OCT-1 function varies with cell lineage but is not influenced by BCR-ABL

Jane R. Engler,[1,2,3] Andrew C.W. Zannettino,[1,2,3] Charles G. Bailey,[4] John E.J. Rasko,[4,5] Timothy P. Hughes,[1,2,3] and Deborah L. White[1,2,3]

[1]Department of Haematology, SA Pathology (RAH Campus), Adelaide; [2]School of Medicine, The University of Adelaide, Adelaide; [3]Centre for Cancer Biology, Adelaide; [4]Centenary Institute, Sydney; [5]Cell and Molecular Therapies, Royal Prince Alfred Hospital, Sydney, Australia

Acknowledgments: the authors would like to thank Don Anson for providing the pHIV-1SDm lentiviral construct; Kate Pilkington for performing FACS; Sharon Paton for her assistance in morphological assessment; Teresa Sadras for her assistance in cell cycle analysis and members of DCP for performing Wright's stains. We would also like to thank Novartis Pharmaceuticals for the provision of imatinib and [14C]-imatinib. J Engler is supported by a PhD scholarship from the Leukaemia Foundation of Australia.

Manuscript received on September 9, 2010. Revised version arrived on October 17, 2010. Manuscript accepted on October 21, 2010.

Deborah L. White, Department of Haematology, SA Pathology (RAH Campus), Frome Road, Adelaide. Australia. Phone: international +61.08.82223458. Fax: international +61.08.82223162 E-mail: deborah.white@health.sa.gov.au

The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

Despite the excellent responses to imatinib therapy observed in patients with chronic phase chronic myeloid leukemia, approximately 25% of patients display primary resistance or suboptimal response. The OCT-1 activity in mononuclear cells reflects the efficiency of active influx of imatinib. OCT-1 activity in mononuclear cells is highly variable between patients and significantly correlates with a patient's molecular response to imatinib treatment and overall survival. The present study examined whether cell lineage and BCR-ABL expression influenced OCT-1 activity.

Design and Methods

The OCT-1 activity and OCT-1 mRNA expression was assessed in pure populations of neutrophils, monocytes and lymphocytes recovered from chronic myeloid leukemia patients at diagnosis, in cytogenetic remission and normal individuals. The role of BCR-ABL on OCT-1 activity and differentiation was examined in a cell line model of ectopic BCR-ABL expression.

Results

The OCT-1 activity and OCT-1 mRNA expression was highest in the neutrophil population and lowest in lymphocytes ($P<0.05$). This was observed for patients at diagnosis, in cytogenetic remission and normal individuals. Interestingly, neutrophil OCT-1 activity was not significantly different between patients at diagnosis, in remission and normal donors. This was also observed for monocytes and lymphocytes. Furthermore, OCT-1 activity in mononuclear cells was significantly correlated with the OCT-1 activity in neutrophils ($P=0.001$). In a cell line model in which BCR-ABL was ectopically expressed, we found no evidence that BCR-ABL directly affected OCT-1 expression and function. However, BCR-ABL stimulated granulocyte differentiation which, in turn, led to significantly increased OCT-1 activity ($P=0.024$).

Conclusions

These studies suggest that the predictive OCT-1 activity in patient mononuclear cells is strongly related to cell lineage, particularly the presence of neutrophils in the peripheral blood. Furthermore, BCR-ABL expression is unlikely to directly influence OCT-1 activity but may have an indirect role by enhancing granulocyte differentiation.

Key words: chronic myeloid leukemia, OCT-1, OCT-1 activity, BCR-ABL, imatinib.

Citation: Engler JR, Zannettino ACW, Bailey CG, Rasko JEJ, Hughes TP and White DL. OCT-1 function varies with cell lineage but is not influenced by BCR-ABL. Haematologica 2011;96(2):213-220. doi:10.3324/haematol.2010.033290

Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disease characterized by the reciprocal translocation between chromosomes 9 and 22, resulting in the formation of a constitutively active tyrosine kinase, BCR-ABL.[1,2] The resultant BCR-ABL oncoprotein is essential for chronic myeloid leukemia pathogenesis,[3] and mediates an increase in cell proliferation, a suppression of apoptosis, altered cell adhesion and genetic instability.[4-6]

Imatinib mesylate (Novartis Pharmaceuticals™) competitively binds to the ATP-binding domain of BCR-ABL,[7] rendering it inactive and preventing downstream signaling.[8] As frontline therapy, imatinib results in excellent cytogenetic and molecular responses for the majority of chronic phase chronic myeloid leukemia (CP-CML) patients.[9] However, approximately 25% to 35% of patients display primary resistance or sub-optimal response to imatinib.[10] Therefore, identifying prognostic indicators of poor response to imatinib may enable tailored therapy for patients at risk.[11,12]

The organic cation transporter 1 (OCT-1, also known as SLC22A1) is the major active influx transporter for imatinib in chronic myeloid leukemia cells.[13,14] A number of studies have found a relationship between OCT-1 mRNA expression and response to imatinib treatment;[15-17] however, this has not been confirmed in other studies.[18,19] We have previously demonstrated that the functional activity of OCT-1 (OCT-1 activity) measured in mononuclear cells (MNC) from CP-CML patients at diagnosis is associated with their molecular response to imatinib treatment at both 24 months[20] and at five years.[19] Furthermore, we have established that OCT-1 activity is a strong predictor of event-free and transformation-free survival following five years of imatinib treatment.[19]

It has recently been suggested that OCT-1 mRNA expression is increased in polymorphonuclear cells compared to mononuclear cells[21,22] and that BCR-ABL may have an inhibitory effect on OCT-1 expression.[21] Given the strong predictive value of OCT-1 activity, the present study was aimed at identifying factors which may influence patient variation in OCT-1 activity. Specifically, the effects of cell lineage, cell maturity and BCR-ABL expression on OCT-1 activity were assessed.

Design and Methods

Patient samples

Peripheral Blood (PB) was obtained from CP-CML patients at diagnosis and once they had entered complete cytogenetic remission. Normal peripheral blood was obtained from healthy donors. All samples were collected with informed consent in accordance with the Institutional Ethics approved protocols and with reference to the Declaration of Helsinki.

Cell isolation

Mononuclear cells were isolated from peripheral blood by Ficoll density gradient centrifugation according to the manufacturer's instructions (Lymphoprep; Axis Shield, Oslo, Norway). For chronic myeloid leukemia diagnosis samples: mononuclear cells were enriched for monocytes using a Percoll continuous gradient (GE Healthcare, Uppsala, Sweden) and pure monocytes were then isolated using CD14 microbeads according to the manufacturer's instructions by magnetic-activated cell sorting (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Pure neutrophils and lymphocytes were also isolated from the mononuclear cell compartment using CD16 and CD3 MACS microbeads, respectively. For chronic myeloid leukemia remission samples and normal donors, monocytes and lymphocytes were enriched directly from mononuclear cells using CD14 and CD3 MACS microbeads, respectively. Granulocytes were obtained from the red blood cell layer of the Ficoll and were washed twice in red blood cell lysis buffer. Pure neutrophils were then isolated using CD16 MACS microbeads (see *Online Supplementary Figure S1* for the entire cell lineage enrichment workflow). The purity of all MACS-purified populations was assessed using immunophenotyping and morphology (see *Online Supplementary Figure S2* for purity assessment).

Immunophenotyping

Approximately 1×10^5-5×10^5 cells were incubated for 40 min on ice with PE or FITC-conjugated antibodies (CD3, CD11b, CD14, CD15 and CD16, BD Biosciences, San Jose, CA, USA) or isotype control IgG PE or FITC antibodies (DakoCytomation, Glostrup, Denmark). Cells were then washed in Hanks buffer and re-suspended in fixative and analyzed by flow cytometry (Epics XL-MCL flow cytometer and CXP Analysis Version 2.2 Software; Beckman Coulter, Miami, FL, USA).

Drugs and kinase inhibitors

Imatinib mesylate (STI571) and [^{14}C]-imatinib, were kindly provided by Novartis Pharmaceuticals (Basel, Switzerland). Stock solutions of imatinib were prepared at concentrations of 1 and 10 mM in distilled water, sterile filtered and stored at -70°C. The potent OCT-1 inhibitor prazosin (Sigma-Aldrich, Steinheim, Germany) was used at 100μM in the intracellular uptake and retention (IUR) assay.

OCT-1 activity

OCT-1 activity was calculated from the IUR assay as described previously.[20] In brief, 2×10^5 cells were incubated in the presence or absence of 2μM [^{14}C]-imatinib ± prazosin for 2 h at 37°C. Following this time, tubes were centrifuged to separate the cells from the supernatant and these were added to OptiPlates containing Microscint[20] scintillation fluid (both from PerkinElmer, Boston, MA, USA). Imatinib incorporation was determined using a Top Count Microplate β-Scintillation Counter (PerkinElmer). Imatinib uptake was calculated as ng of imatinib per 200,000 cells. The OCT-1 activity was expressed as the difference between the intracellular uptake and retention in the absence and presence of prazosin, as described earlier.[20] Where intracellular uptake and retention values in the presence of prazosin were equal or higher than the values in the absence of prazosin, these patients were scored as having negligible (0 ng/200,000 cells) OCT-1 activity.

mRNA expression of OCT-1

OCT-1 mRNA expression levels were determined as previously described.[23] Briefly, total RNA was extracted from 1×10^6-1×10^7 cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA was synthesized using random hexamers (GeneWorks Pty. Ltd, Hindmarsh, SA, Australia) and Superscript II Reverse Transcriptase (Invitrogen). OCT-1 and BCR were amplified using PCR primer sequences as previously reported.[23] Quantitative PCR (qPCR) was performed on a RotorGene 3000 real-time PCR thermal cycler (Qiagen, San Francisco, CA, USA) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). OCT-1 mRNA expression was calculated as a percentage relative to BCR expression.

Cell culture

The promyelocytic HL60 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI-1640 media (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), penicillin and streptomycin and L-glutamine at 37°C in a 5% CO_2-humidified incubator.

Viral vector construction and transduction of cell lines

The pHIV-1SDm-based lentiviral vector and four accessory plasmids were used for Tat-dependent lentiviral vector production.[24] The SV40 promoter was exchanged for a CMV promoter and the woodchuck post-transcriptional regulatory element was added to generate pHIV-1SDmCMV.wpre. A control vector (CV) containing enhanced green fluorescent protein (eGFP) was generated by cloning in a *BamHI/NotI* Klenow-blunted fragment from peGFP-1 (BD Biosciences). The 6.1 kb BCR-ABL fusion gene cDNA representing the p210 variant was PCR-amplified from pSIN-p210[25] and fused to eGFP using the porcine teschovirus-1 2A peptide sequence (P2A) represented as the amino acids (SGSGATNFSLLKQAGDVEENPGP). This system allows stoichiometric expression of separate eGFP and BCR-ABL proteins resulting from a ribosomal skipping mechanism.[26] Lentiviral particles comprised of either control vector or wild-type BCR-ABL were generated as previously described.[27] Approximately 2×10^5 HL60 cells were suspended in 500 μL of culture media in a 5 mL FACS tube. Polybrene (8 μg/mL) and concentrated viral particles (10 μL of control vector or 50 μL of BCRABL vector) were added to the cells and centrifuged at 1,500 rpm for three hours. The cells were washed and cultured in 1 mL of culture media for 4-12 days. Transduced cells were FACS sorted based on their expression of eGFP (Epics Altra HyperSort, using Expo MultiComp Software version 1.2B, Beckman Coulter). The control vector cell line underwent one round of sorting and the BCR-ABL cell line underwent four rounds of sorting to obtain pure populations. (See *Online Supplementary Figure S3* for vector diagrams, FACS plot of GFP expression and confirmatory BCR-ABL mRNA expression[28] and phosphorylated Crkl (p-Crkl) levels).

Differentiation assays

Differentiation of the HL60 cell line into granulocytes was induced by adding 1.25% (v/v) DMSO (Dimethyl sulfoxide, Merck, Darmstadt, Germany) for seven days.[29,30] Cell differentiation was monitored by cell growth, cell cycle status and antigen CD11b expression.[31] Furthermore, cytospin preparations were stained with Wright's stain for morphological assessment.

Cell cycle analysis

Following induction of differentiation, 5×10^5 HL60 cells were washed once in phosphate-buffered saline (PBS, Sigma-Aldrich) and fixed in 100% ethanol at 4°C for a minimum of 24 h. Cells were then washed in PBS and incubated at 37°C for 40 minutes in a 1 mL PBS solution containing propidium iodide (40 μg/mL, Sigma-Aldrich), RNase A (100 μg/mL, Qiagen, Doncaster, Australia) and Triton X-100 (0.1% (v/v), Sigma-Aldrich). Cells were analyzed by flow cytometry (Beckman Coulter).

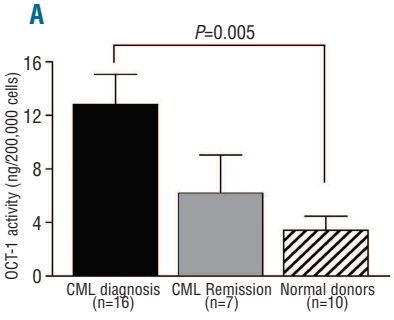
Statistics

All statistics were performed using SigmaStat 3.0 software (SPSS Inc., Chicago, IL, USA). The Mann–Whitney Rank Sum or the Student's t-test were used to determine differences between experimental groups as appropriate. Column graphs show mean plus the standard error of the mean (SEM). Data in tables show mean plus the standard deviation (SD).

Results

MNC OCT-1 activity and cell populations vary between CML diagnosis patients, remission patients and normal donors

Previous studies have shown that OCT-1 activity in mononuclear cells recovered from chronic myeloid leukemia patients at diagnosis is highly variable and that response to imatininb treatment is closely linked to OCT-1 activity.[19] To determine if this variation was a feature of chronic myeloid leukemia or was patient-specific, mononuclear cells were isolated from 16 chronic myeloid leukemia patients at diagnosis; 7 of these patients who had entered complete cytogenetic remission (CCR) following imatinib treatment, and 10 normal individuals. The OCT-1 activity in mononuclear cells recovered from chronic myeloid leukemia patients in complete cytogenetic remission was lower than that observed in mononuclear cells from chronic myeloid leukemia patients at diagnosis and was lowest in mononuclear cells from normal healthy individuals (average: CML diagnosis 12.8; CML remission 6.2; normal 3.4 ng/200,000 cells; Figure 1A). To assess whether the low MNC OCT-1 activity in normal healthy volunteers was due to lineage differences in the mononu-



B

CML diagnosis		CML remission		Normal donors	
CD15+16+ Neutrophils	27±16% (n=15)	CD15+16+ Neutrophils	4±2% (n=6)	CD15+16+ Neutrophils	NT
CD15+ Granulocytes	71±19% (n=15)	CD15+ Granulocytes	5±5% (n=6)	CD15+ Granulocytes	3±1% (n=9)
CD14+ Monocytes	5±4% (n=13)	CD14+ Monocytes	8±8% (n=6)	CD14+ Monocytes	4±2% (n=9)
CD3+ Lymphocytes	4±3% (n=13)	CD3+ Lymphocytes	21±10% (n=6)	CD3+ Lymphocytes	50±16% (n=9)

Figure 1. (A) OCT-1 activity in MNC isolated from the study cohort. The number 'n' of CML patients at diagnosis, CML patients in CCR and normal individuals is indicated, OCT-1 activity is expressed as ng/200,000 cells. Columns represent the mean + SEM. The *P* value demonstrates a significant difference in OCT-1 activity between CML diagnosis and normal individuals. (B) Immunophenotyping and morphology of the MNC compartment from CML patients at diagnosis, CML patients in CCR and normal individuals. Immunophenotype percentages represent the mean ± SD for all individuals tested. Representative photos are taken from one individual in each group (Wright's stain, Olympus BX51, magnification 40x). NT = not tested.

clear cell population or related to the absence of BCR-ABL signaling, mononuclear cell populations for each group were examined by immunophenotyping and morphological assessment. The percentage of $CD15^+$ granulocytes, $CD15^+16^+$ neutrophils, $CD14^+$ monocytes and $CD3^+$ lymphocytes in each sample was evaluated by flow cytometry. It was observed that the mononuclear cell layer in chronic myeloid leukemia patients at diagnosis consisted primarily of maturing and mature granulocytes (average of 71% $CD15^+$, Figure 1B). Of interest, only 27% of the cells were positive for both CD15 and CD16 (mature neutrophil makers) indicating the presence of immature myelocytes and metamyelocytes. In contrast, the major populations present within remission chronic myeloid leukemia and normal mononuclear cells were found to be lymphoid in nature (average of 21% and 50% $CD3^+$ lymphocytes, respectively). Morphological analysis confirmed these findings. These data suggest that the intrinsic differences between myeloid and lymphoid cells may account for the differences in imatinib uptake and hence OCT-1 activity in the mononuclear cell compartment of the various sample types.

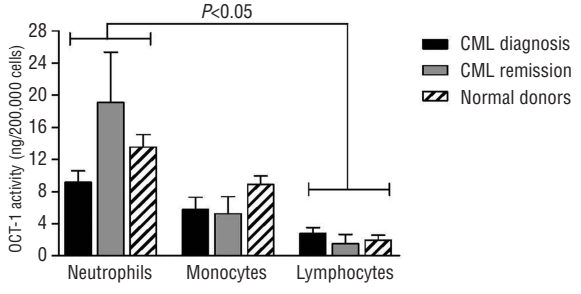


Figure 2. OCT-1 activity in neutrophils, monocytes and lymphocytes isolated from the study cohort. Data from CML patients at diagnosis, CML patients in CCR and normal individuals is shown, OCT-1 activity is expressed as ng/200,000 cells. Columns represent the mean + SEM. The *P* value demonstrates a significant difference in OCT-1 activity between neutrophils and lymphocytes for all groups.

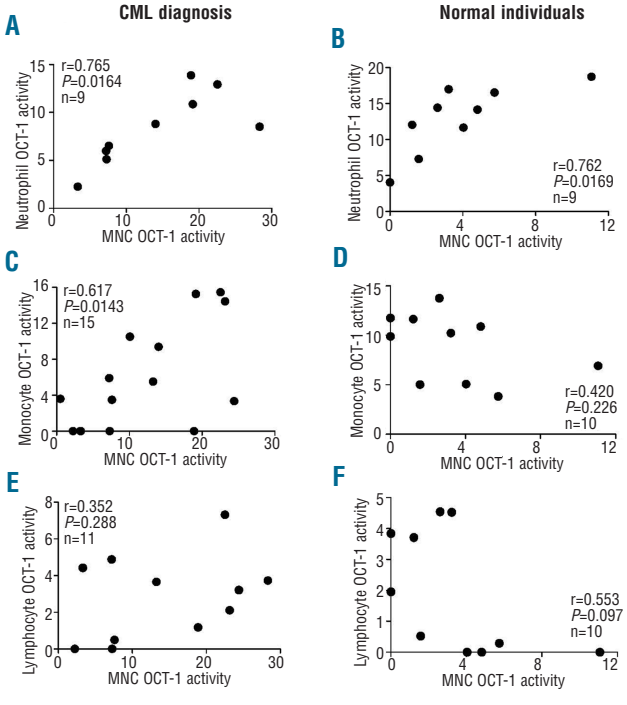


Figure 3. In CML diagnosis patients (A, C and E) and normal donors (B, D and F) the OCT-1 activity measured in individuals' MNC was associated with the OCT-1 activity in their neutrophil (A and B), monocyte (C and D) and lymphocyte (E and F) populations. A significant correlation was seen between neutrophil OCT-1 activity and MNC OCT-1 activity in both CML diagnosis patients and normal donors. A significant correlation was also found between monocyte OCT-1 activity and MNC OCT-1 activity in diagnosis patients. The Pearson's product moment was used to assess the correlation.

OCT-1 activity is highest in the neutrophil population and is not influenced by BCR-ABL

The intrinsic OCT-1 activity of lineage marker-defined cell populations was examined following the isolation of populations enriched for $CD15^+16^+$ neutrophils, $CD14^+$ monocytes or $CD3^+$ lymphocytes from CML patients and normal donors (*Online Supplementary Figures S1 and S2*). After examining samples from CML diagnosis, CML remission, and normal individuals, neutrophils were found to express the highest OCT-1 activity (means 9.1, 19.1, and 13.5 ng/200,000 cells, respectively), monocytes exhibited intermediate OCT-1 activity (means 5.8, 5.2, and 9.0 ng/200,000 cells, respectively), while lymphocytes exhibited the lowest OCT-1 activity (means 2.8, 1.5, and 2.0 ng/200,000 cells, respectively; Figure 2). Furthermore, in all groups, OCT-1 activity in neutrophils was significantly higher than lymphocytes ($P<0.001$ for CML diagnosis, $P=0.012$ for CML remission, and $P<0.001$ for normal individuals). These data suggest that high MNC OCT-1 activity in chronic myeloid leukemia patients at diagnosis may be due to the predominance of granulocytes in the mononuclear cell population. Furthermore, the low mononuclear cell OCT-1 activity in CML remission and normal individuals may reflect the presence of greater numbers of lymphocytes. Consistent with this notion, there was no significant difference in neutrophil OCT-1 activity in samples collected from CML diagnosis, CML remission and normal individuals. This finding was also consistent in monocytes and lymphocytes, suggesting that BCR-ABL expression, absent or low in remission and absent in normal individuals, is unlikely to be affecting OCT-1 activity.

MNC OCT-1 activity is related to neutrophil OCT-1 activity

The relationship between MNC OCT-1 activity and OCT-1 activity in neutrophils, monocytes and lymphocytes was examined (Figure 3). A significant correlation between MNC OCT-1 activity and neutrophil OCT-1 activity was observed in chronic myeloid leukemia

Table 1. OCT-1 mRNA expression between cell types.

	MNC	Neutrophils	Monocytes	Lymphocytes
CML Diagnosis	10% (10%) *	54% (33%)	13% (12%)*	3% (2.2%)*
CML Remission	5.5% (1.2%)*	198% (163%)	11% (9%)*	1.2% (1.2%)*
Normal Donors	1.2% (1%)	76% (121%)	2% (1.2%)	0.5% (0.5%)

*CML: chronic myeloid leukemia; MNC: mononuclear cells. Average OCT-1 mRNA as % of BCR expression (SD). * Represents P<0.05 compared to normal donors.*

patients at diagnosis and normal individuals (r=0.765, *P*=0.016 and r=0.762, *P*=0.016, respectively; Figure 3A and B). A significant correlation was also found between CML diagnosis mononuclear cell OCT-1 activity and monocyte OCT-1 activity (r=0.617, *P*=0.014; Figure 3C). No relationship was observed between the OCT-1 activity in lymphocytes and mononuclear cells (Figure 3E and F).

OCT-1 mRNA expression is highest in the neutrophil population

In order to determine whether messenger RNA levels may contribute to the variation in OCT-1 activity observed in the different cell lineages, qPCR was employed. As demonstrated in Table 1, the highest level of OCT-1 mRNA expression was seen in the neutrophil population, while the lowest expression was observed in the lymphocytes (*P*=0.026 for CML diagnosis, *P*=0.016 for CML remission and *P*<0.001 for normal donors). No difference in neutrophil OCT-1 mRNA expression was observed in samples recovered from CML diagnosis patients, CML remission patients and normal healthy individuals. However, monocytes and lymphocytes from CML diagnosis and remission patients exhibited significantly higher OCT-1 mRNA than normal individuals (*P*<0.05).

BCR-ABL drives myeloid differentiation and hence increases OCT-1 activity

To more clearly investigate the role that BCR-ABL plays in OCT-1 activity, the promyeloblastic HL60 cell line was transduced with lentivirus constitutively expressing a 'wild-type' BCR-ABL 2A-peptide-linked to eGFP (CMV.BCRABL) or control vector (CMV.CV) and then purified by FACS. BCR-ABL expression and signaling was confirmed in the CMV.BCRABL HL60 cell line using quantitative PCR and Western blot for phosphorylated Crkl (p-Crkl, *Online Supplementary Figure S3*). BCR-ABL expression was found to be expressed at normal levels by comparison with the CML cell line K562. As shown in Figure 4, no difference in OCT-1 activity or OCT-1 mRNA expression

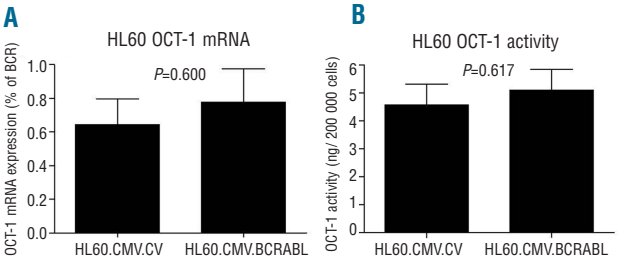
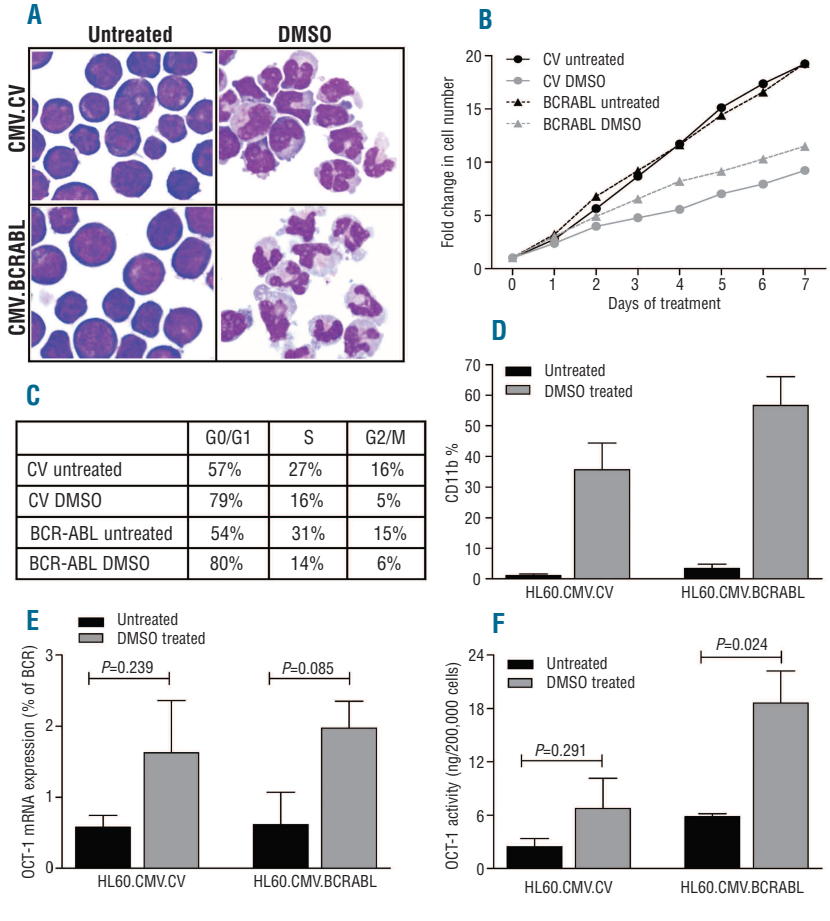


Figure 4. The HL60 cell line was stably transfected with lentivirus containing control vector or BCR-ABL. (A) OCT-1 mRNA expression (n=7) and (B) OCT-1 activity (n=14) was measured in these two cell lines. OCT-1 activity is expressed as ng/200,000 cells. Columns represent the mean + SEM. n represents the number of times the assay was repeated for each stable cell line. The *P* values demonstrate a lack of difference between control vector and BCR-ABL.



	G0/G1	S	G2/M
CV untreated	57%	27%	16%
CV DMSO	79%	16%	5%
BCR-ABL untreated	54%	31%	15%
BCR-ABL DMSO	80%	14%	6%

Figure 5. Cell differentiation of HL60 cells transduced with control vector or BCR-ABL. Granulocyte differentiation was induced with 1.25% (v/v) DMSO for seven days. (A) Examination of cell morphology on cytospin preparations (Wright's stain, Olympus BX51, magnification 40x). (B) Relative cell proliferation of untreated (black lines) and DMSO treated (gray line) HL60 cells. (C) Cell cycle analysis of untreated and DMSO treated HL60 cells. (D) Cell surface expression of the myeloid differentiation marker CD11b on untreated and DMSO treated HL60 cells. (E) OCT-1 mRNA expression (expressed as % of BCR) in untreated and DMSO treated HL60 cells. (F) OCT-1 activity in the HL60 cell line with and without DMSO treatment. All results show the average of 3 individual experiments.

was observed between the two cell lines. These findings suggest that the presence of BCR-ABL alone has no direct effect on OCT-1 activity or expression.

To induce granulocyte differentiation, vector control and BCR-ABL-transduced HL60 cell lines were cultured in media supplemented with 1.25% DMSO for seven days. As expected, DMSO treatment resulted in changes in cell morphology as evidenced by a more lobular nuclei (Figure 5A), a reduction in cell growth (Figure 5B), an accumulation in the G0/G1 cell cycle phase (Figure 5C) and an increase in CD11b cell surface expression (Figure 5D), all consistent with granulocytic differentiation. Notably, CMV.BCRABL HL60 were found to differentiate more than vector control cells as evidenced by increased nuclear segmentation (Figure 5A) and increased CD11b expression (Figure 5D). Differentiation was associated with increased OCT-1 mRNA expression in both cell lines, although this was not significant (Figure 5E). Interestingly, while the OCT-1 activity in the control vector line did not significantly change following DMSO treatment, the OCT-1 activity in HL60 CMV.BCRABL significantly increased from a mean of 5.8 to 18.6 ng/200,000 cells (Figure 5F, P=0.024).

Therefore, whilst BCR-ABL alone appears not to have an effect on OCT-1 expression or activity, the presence of BCR-ABL clearly plays a role in driving granulocyte differentiation which in turn results in an increase in OCT-1 activity.

Discussion

OCT-1 activity, measured in mononuclear cells at diagnosis, is a strong predictive test to identify patients who will respond well to treatment and, more importantly, to identify patients who are most likely to fail treatment or progress. An understanding of the biology of OCT-1 activity is central to identifying those patients who are likely to be poor responders to imatinib therapy and in developing new strategies to better target these patients at risk.

The present study identified that the mononuclear cell compartment in CML diagnosis patients is comprised predominantly of a mixture of granulocytes at varying stages of differentiation. Notably, purified populations of neutrophils, monocytes and lymphocytes exhibited differences in imatinib uptake, with neutrophils showing the greatest OCT-1 activity and lymphocytes the lowest. A recent study suggested that differences in imatinib uptake between patient cells and cell lines may be associated with cell volume.[32] Given the considerable size differences between neutrophils and lymphocytes it is possible that reduced cell volume may contribute to the lower OCT-1 activity seen in lymphocytes. When MNC OCT-1 activity was correlated with OCT-1 activity in neutrophils, monocytes and lymphocytes, the strongest significant relationship was observed between MNC OCT-1 activity and neutrophil OCT-1 activity. This was found in both chronic myeloid leukemia patients at diagnosis and normal individuals. As a result, individuals with high MNC OCT-1 activity tend to have high OCT-1 activity in their neutrophils but not necessarily in their monocytes or lymphocytes. Therefore, OCT-1 activity in the granulocyte lineage is likely to be a key contributor to MNC OCT-1 activity in chronic myeloid leukemia patients at diagnosis.

Variation in OCT-1 activity may be a result of variation in OCT-1 mRNA expression. Two recent studies have demonstrated that polymorphonuclear cells had a higher OCT-1 mRNA expression compared with mononuclear cells in normal peripheral blood.[21,22] Studies presented here show that neutrophils express significantly higher levels of OCT-1 mRNA than lymphocytes in normal, CML diagnosis and CML remission peripheral blood and that mRNA expression relates closely to OCT-1 activity. Bazeos *et al.*[21] subsequently examined OCT-1 mRNA expression in total white blood cells between CML diagnosis, CML remission and normal donors and found that OCT-1 mRNA expression was increased in CML remission and normal individuals compared to CML diagnosis. This was attributed to an inhibitory effect of BCR-ABL on OCT-1 expression. However, it is important to remember that between these groups, the total white blood cell population may be comprised of markedly different cell populations/cell ratios. In normal peripheral blood, total white blood cells consist of approximately 70% neutrophils, which would be similar to what would be observed in chronic myeloid leukemia patients in complete cytogenetic remission. However, as we and others[22] have demonstrated, CML diagnosis peripheral blood contains more immature forms of granulocytes (i.e. myelocytes and metamyelocytes) which are not seen in normal peripheral blood. These differences in the total white blood cell population may be influencing the results of Bazeos *et al.*[21] To overcome this, we have compared OCT-1 activity and mRNA expression in the mature neutrophil population recovered from CML diagnosis, CML remission and normal donors and have found no significant difference in OCT-1 activity or expression. Furthermore, we found no difference in OCT-1 activity and expression in the HL60 cell line transfected with control vector or BCR-ABL, indicating that BCR-ABL is unlikely to be affecting OCT-1 activity or expression. Of note, there appears to be a trend of increased OCT-1 activity and expression in the neutrophil population recovered from CML remission patients compared to both CML diagnosis and normal individuals. While not reaching statistical significance, possibly due to inadequate numbers, it is tempting to speculate that imatinib itself may have an effect on OCT-1 expression and/or function.

We and others have previously shown that primitive $CD34^+$ cells from chronic myeloid leukemia patients have reduced levels of OCT-1 activity[23] and expression[23,33] compared to mature CD34- cell indicating a role for cell maturation in OCT-1 regulation. We now substantiate these findings in a cell line model in which we have shown that maturation of the HL60 cell line from a blastic state to a more differentiated granulocyte is associated with an increase in OCT-1 activity and expression. The level of differentiation is enhanced by the presence of BCR-ABL which in turn further increases the OCT-1 activity. This data is in agreement with studies of induced BCR-ABL expression in cord blood $CD34^+$ cells[34] and mouse embryonic stem cells,[35] where BCR-ABL expression induced significant increases in myeloid colonies. While other studies suggest that increased BCR-ABL expression in blast crisis mediates a halt in differentiation, this is usually associated with additional genetic defects such as reduced p5336, and reduced C/EBP-α.[37,38] Overexpression of BCR-ABL in the pluripotent primitive hematopoietic cell line, FDCP-Mix, resulted in a cell line with characteristics similar to that observed in cells recovered from "chronic phase" chronic

myeloid leukemia patients.[39] In this cell line, expression of BCR-ABL along with culture in myeloid differentiation media resulted in preferential granulocyte differentiation. Furthermore, BCR-ABL expression did not induce a block in differentiation as blast cell numbers decreased dramatically. Lastly, a reduction in granulocyte differentiation has been observed in a chronic myeloid leukemia cell line following imatinib treatment[40].

In conclusion, OCT-1 activity and expression appears to be strongly related to cell lineage and maturity. BCR-ABL does not appear to directly influence OCT-1 activity but may do so by facilitating granulocyte differentiation. Therefore, variation in MNC OCT-1 activity, and hence response to imatinib therapy in chronic myeloid leukemia patients, may be related to factors associated with the majority cell population at diagnosis.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

1. Heisterkamp N, Groffen J, Stephenson JR, Spurr NK, Goodfellow PN, Solomon E, et al. Chromosomal localization of human cellular homologues of two viral oncogenes. Nature. 1982;299(5885):747-9.
2. de Klein A, van Kessel AG, Grosveld G, Bartram CR, Hagemeijer A, Bootsma D, et al. A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. Nature. 1982;300(5894):765-7.
3. Daley GQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. Science. 1990;247(4944):824-30.
4. Bedi A, Zehnbauer BA, Barber JP, Sharkis SJ, Jones RJ. Inhibition of apoptosis by BCR-ABL in chronic myeloid leukemia. Blood. 1994;83(8):2038-44.
5. Steelman LS, Pohnert SC, Shelton JG, Franklin RA, Bertrand FE, McCubrey JA. JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. Leukemia. 2004;18(2):189-218.
6. Van Etten RA. Mechanisms of transformation by the BCR-ABL oncogene: new perspectives in the post-imatinib era. Leuk Res. 2004;28 (Suppl 1):S21-8.
7. Schindler T, Bornmann W, Pellicena P, Miller WT, Clarkson B, Kuriyan J. Structural mechanism for STI-571 inhibition of abelson tyrosine kinase. Science. 2000;289 (5486):1938-42.
8. Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med. 1996;2(5):561-6.
9. Hochhaus A, O'Brien SG, Guilhot F, Druker BJ, Branford S, Foroni L, et al. Six-year follow-up of patients receiving imatinib for the first-line treatment of chronic myeloid leukemia. Leukemia. 2009;23(6):1054-61.
10. Marin D, Milojkovic D, Olavarria E, Khorashad JS, de Lavallade H, Reid AG, et al. European LeukemiaNet criteria for failure or suboptimal response reliably identify patients with CML in early chronic phase treated with imatinib whose eventual outcome is poor. Blood. 2008;112(12):4437-44.
11. Villuendas R, Steegmann JL, Pollan M, Tracey L, Granda A, Fernandez-Ruiz E, et al. Identification of genes involved in imatinib resistance in CML: a gene-expression profiling approach. Leukemia. 2006;20(6):1047-54.
12. McWeeney SK, Pemberton LC, Loriaux MM, Vartanian K, Willis SG, Yochum G, et al. A gene expression signature of CD34+ cells to predict major cytogenetic response in chronic-phase chronic myeloid leukemia patients treated with imatinib. Blood. 2010;115(2):315-25.
13. Thomas J, Wang L, Clark RE, Pirmohamed M. Active transport of imatinib into and out of cells: implications for drug resistance. Blood. 2004;104(12):3739-45.
14. White DL, Saunders VA, Dang P, Engler J, Zannettino AC, Cambareri AC, et al. 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(2):697-704.
15. Crossman LC, Druker BJ, Deininger MW, Pirmohamed M, Wang L, Clark RE. hOCT 1 and resistance to imatinib. Blood. 2005;106(3):1133-4; author reply 4.
16. Wang L, Giannoudis A, Lane S, Williamson P, Pirmohamed M, Clark RE. Expression of the uptake drug transporter hOCT1 is an important clinical determinant of the response to imatinib in chronic myeloid leukemia. Clin Pharmacol Ther. 2008;83(2):258-64.
17. Marin D, Bazeos A, Mahon FX, Eliasson L, Milojkovic D, Bua M, et al. Adherence is the critical factor for achieving molecular responses in patients with chronic myeloid leukemia who achieve complete cytogenetic responses on imatinib. J Clin Oncol. 2010;28(14):2381-8.
18. Zhang WW, Cortes JE, Yao H, Zhang L, Reddy NG, Jabbour E, et al. Predictors of primary imatinib resistance in chronic myelogenous leukemia are distinct from those in secondary imatinib resistance. J Clin Oncol. 2009;27(22):3642-9.
19. White DL, Dang P, Engler J, Frede A, Zrim S, Osborn M, et al. Functional activity of the OCT-1 protein is predictive of long-term outcome in patients with chronic-phase chronic myeloid leukemia treated with imatinib. J Clin Oncol. 2010;28 (16):2761-7.
20. White DL, Saunders VA, Dang P, Engler J, Venables A, Zrim S, et al. 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(12):4064-72.
21. Bazeos A, Marin D, Reid AG, Gerrard G, Milojkovic D, May PC, et al. hOCT1 transcript levels and single nucleotide polymorphisms as predictive factors for response to imatinib in chronic myeloid leukemia. Leukemia. 2010;24(6):1243-5.
22. Racil Z, Razga F, Buresova L, Jurcek T, Dvorakova D, Zackova D, et al. The assessment of human organic cation transporter 1 (hOCT1) mRNA expression in patients with chronic myelogenous leukemia is affected by the proportion of different cells types in the analyzed cell population. Am J Hematol. 2010;85(7):525-8.
23. Engler JR, Frede A, Saunders VA, Zannettino AC, Hughes TP, White DL. Chronic myeloid leukemia CD34+ cells have reduced uptake of imatinib due to low OCT-1 activity. Leukemia. 2010;24(4):765-70.
24. Koldej R, Cmielewski P, Stocker A, Parsons DW, Anson DS. Optimisation of a multipartite human immunodeficiency virus based vector system; control of virus infectivity and large-scale production. J Gene Med. 2005;7(11):1390-9.
25. Dugray A, Geay JF, Foudi A, Bonnet ML, Vainchenker W, Wendling F, et al. Rapid generation of a tetracycline-inducible BCR-ABL defective retrovirus using a single autoregulatory retroviral cassette. Leukemia. 2001;15(10):1658-62.
26. Holst J, Szymczak-Workman AL, Vignali KM, Burton AR, Workman CJ, Vignali DA. Generation of T-cell receptor retrogenic mice. Nat Protoc. 2006;1(1):406-17.
27. Anson DS, Fuller M. Rational development of a HIV-1 gene therapy vector. J Gene Med. 2003;5(10):829-38.
28. Branford S, Hughes TP, Rudzki Z. Monitoring chronic myeloid leukaemia therapy by real-time quantitative PCR in blood is a reliable alternative to bone marrow cytogenetics. Br J Haematol. 1999;107(3):587-99.
29. Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc Natl Acad Sci USA. 1978;75(5):2458-62.
30. Jiang G, Bi K, Tang T, Wang J, Zhang Y, Zhang W, et al. Down-regulation of TRRAP-dependent hTERT and TRRAP-independent CAD activation by Myc/Max contributes to the differentiation of HL60 cells after exposure to DMSO. Int Immunopharmacol. 2006;6(7):1204-13.
31. Wang X, Studzinski GP. Inhibition of p38MAP kinase potentiates the JNK/SAPK pathway and AP-1 activity in monocytic but not in macrophage or granulocytic differentiation of HL60 cells. J Cell Biochem.

2001;82(1):68-77.
32. Hatziieremia S, Jordanides NE, Holyoake TL, Mountford JC, Jorgensen HG. Inhibition of MDR1 does not sensitize primitive chronic myeloid leukemia CD34+ cells to imatinib. Exp Hematol. 2009;37(6):692-700.
33. Jiang X, Zhao Y, Smith C, Gasparetto M, Turhan A, Eaves A, et al. Chronic myeloid leukemia stem cells possess multiple unique features of resistance to BCR-ABL targeted therapies. Leukemia. 2007;21(5): 926-35.
34. Zhao RC, Jiang Y, Verfaillie CM. A model of human p210(bcr/ABL)-mediated chronic myelogenous leukemia by transduction of primary normal human CD34(+) cells with a BCR/ABL-containing retroviral vector. Blood. 2001;97(8):2406-12.
35. Era T, Witte ON. Regulated expression of P210 Bcr-Abl during embryonic stem cell differentiation stimulates multipotential progenitor expansion and myeloid cell fate. Proc Natl Acad Sci USA. 2000;97(4):1737-42.
36. Pierce A, Spooncer E, Wooley S, Dive C, Francis JM, Miyan J, et al. Bcr-Abl protein tyrosine kinase activity induces a loss of p53 protein that mediates a delay in myeloid differentiation. Oncogene. 2000; 19(48):5487-97.
37. Perrotti D, Cesi V, Trotta R, Guerzoni C, Santilli G, Campbell K, et al. BCR-ABL suppresses C/EBPalpha expression through inhibitory action of hnRNP E2. Nat Genet. 2002;30(1):48-58.
38. Chang JS, Santhanam R, Trotta R, Neviani P, Eiring AM, Briercheck E, et al. High levels of the BCR/ABL oncoprotein are required for the MAPK-hnRNP-E2 dependent suppression of C/EBPalpha-driven myeloid differentiation. Blood. 2007;110(3):994-1003.
39. Pierce A, Owen-Lynch PJ, Spooncer E, Dexter TM, Whetton AD. p210 Bcr-Abl expression in a primitive multipotent haematopoietic cell line models the development of chronic myeloid leukaemia. Oncogene. 1998;17(5):667-72.
40. Kobayashi S, Kimura F, Ikeda T, Osawa Y, Torikai H, Kobayashi A, et al. BCR-ABL promotes neutrophil differentiation in the chronic phase of chronic myeloid leukemia by downregulating c-Jun expression. Leukemia. 2009;23(9):1622-7.