Supplementary methods to:

Cellular uptake of imatinib into leukemic cells is independent of human organic cation transporter 1 (OCT1)

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Supplementary Methods

Chemicals

[^{3}H]1-Methyl-4-phenylpyridinium ([^{3}H]MPP, 2.96 TBq/mmol) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). [1-^{14}C]Tetraethylammonium bromide ([^{14}C]TEA, 130 MBq/mmol) was from PerkinElmer (Boston, MA, USA). [^{14}C]imatinib (1.95 GBq/mmol; Gleevec, formerly STI571) was a kind gift from Novartis Pharma AG (Basel, Switzerland), and [^{3}H]imatinib was from Moravek Biochemicals (Brea, CA). Nonradiolabeled imatinib was from Sequoia Research Products Ltd (Pangbourne, UK). *N*-Desmethyl imatinib, imatinib-d_8, and *N*-desmethyl imatinib-d_8 were from Toronto Research Chemicals (North York, Canada). All other chemicals were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO).

Transport studies using OCT1-expressing oocytes

Oocytes of *Xenopus laevis* were isolated, collagenase-treated and stored in Ori buffer as previously described (1). 50 nl of H_2O containing 10 ng of human *OCT1/SLC22A1* cRNA were injected per oocyte. Oocytes were then stored for 3 days in Ori buffer at 16°C. For transport measurements, OCT1-expressing oocytes and non-injected control oocytes were incubated at room temperature for 30 minutes in Ori buffer containing 12 nmol/L [^{3}H]MPP or 1 µmol/L [^{14}C]imatinib in the absence or presence of the OCT1 inhibitor tetrabutylammonium (TBuA). The oocytes were then washed 3 times in ice-cold Ori buffer, solubilized in 5% sodium dodecyl sulfate (SDS), and intracellular radioactivity was analyzed by liquid scintillation counting (Packard TriCarb 1600; PerkinElmer, Waltham, MA, USA).

Generation of a HEK cell line expressing OCT1 variant p.V408

Generation of the vector with the *SLC22A1* reference sequence NM_003057 has been previously described (2). The *SLC22A1* cDNA was excised from this vector using the XhoI/BamHI restriction sites and cloned into expression vector pcDNA3.1(-) (Invitrogen/Life Technologies, Grand Island, NY, USA). This vector was used as template for mutagenesis. Variant SLC22A1 vector encoding OCT1-c.1222A>G (rs628031, NP_003048:p.Met408Val), was constructed using the QuikChange multi site-directed mutagenesis kit (Stratagene/Agilent Technologies, Santa Clara, CA, USA). The vector was sequenced to confirm presence of the intended variant and absence of any other nucleotide exchange in the *SLC22A1* cDNA. Human embryonic kidney 293 (HEK) cells (CRL-1573; American Type Culture Collection, Manassas, VA) were grown in DMEM (Sigma) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin, and 100 µg/ml streptomycin (Lonza, Basel, Switzerland) at 37°C and 5% CO_2 and transfected with Metafectene Pro (Biontex, München, Germany) as described (2). Cell clones stably expressing OCT1 variant were selected with 800 µg/ml G418.

Transport studies using OCT1-expressing and CML cell lines

For analyzing the inhibition of probe substrate uptake by imatinib, confluent CHO-OCT1 cells were washed with Dulbecco's phosphate-buffered saline (D-PBS), suspended by shaking, collected by centrifugation and suspended at 37°C in D-PBS as described (3). Cells were incubated with 0.1 µmol/L [^{3}H]MPP and different imatinib concentrations from 1 nmol/L to 200 µmol/L covering a wide range to adequately determine the IC_{50}. As previously described (3,4) uptake was stopped after 1 sec by addition of ice-cold PBS containing 100 µM quinine (stop solution) as described. Cells were washed 3 times with ice-cold stop solution, solubilized with 4 mol/L guanidine thiocyanate and analyzed for radioactivity by liquid

scintillation counting (Packard TriCarb 1600). Apparent IC_{50} values were obtained by fitting the Hill equation to uptake measurements. The IC_{50} value is virtually identical to the K_i value because the employed MPP concentration was >100 times lower than the K_m value for MPP (5).

In general, uptake experiments with probe substrates, imatinib or *N*-desmethyl imatinib into OCT1-transfected cells grown to confluence in a monolayer were performed following previously published procedures (2,3,6). K562, Meg-01, and LAMA-84 cells growing in suspension were seeded at a density of 200000 cells/500 µl in a 24-well cell culture plate. Cell line authentication was performed using the PowerPlex 21 kit (Promega, Madison, USA). Transport was initiated by adding 500 µl of medium containing either probe substrate or imatinib. Uptake was stopped after 10 minutes by centrifuging the cells at 290 *x*g (5 minutes) and washing the cell pellet 2 times with ice-cold PBS PBS as described previously (3,4). All uptake experiments were performed at 37°C. Uptake was stopped either within the initial uptake phase (up to 15 minutes) or after 120 minutes.

For determination of intracellular accumulation of radiolabeled substances, cells were lysed with 0.2% SDS and radioactivity was determined as described (6). For determination of intracellular accumulation of unlabeled imatinib and *N*-desmethyl imatinib, cells were lysed with 1% formic acid in acetonitrile/uptake buffer (50/50; vol/vol) containing 1 µmol/L imatinib-d_8 and 1 µmol/L *N*-desmethyl imatinib-d_8, respectively, as internal standard. Cells were frozen 3 times in liquid nitrogen and rapidly thawed at 37°C. Cell solutions were then subjected to ultrasound treatment using 3 pulses of 3 sec at 4°C (Sonopuls HD200, Bandelin GmbH, Berlin, Germany) and centrifuged at 15000 *x*g (5 minutes, 4°C). Imatinib was measured in the supernatants by LC-MS-MS using a triple quadrupole mass spectrometer coupled to an HPLC system.

Uptake of imatinib was also continuously monitored at 37 °C using the scintillation proximity assay (7) and a microplate reader with a heatable stage (Plate Chameleon V, Hidex Oy, Turku, Finland). For this, HEK-OCT1 cells were grown as described (6) on Cytostar-T 96-well scintillating plates (PerkinElmer) and washed with uptake buffer prior to transport experiments. Uptake was initiated by replacing this solution with uptake buffer containing 10 µmol/L [^{14}C]imatinib. After 50 minutes, cells were washed 3 times with ice-cold uptake buffer and lysed with 0.2% SDS.

Protein content in lysed cells was measured as described (2,6).

Processing of mouse tissue for imatinib analysis

Liver and plasma samples were processed as follows and spiked with imatinib-d_8 (500 ng/sample) as internal standard. Liver samples (20 – 30 mg) were homogenized in 0.9% NaCl using a pestle and centrifuged (10 minutes, 14000 *x*g). The supernatants and the plasma samples were deproteinized with a 3-fold volume of acetonitrile. After dilution of the supernatant with a 3-fold volume of water and an additional centrifugation step, imatinib was quantified by an assay based on LC-MS-MS. Calibration samples were prepared by adding varying amounts of imatinib (10 - 1000 ng) to 10 µl of blank plasma or 50 to 4000 ng per 20 mg of untreated liver tissue.

Calibration samples for *N*-desmethyl imatinib were prepared in acetonitrile/uptake buffer (50/50; vol/vol) with 1 % formic acid containing 1 µmol/L *N*-desmethyl imatinib-d_8 as internal standard in the concentration range 20 nmol/L to 1000 nmol/L.

Calibration samples were prepared as the samples, and analyzed together with the unknown samples. Calibration curves based on internal standard calibration were obtained by weighted ($1/x^2$) linear regression for the peak area ratio of the analyte to the respective

internal standard against the amount of the analyte. The concentration in unknown samples was obtained from the regression line.

LC-MS-MS analysis of imatinib

An Agilent 6460 triple quadrupole mass spectrometer (Agilent, Waldbronn, Germany) coupled to an Agilent 1200 HPLC system consisting of degasser G1379B, binary pump G1312B, well-plate sampler G1367D and column thermostat G1316B was used. Ionization mode was electrospray (ESI), polarity positive. Electrospray jetstream conditions were as follows: capillary voltage 3500 V, nozzle voltage 1000 V, drying gas flow 10 l/minute nitrogen, drying gas temperature 325 °C, nebulizer pressure 20 psi, sheath gas temperature 350 °C, sheath gas flow 11 l/minute. HPLC separation was achieved on a SymmetryShield RP8 column (50×2.1 mm I.D., 3.5 µm particle size, Waters, Eschborn, Germany) using (A) 10 mmol/L ammonium formate and 0.2% formic acid in water, pH 3 and (B) 0.2% formic acid in acetonitrile as mobile phases at a flow rate of 0.4 ml/minute. Gradient runs for imatinib and *N*-desmethyl imatinib were programmed as follows: 10% B from 0 to 1 minute, increase to 40% B to 3 minutes, remaining at 40% B to 8 minutes, then re-equilibration. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. MRM transitions, dwell time, fragmentor voltage and collision energy for analytes and internal standards are summarized in Supplementary Table S2.

RNA isolation and quantification

Total RNA was isolated from CML cell lines, primary $CD34^+$ cells and transfected HEK cells using the mirVana isolation kit according to the manufacturer's protocol (Applied Biosystems/Ambion, Foster City, CA). RNA quality of each sample was controlled with the Agilent 2100 Bioanalyzer using the RNA6000 Nano Lab Chip kit (Agilent, Santa Clara, CA).

One µg of total RNA was reverse-transcribed as described (3). For isolation of total RNA from whole blood or bone marrow samples the mirVana kit was used as well. 200 ng total RNA was reverse transcribed in a final volume of 20 µl using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Quantification was performed by RT-PCR using TaqMan technology and an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). *OCT1/SLC22A1* transcript levels were determined with a primer-probe assay as previously described (3). For screening of mRNA expression of 55 selected drug transporters of the solute carrier (SLC) family, considered to be important for drug uptake by the PharmaADME Consortium (Supplementary Table S3), cDNAs from primary $CD34^+$ cells from CP-CML Ph^+ patients or from Ph^- non-CML donors were pooled and measured by pre-developed TaqMan assays (Applied Biosystems). Assay information is available on request. Samples were analyzed in triplicate and mean values were normalized to the expression of *β-actin* mRNA.

shRNA knockdown

Three different *OCT1/SLC22A1* specific shRNAs and scrambled control were purchased from OriGene (Rockville, MD, USA), and gene silencing was performed on MV4-11 cells using Amaxa nucleofector and cell line nucleofector kit L (Lonza, NJ, USA). After 72-h incubations, knockdown efficiency was confirmed by RT-PCR. In addition, efficient knockdown of protein expression was confirmed according to the manufacturer's instructions. For this, HEK cells were seeded into 6-well plates and transiently transfected with 1 µg of the previously-generated *SLC22A1* expression plasmid (2) and 1 µg of respective shRNA plasmid (shRNA(1): TR309380B, shRNA(2): TR309380C, shRNA(3): TR309380D, scrambled: TR30012) using TurboFection 8.0 solution (OriGene). After 72-h incubations, membrane fractions were prepared and immunoblotting onto nitrocellulose membranes was performed as described (3). OCT1 was detected with the previously-described OCT1 antiserum (1:3000

dilution) (3). The nitrocellulose membrane was then stripped by 30 min incubation with Restore™ Western Blot Stripping Buffer (ThermoScientific, Rockford, IL, USA) and then incubated with a mouse monoclonal anti-β-actin antibody (Sigma; 1:5000, 16 h at 4 °C) and, after washing with TBS-T, with secondary goat anti-mouse horseradish peroxidase antibody (Santa Cruz Biotechnology, Santa Cruz, USA; 1:5000, 1 h at room temperature). The OCT1-specific shRNA plasmids efficiently silenced OCT1 protein expression (Supplementary Figure S5).

Flow cytometry and confocal laser scanning microscopy

CML cell lines, primary $CD34^+$ from CP-CML Ph^+ and Ph^- non-CML donors, cultured as described (8), and transfected HEK cells were immunolabeled with a previously-validated OCT1-specific polyclonal rabbit antiserum, able to distinguish graded levels of cellular OCT1 protein (3), and the corresponding pre-immune serum (negative control), both at a 1:3000 dilution, using the Fix&Perm staining kit according to the manufacturer's protocol (An der Grub Research GmbH, Kaumberg, Austria). An AlexaFluor488-conjugated goat anti-rabbit IgG was the secondary antibody (Invitrogen/Molecular Probes, Carlsbad, CA). Cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA). For each cell type, the median fluorescence with the pre-immune serum was subtracted from the median fluorescence with the OCT1 antiserum. In a parallel sample, DNA of immunolabeled cells was counterstained with TO-PRO-3 iodide (1:2000, Invitrogen/Molecular Probes). Pictures were taken with a confocal laser scanning microscope (TCS NT Confocal System, Leica Microsystems, Wetzlar, Germany).

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