injection analysis vs. ultra high performance liquid chromatography coupled with tandem mass spectrometry for determination of imatinib in human plasma. Clin Chim Acta. 2010;411:1957-62 b.

Mičová K, Friedecký D, Polýnková A, Faber E, Adam T. Determination of tyrosine kinase levels by UHPLC-MS/MS. Chem Papers 2010;104:31-34 a.

Mitelman F. The cytogenetic scenario of chronic myeloid leukemia. Leuk Lymphoma. 1993;11(Suppl 1):11-5.

Mlejnek P, Novak O, Dolezel P. A non-radioactive assay for precise determination of intracellular levels of imatinib and its main metabolite in Bcr-Abl positive cells. Talanta. 2011;83:1466-71.

Moraes MO, Lerner FE, Corso G, Bezerra FA, Moraes ME, De Nucci G. Fluoxetine bioequivalence study: quantification of fluoxetine and norfluoxetine by liquid chromatography coupled to mass spectrometry. J Clin Pharmacol. 1999;39(10):1053-61.

Muhlmann J, Thaler J, Hilbe W, Bechter O, Erdel M, Utermann G, Duba HC. Fluorescence in situ hybridization (FISH) on peripheral blood smears for monitoring Philadelphia chromosome-positive chronic myeloid leukemia (CML) during interferon treatment: a new strategy for remission assessment. Genes Chromosomes Cancer. 1998;21:90-100.

Nagar B, Bornmann WG, Pellicena P, Schindler T, Veach DR, Miller WT, Clarkson B, Kuriyan J. Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571). Cancer Res. 2002;62:4236–43.

Ni LN, Li JY, Miao KR, Qiao C, Zhang SJ, Qiu HR, Qian SX. Multidrug resistance gene (MDR1) polymorphisms correlate with imatinib response in chronic myeloid leukemia. Med Oncol. 2011;28(1):265-9.

Nirogi RV, Kandikere VN, Shukla M, Mudigonda K, Maurya S, Boosi R, Yerramilli A. Development and validation of a sensitive liquid chromatography/electrospray tandem mass spectrometry assay for the quantification of olanzapine in human plasma. J Pharm Biomed Anal. 2006;41(3):935-42.

Nowell P, Hungerford D. A minute chromosome in human chronic granulocytic leukemia. Science. 1960;132:1497-501.

O'Brien SG, Guilhot F, Larson RA, Gathmann I, Baccarani M, Cervantes F, Cornelissen JJ, Fischer T, Hochhaus A, Hughes T, Lechner K, Nielsen JL, Rousselot P, Reiffers J, Saglio G, Shepherd J, Simonsson B, Gratwohl A, Goldman JM, Kantarjian H, Taylor K, Verhoef G, Bolton AE, Capdeville R, Druker BJ; IRIS Investigators. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. N Engl J Med. 2003;348:994- 1004.

Ohnishi K, Nakaseko C, Takeuchi J, Fujisawa S, Nagai T, Yamazaki H, Tauchi T, Imai K, Mori N, Yagasaki F, Maeda Y, Usui N, Miyazaki Y, Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix

effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem. 2003;75:3019-3030.

Osborn MP, White DL, Saunders VA, Cambareri B, Branford S, Menelaou A, et al. Early dose-escalation in chronic myeloid leukaemia patients with low plasma imatinib levels leads to equivalent BCR-ABL values and drug levels at 6 months to those with optimal drug levels: first analysis from the TIDEL II trial of de-novo patients treated with 600mg imatinib. Blood. 2009;114(22):Abstract 1131.

Ottmann OG, Druker BJ, Sawyers CL, Goldman JM, Reiffers J, Silver RT, Tura S, Fischer T, Deininger MW, Schiffer CA, Baccarani M, Gratwohl A, Hochhaus A, Hoelzer D, Fernandes-Reese S, Gathmann I, Capdeville R, O'Brien SG. A phase 2 study of imatinib in patients with relapsed or refractory Philadelphia chromosome-positive acute lymphoid leukemias. Blood. 2002;100:1965-71.

Pane F, Frigeri F, Sindona M, Luciano L, Ferrara F, Cimino R, Meloni G, Saglio G, Salvatore F, Rotoli B. Neutrophilic-chronic myeloid leukemia: a distinct disease with a specific molecular marker (BCR/ABL with C3/A2 junction). Blood. 1996;88(7):2410-4.

Parise RA, Ramanathan RK, Hayes MJ, Egorin MJ. Liquid chromatographic-mass spectrometric assay for quantitation of imatinib and its main metabolite (CGP 74588) in plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2003;791:39-44.

Park JH, Jhee OH, Park SH, Lee JS, Lee MH, Shaw LM, Kim KH, Lee JH, Kim YS, Kang JS. Validated LC-MS/MS method for quantification of gabapentin in human plasma: application to pharmacokinetic and bioequivalence studies in Korean volunteers. Biomed Chromatogr. 2007;21(8):829-35.

Park JH, Park YS, Lee MH, Rhim SY, Song JC, Lee SJ, Kim JM, Shaw LM, Kang JS. Determination of plasma topiramate concentration using LC-MS/MS for pharmacokinetic and bioequivalence studies in healthy Korean volunteers. Biomed Chromatogr. 2008;22(8):822-9.

161

Patel BN, Sharma N, Sanyal M, Shrivastav PS. Liquid chromatography tandem mass spectrometry assay for the simultaneous determination of venlafaxine and O-desmethylvenlafaxine in human plasma and its application to a bioequivalence study. J Pharm Biomed Anal. 2008;47(3):603-11.

Pavlovsky C, Kantarjian H, Cortes JE. First-line therapy for chronic myeloid leukemia: Past, present, and future. Am J Hematol 2009;84:287-93.

Peng B, Hayes M, Resta D, Racine-Poon A, Druker BJ, Talpaz M, Sawyers CL, Rosamilia M, Ford J, Lloyd P, Capdeville R. Pharmacokinetics and pharmacodynamics of imatinib in a phase I Trial with chronic myeloid leukemia patients. J Clin Oncol 2004;22:935-42.

Picard S, Titier K, Etienne G, Teilhet E, Ducint D, Bernard MA, Lassalle R, Marit G, Reiffers J, Begaud B, Moore N, Molimard M, Mahon FX. Trough imatinib plasma levels are associated with both cytogenetic and molecular responses to standard-dose imatinib in chronic myeloid leukemia. Blood. 2007;109:3496-9.

Potěšil D, Rázga F, Stejskal S, Tesařová L, Šimara P, Peterková M, Dvořáková D, Malášková L, Koutná I, Ráčil Z, Mayer J, Zdráhal Z. Determination of intracellular imatinib concentration at CML patients. XXIV Olomouc Hematology Days, June 24-27, 2010, Olomouc, Czech Republic (poster)

Pursche S, Ottmann OG, Ehninger G, Schleyer E. High-performance liquid chromatography with ultraviolet detection for the quantification of the BCR-ABL inhibitor nilotinib (AMN107) in plasma, urine, culture medium and cell preparations. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;852:208-216.

Quintás-Cardama A, Kantarjian HM, Cortes JE. Mechanisms of primary and secondary resistance to imatinib in chronic myeloid leukemia. Cancer Control 2009;16:122-31.

Raitano AB, Whang YE, Sawyers CL. Signal transduction by wild-type and leukemogenic Abl proteins. Biochim Biophys Acta. 1997;1333(3):F201-16.

Ramirez P, DiPersio P. Therapy Options in Imatinib Failures The Oncologist. 2008;13:424–434.

Rochat B, Fayet A, Widmer N, Lahrichi SL, Pesse B, Décosterd LA, Biollaz J. Imatinib metabolite profiling in parallel to imatinib quantification in plasma of treated patients using liquid chromatography-mass spectrometry. J. Mass Spectrom. 2008;43(6):736-52.

Roche S, McMahon G, Clynes M, O'Connor R. Development of a high-performance liquid chromatographic-mass spectrometric method for the determination of cellular levels of the tyrosine kinase inhibitors lapatinib and dasatinib. J Chromatogr B Analyt Technol Biomed Life Sci. 2009;877(31):3982-90.

Rodríguez-Flores J, Berzas JJ, Castaneda G, Rodríguez N. Direct and fast capillary zone electrophoresic Method for the determination of Gleevec and its main metabolite in human urine. J Chromatogf B Analyt Technol Biomed Life Sci. 2003;794:381-388.

Rodríguez-Flores J, Berzas Nevado JJ, Contento Salcedo AM, Cabello Díaz MP. Nonaqueous capillary electrophoresis metod for the analysis of gleevec and its main metabolite in human urine. J Chromatogr A. 2005;1068:175-182.

Roskoski R Jr. STI-571: an anticancer protein-tyrosine kinase inhibitor. Biochem Biophys Res Commun. 2003;309:709-17.

Roth O, Spreux-Varoquaux O, Bouchet S, Rousselot P, Castaigne S, Rigaudeau S, Raggueneau V, Therond P, Devillier P, Molimard M, Maneglier B. Imatinib assay by HPLC with photodiode-array UV detection in plasma from patients with chronic myeloid leukemia: Comparison with LC-MS/MS. Clin Chim Acta. 2010;411:140-6.

Rouzes A, Berthoin K, Xuereb F, Djabarouti S, Pellegrin I, Pellegrin JL, Coupet AC, Augagneur S, Budzinski H, Saux MC, Breilh D. Simultaneous determination of the antiretroviral agents: amprenavir, lopinavir, ritonavir, saquinavir and efavirenz in human peripheral blood mononuclear cells by

high-performance liquid chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2004;813(1-2):209-16.

Rowley JD. A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature. 1973;243:290-3.

Rumpold H, Webersinke G. Molecular pathogenesis of Philadelphia-positive chronic myeloid leukemia - is it all BCR-ABL? Curr Cancer Drug Targets. 2011;11(1):3-19.

Sakai M, Miyazaki Y, Matsuo E, Moriuchi Y, Hata T, Fukushima T, Imaizumi Y, Imanishi D, Taguchi J, Iwanaga M, Tsushima H, Inoue Y, Takasaki Y, Tsuchiya T, Komoda M, Ando K, Horio K, Moriwaki Y, Tominaga S, Itonaga H, Nagai K, Tsukasaki K, Tsutsumi C, Sawayama Y, Yamasaki R, Ogawa D, Kawaguchi Y, Ikeda S, Yoshida S, Onimaru Y, Tawara M, Atogami S, Koida S, Joh T, Yamamura M, Matsuo Y, Soda H, Nonaka H, Jinnai I, Kuriyama K, Tomonaga M. Long-term efficacy of imatinib in a practical setting is correlated with imatinib trough concentration that is influenced by body size: a report by the Nagasaki CML Study Group. Int J Hematol. 2009;89(3):319-25.

Savage DG, Szydlo RM, Goldman JM. Clinical features at diagnosis in 430 patients with chronic myeloid leukemia seen at a referral centre over a 16-year period. Br J Haematol. 1997;96:111-6.

Sawyers CL, Hochhaus A, Feldman E, Goldman JM, Miller CB, Ottmann OG, Schiffer CA, Talpaz M, Guilhot F, Deininger MW, Fischer T, O'Brien SG, Stone RM, Gambacorti-Passerini CB, Russell NH, Reiffers JJ, Shea TC, Chapuis B, Coutre S, Tura S, Morra E, Larson RA, Saven A, Peschel C, Gratwohl A, Mandelli F, Ben-Am M, Gathmann I, Capdeville R, Paquette RL, Druker BJ. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. Blood. 2002;99:3530-39.

Shah VP, Midha KK, Findlay JWA, Hill HM, Hulse JD, McGilveray IJ, McKay G, Miller KJ, Patnaik RN, Powell ML, Tonelli A, Viswanathan CT, Yacobi A. Bioanalytical Method Validation – A Revisit with a Decade of Progress. Pharmaceut Res. 2000;17(12):1551-1557.

Shah NP, Nicoll JM, Nagar B, Gorre ME, Paquette RL, Kuriyan J, Sawyers CL. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. Cancer Cell. 2002;2(2):117-25.

Shah NP, Witte ON, Denny CT. Characterization of the BCR promoter in Philadelphia chromosome-positive and -negative cell lines. Mol Cell Biol. 1991;11(4):1854-60.

Simonsen KW, Steentoft A, Buck M, Hansen L, Linnet K. Screening and quantitative determination of twelve acidic and neutral pharmaceuticals in whole blood by liquid-liquid extraction and liquid chromatography-tandem mass spectrometry. J Anal Toxicol. 2010;34(7):367-73.

Singh N, Kumar L, Meena R, Velpandian T. Drug monitoring of imatinib levels i patiens undergoing therapy for chronic myeloid leukaemia: comparing plasma levels of responders and non-responders. Eur J Clin Pharmacol. 2009;65:545-9.

Slattery JT, Clift RA, Buckner CD, Radich J, Storer B, Bensinger WI, Soll E, Anasetti C, Bowden R, Bryant E, Chauncey T, Deeg HJ, Doney KC, Flowers M, Gooley T, Hansen JA, Martin PJ, McDonald GB, Nash R, Petersdorf EW, Sanders JE, Schoch G, Stewart P, Storb R, Sullivan KM, Thomas ED, Witherspoon RP, Appelbaum FR. Marrow transplantation for chronic myeloid leukemia: the influence of plasma busulfan levels on the outcome of transplantation. Blood. 1997;89(8):3055-60.

Sokal JE, Cox EB, Baccarani M, Tura S, Gomez GA Robertson JE, Tso CY, Braun TJ, Clarkson BD, Cervantes F. Prognostic discrimination in "good-risk" chronic granulocytic leukemia. Blood. 1984;63(4):789-799.

165

Strathmann FG, Hoofnagle AN. Current and future applications of mass spectrometry to the clinical laboratory. Am J Clin Pathol. 2011;136(4):609-16.

Stearns V, Johnson MD, Rae JM, Morocho A, Novielli A, Bhargava P, Hayes DF, Desta Z, Flockhart DA. Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. J Natl Cancer Inst. 2003;95(23):1758-63.

Streit F, Armstrong VW, Oellerich M. Rapid Liquid Chromatography–Tandem Mass Spectrometry Routine Method for Simultaneous Determination of Sirolimus, Everolimus, Tacrolimus, and Cyclosporin A in Whole Blood. Clin Chem. 2002;48(6):955-58.

Takahashi N, Wakita H, Miura M, Scott SA, Nishii K, Masuko M, Sakai M, Maeda Y, Ishige K, Kashimura M, Fujikawa K, Fukazawa M, Katayama T, Monma F, Narita M, Urase F, Furukawa T, Miyazaki Y, Katayama N, Sawada K. Correlation between imatinib pharmacokinetics and clinical response in Japanese patients with chronic-phase chronic myeloid leukemia. Clin Pharmacol Ther. 2010;88(6):809-13.

Talpaz M, Silver RT, Druker BJ, Goldman JM, Gambacorti-Passerini C, Guilhot F, Schiffer CA, Fischer T, Deininger MW, Lennard AL, Hochhaus A, Ottmann OG, Gratwohl A, Baccarani M, Stone R, Tura S, Mahon FX, Fernandes-Reese S, Gathmann I, Capdeville R, Kantarjian HM, Sawyers CL. Imatinib induces durable hematologic and cytogenetic responses in patients with accelerated phase chronic myeloid leukemia: results of a phase 2 study. Blood. 2002;99:1928-37.

Taylor PJ. Therapeutic drug monitoring of immunosuppressant drugs by high-performance liquid chromatography-mass spectrometry. Ther Drug Monit. 2004;26(2):215-9.

The Italian Cooperative Study Group on chronic myeloid leukemia. Interferon alfa-2a as compared with conventional chemotherapy for the treatment of chronic myeloid leukemia. N Engl J Med. 1994;330:820-5.
166

Thielen N, van der Holt B, Verhoef GE, Ammerlaan RA, Sonneveld P, Janssen JJ, Deenik W, Falkenburg JH, Kersten MJ, Sinnige HA, Schipperus M, Schattenberg A, van Marwijk Kooy R, Smit WM, Chu IW, Valk PJ, Ossenkoppele GJ, Cornelissen JJ. High-dose imatinib versus high-dose imatinib in combination with intermediate-dose cytarabine in patients with first chronic phase myeloid leukemia: a randomized phase III trial of the Dutch-Belgian HOVON study group. Ann Hematol. 2013;92(8):1049-56.

Thomas J, Wang L, Clark RE, Pirmohamed M. Active transport of imatinib into and out of cells: implications for drug resistance. Blood. 2004;104:3739-45.

Titier K, Picard S, Ducint D, Teilhet E. Moore N, Berthaud P, Mahon FX, Molimard M. Quantification of imatinib in human plasma by high-performance liquid chromatography-tandem mass spectrometry. Ther Drug Monit. 2005;27:634-40.

van Erp NP, Gelderblom H, Guchelaar HJ. Clinical pharmacokinetics of tyrosine kinase inhibitors. Cancer Treat Rev. 2009;35:692-706.

van Rhee F, Hochhaus A, Lin F, Melo JV, Goldman JM, Cross NC. p190 BCR-ABL mRNA is expressed at low levels in p210-positive chronic myeloid and acute lymphoblastic leukemias. Blood. 1996;87(12):5213-7.

Van Rooyen GF, Badenhorst D, Swart KJ, Hundt HK, Scanes T, Hundt AF. Determination of carbamazepine and carbamazepine 10,11-epoxide in human plasma by tandem liquid chromatography-mass spectrometry with electrospray ionisation. J Chromatogr B Analyt Technol Biomed Life Sci. 2002;769(1):1-7.

Velpandian T, Mathur R, Agarwal NK, Arora B, Kumar L, Gupta SK. Development and validation of simple liquid chromatographic method with ultraviolet detection for the determination of imatinib in biological samples. J Chromatogr B Analyt Technol Biomed Life Sci. 2004;804:431-434.

Vivekanand VV, Sreenivas Rao D, Vaidyanathan G, Sekhar NM, Avijit Kelkar S, Ramachandra Puranik P. A validated LC method for imatinib mesylate. J Pharm Biomed Anal. 2003;33:879-89.

Vogeser M, Seger C. A decade of HPLC-MS/MS in the routine clinical laboratory-goals for further developments. Clin Biochem. 2008;41(9):649-62.

Vogeser M, Seger C. Pitfalls associated with the use of liquid chromatography-tandem mass spectrometry in the clinical laboratory. Clin Chem. 2010;56(8):1234-44.

Voncken JW, Kaartinen V, Pattengale PK, Germeraad WT, Groffen J, Heisterkamp N. BCR/ABL P210 and P190 cause distinct leukemia in transgenic mice. Blood. 1995;86:4603-11.

Wang JY. Abl tyrosine kinase in signal transduction and cell-cycle regulation. Curr Opin Genet Dev. 1993;3(1):35-43.

Widmer N, Béguin A, Rochat B, Buclin T, Kovacsovics T, Duchosal MA, Leyvraz S, Rosselet A, Biollaz J, Decosterd LA. Determination of imatinib (Gleevec) in human plasma by solid-phase extraction-liquid chromatography-ultraviolet absorbance detection. J Chromatogr B Analyt Technol Biomed Life Sci. 2004;803:285-92.

Widmer N, Decosterd LA, Csajka C, Leyvraz S, Duchosal MA, Rosselet A, Rochat B, Eap CB, Henry H, Biolazz J, Buclin T. Population pharmacokinetics of imatinib and the role of α1-acid glycoprotein. Br J Clin Pharmacol. 2006;62:97-112.

White DL, Saunders VA, Dang P, Engler J, Zannettino ACW, Cambareri AC, Quinn SR, Manley PW, Hughes TP. OCT-1 mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib (AMN107): reduced OCT-1 activity is the cause of low in vitro sensitivity to imatinib. Blood. 2006;108:697-704.

White DL, Saunders VA, Dang P, Engler J, Venables A, Zrim S, Zannettino A, Lynch K, Manley PW, Hughes T. Most CML patients who have a suboptimal response to imatinib have low OCT-1 activity: higher doses of imatinib may overcome the negative impact of low OCT-1 activity. Blood. 2007;110:4064-72.

Xian F, Hendrickson CL, Marshall AG. High resolution mass spectrometry. Anal Chem. 2012;84(2):708-19.

Xiao W, Chen B, Yao S, Cheng Z. Simultaneous determination of erythromycin propionate and base in human plasma by high-performance liquid chromatography-electrospray mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2005;817(2):153-8.

Zhang Y, Mehrotra N, Budha NR, Christensen ML, Meibohm B. A tandem mass spectrometry assay for the simultaneous determination of acetaminophen, caffeine, phenytoin, ranitidine, and theophylline in small volume pediatric plasma specimens. Clin Chim Acta. 2008;398(1-2):105-12.

Zhong JS, Meng FY, Xu D, Zhou HS, Dai M. Correlation between imatinib trough concentration and efficacy in Chinese chronic myelocytic leukemia patients. Acta Haematol. 2012;127(4):221-7.

Zhu Z, Neirinck L. High-performance liquid chromatography-mass spectrometry method for the determination of paroxetine in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2002;780(2):295-300.

Zubarev RA, Makarov A. Orbitrap mass spectrometry. Anal Chem. 2013;85(11):5288-96.

http://www.cancer.gov/cancertopics/pdq/treatment/CML/Patient/page1 (8.8.2014)

http://www.cixip.com/index.php/page/content/id/612

(8.8.2014)

http://www.magnet.fsu.edu/education/tutorials/tools/ionization_esi.html
(8.8.2014)

http://www.recipe.de/en/index.html
(8.8.2014)

http://chromsystems.com/en-gb/produkte/therapeutisches-drug-monitoring
(8.8.2014)

http://www.pharmatutor.org/articles/overview-liquid-chromatography-coupled-tandem-mass-spectroscopy-lc-ms
(8.8.2014)

http://www.hematology.org/Patients/Cancers/Leukemia.aspx
(8.8.2014)

http://www.cancer.gov/researchandfunding/snapshots/leukemia
(8.8.2014)

http://www.chemheritage.org/discover/online-resources/chemistry-in-history/themes/pharmaceuticals/restoring-and-regulating-the-bodys-biochemistry/hitchings--elion.aspx
(8.8.2014)

8. List of author's publications

A) Publications associated with the thesis

<u>A1) Original research articles published in journals with IF</u>

- **Micova K.,** Friedecky D., Faber E., Polynkova A., Adam T. Flow injection analysis vs. ultra high performance liquid chromatography coupled with tandem mass spectrometry for determination of imatinib in human plasma. Clin Chim Acta. 2010;411:1957-1962. IF(2010) = 2.85

- **Mičová K.**, Friedecký D., Polýnková A., Faber E. a Adam T.: Stanovení hladiny tyrosinkinasových inhibitorů metodou UHPLC-MS/MS. Chem. Listy. 2010;104:31-34. IF(2010) = 0.754

- Faber E., Friedecky D., **Micova K.**, Divoka M., Katrincsakova B., Rozmanova S., Jarosova M., Indrak K., Adam T: Imatinib dose escalation in two patients with chronic myeloid leukemia, with low trough imatinib plasma levels measured at various intervals from the beginning of the therapy and with suboptimal treatment response, leads to the achievement of higher plasma levels and major molecular response. Int J Hematol. 2010;9(15):897-902. IF(2010) = 1.324

- **Micova K.,** Friedecky D., Faber E., Adam T. Isotope dilution direct injection mass spectrometry method for determination of four tyrosine kinase inhibitors in human plasma. Talanta. 2012;93:307-13. IF(2012)=3.498

- Faber E., Friedecky D., **Micova K.**, Rozmanova S., Divoka M., Jarosova M., Indrak K., Adam T. Imatinib trough plasma levels do not correlate with the response to therapy in patients with chronic myeloid leukemia in routine clinical setting. Ann Hematol. 2012;91:923-9. IF(2012) = 2.866

<u>A2) Original research articles published in reviewed journals</u>

- **Mičová K.,** Friedecký D., Faber E., Adam T.: Rutinní terapeutické monitorování tyrosinkinázových inhibitorů

metodou LC-MS/MS. Klin. Biochem. Metab. 2012;20(41): 222–225.

A3) Published abstracts

- Faber E, Friedecky D, Micova K, Skoumalova I, Rohon P, Rozmanova S, Divoka M, Jarosova M, Indrak K, Adam T. Measurement of trough imatinib plasma levels in patients with CML does not significantly correlate with treatment response but may be succesfully used in selected patients for dosage adjustment. Haematologica – The Hematology Journal. 2010;95:343-344, Suppl. 2, Meeting Abstract 0821

- Friedecký, D., Faber, E., Micova, K., Indrak, K. & Adam, T. Hightroughput Blood Level Testing of Imatinib by Flow Injection Analysis - Tandem Mass Spectrometry. Haematol-Hematol J. 2010;95:535-535. IF(2010) = 6.532

A4) Poster presentations

- Mičová K., Polýnková A., Friedecký D., Adam T.: Determination of tyrosine kinase inhibitors in human plasma by UHPLC-MS/MS. 25th International symposium on microscale bioseparations, Praha, Czech Republic 21. - 25. 3. 2010 Final program & Book of abstracts, p. 113, ISBN 978-80-254-6631-5.

- Mičová K, Friedecký D, Adam T. Determination of tyrosine kinase inhibitors in human plasma by UHPLC-MS/MS. FONS 2010, Hradec Králové, Czech Republic 20.–21. 9. 2010 Final program & Book of abstracts, p. 103, ISBN 978-80-903879-8-0.

- Mičová K, Friedecký D and Adam T. Determination of tyrosine kinase inhibitors in human plasma by UHPLC-MS/MS. Advances in Metabolic Profiling, European Biomarkers Summit, Mass Spec Europe and Advances in Protein Crystallography 2010 conferences. Florence, Italy 9.–7. 11. 2010 Book of abstracts, p. 316.

172

- Mičová K, Friedecký D, Faber E, Adam T. Flow injection analysis coupled with tandem mass spectrometry for determination of tyrosine kinase inhibitors in human plasma. 1. konference České Společnosti pro Hmotnostní spektrometrii. Hradec Králové, Czech Republic 19.-21. 10. 2011, Sborník příspěvků, s. 61, ISBN 978-80-905045-0-9.

- Friedecky D, Faber E, Micova K, Indrák K, Adam T. Hightroughput analysis of imatinib by flow injection analysis – tandem mass spectrometry. 1st AB SCIEX European conference on MS/MS. Noordwijkerhout, The Netherlands 6.–7. 10. 2010 Book of abstracts, p. 3

- Micová K, Friedecky D, Faber E, Adam T. Isotope dilution direct injection mass spectrometry method for determination of tyrosine kinase inhibitors in human plasma; 27th International symposium on microscale bioseparations and analyses, Geneva, Switzerland 12.–15. 2. 2012 Book of abstracts, p. 019.

- Mičová K, Friedecký D, Faber E, Adam T. Isotope dilution direct injection mass spectrometry for therapeutic drug monitoring in clinical laboratories; 30th Informal Meeting on Mass Spectrometry. Olomouc, 29.4. – 3.5. 2012 Acta Universitatis Palackianae Olomucensis - Chemica 49S, p. 89, ISBN 978-80-244-3047-8.

- Mičová K, Friedecký D, Faber E, Adam T. Isotope dilution direct injection mass spectrometry for therapeutic drug monitoring. XXXIII Nordic Congress in Clinical Chemistry; Reykjavik, Island 12.–15. 6. 2012, Book of abstracts, p. 53.

- Mičová K, Friedecký D, Faber E, Hrdá M, Adam T. Biotransformations of tyrosine kinase inhibitor - imatinib by high resolution mass spectrometry; Asilomar Conference on Mass Spectrometry in Environmental Chemistry, Toxicology and Health, 18–22.10. 2013, Monterey, CA, USA.

173

A5) Oral presentations

- Mičová K, Friedecký D, Tomková J, Semeniuk T, Peřinová J, Zábranská A, Ševčíková J, Kittlová L, Růžičková V, Kapustová M, Ludvová M, Adam T. Routine tandem mass specrtrometry in quantitative drug analysis. 13th Training of mass spectrometry, 2.–7. September 2012. Srní, Czech Republic, Book of abstracts, p. C1. ISBN 978-80-7395-523-6

- Mičová K, Friedecký D, Faber E, Adam T. Routine TDM of tyrosine kinase inhibitors. LC/MS for drug determination and IMD diagnostics. Workshop of Department of Clinical Biochemistry, University Hospital Olomouc, 5. 10. 2011, Olomouc, Czech Republic

- Mičová K, Friedecký D, Faber E, Adam T. Flow injection analysis coupled with tandem mass spectrometry for determination of tyrosine kinase inhibitors in human plasma. Conference of scientific work of students DSP 2011, Olomouc, 6.–7. 9. 2011. Abstracts, p. 51, ISBN 978-80-244-2847-5.

- Mičová K, Friedecký D, Faber E, Hrdá M, Adam T. Imatinib metabolite profiling using high-resolution mass spectrometry. Conference of scientific work of students DSP 2013, Olomouc, 16.–17. 12. 2013. Abstracts, p. 33.

B) **Other publications**

B1) Original research articles published in journals with IF

- Mičová K., Friedecký D., Faber E., Adam T. Determination of imatinib levels using capillary electrophoresis in patients with chronic myeloid leukaemia. Chem Papers. 2009;103:185-88. IF(2009) = 0.791

B2) Original research articles published in reviewed journals

- Ondra P., Válka I., Mičová K., Dobiáš M., Vitovják M.: Úmrtí mladé ženy po transdermální aplikaci fentanylu. Folia Societatis Medicinae Legalis Slovacae. 2013;3(1):22-26.

B3) Published abstracts

- Faber, E., Friedecky, D., Micova, K., Adam, T., Indrak, K. Determination of Imatinib in Plasma by High Performance Capillary Electrophoresis. Haematol-Hematol J. 2008;93:442-443. IF(2008) = 5.978