Increased peroxisome proliferator-activated receptor γ activity reduces imatinib uptake and efficacy in chronic myeloid leukemia mononuclear cells

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ABSTRACT

Imatinib is actively transported by organic cation transporter-1 (OCT-1) influx transporter, and low OCT-1 activity in diagnostic chronic myeloid leukemia blood mononuclear cells is significantly associated with poor molecular response to imatinib. Herein we report that, in diagnostic chronic myeloid leukemia mononuclear cells and BCR-ABL + cell lines, peroxisome proliferator-activated receptor γ agonists (GW1929, rosiglitazone, pioglitazone) significantly decrease OCT-1 activity; conversely, peroxisome proliferator-activated receptor γ antagonists (GW9662, T0070907) increase OCT-1 activity. Importantly, these effects can lead to corresponding changes in sensitivity to BCR-ABL kinase inhibition. Results were confirmed in peroxisome proliferator-activated receptor γ-transduced K562 cells. Furthermore, we identified a strong negative correlation between OCT-1 activity and peroxisome proliferator-activated receptor γ transcriptional activity in diagnostic chronic myeloid leukemia patients (n=84; P<0.0001), suggesting that peroxisome proliferator-activated receptor γ activation has a negative impact on the intracellular uptake of imatinib and consequent BCR-ABL kinase inhibition. The inter-patient variability of peroxisome proliferator-activated receptor γ activation likely accounts for the heterogeneity observed in patient OCT-1 activity at diagnosis. Recently, the peroxisome proliferator-activated receptor γ agonist pioglitazone was reported to act synergistically with imatinib, targeting the residual chronic myeloid leukemia stem cell pool. Our findings suggest that peroxisome proliferator-activated receptor γ ligands have differential effects on circulating mononuclear cells compared to stem cells. Since the effect of peroxisome proliferator-activated receptor γ activation on imatinib uptake in mononuclear cells may counteract the clinical benefit of this activation in stem cells, caution should be applied when combining these therapies, especially in patients with high peroxisome proliferator-activated receptor γ transcriptional activity.

Introduction

The first-generation Abl kinase inhibitor imatinib has proven effective in chronic phase chronic myeloid leukemia (CP-CML) patients with minimal toxic side effects. While responses to imatinib are generally excellent, 20-30% of patients will demonstrate suboptimal response / tyrosine kinase inhibitor (TKI) resistance, and 5-10% will still progress to the generally fatal blast crisis stage, despite TKI therapy.1-3 Hence, inter-patient variability in response to TKIs is evident despite the
universal presence of the driving oncoprotein BCR-ABL. On this basis, there is growing scientific and clinical interest to define factors that underpin this response variability.

Peroxisome proliferator-activated receptors (PPARs) are a family of transcription factors that regulate several metabolic pathways in a tissue-selective manner. Of the three PPAR subtypes, PPARγ has been studied most extensively in diverse biological pathways and disease conditions, including adipocyte differentiation/metabolism, insulin sensitivity, and inflammation. PPARγ has two isoforms, PPARγ1 and PPARγ2. While PPARγ2 is mostly found in adipose tissue,2 PPARγ1 is ubiquitously expressed in many tissues and cell types, including immune cells (i.e., activated macrophages, lymphocytes and dendritic cells).4 PPARγ and its agonists have been implicated in hematological malignancies playing antitumor roles, such as inhibiting cell proliferation, inducing cell differentiation, and inducing apoptosis.5-7 Frost et al. recently demonstrated that the PPARγ agonist, pioglitazone, could target the residual CML stem cell pool by suppressing signal transducer and activator of transcription 5 (STAT5) and its downstream targets HIP2 and CITED2.8 This was supported by the work of Glodkowska-Mrowka et al., suggesting the clinical potential of the combination of pioglitazone and second- or third-generation TKIs in CML.9 The importance of the PPAR complex has also been demonstrated by several groups, indicating PPARα/PPARγ activation can increase organic cation uptake by inducing human SLC22A1 (encoding OCT-1) or murine Slc22a1 messenger ribonucleic acid (mRNA) expression.10,11

The functional activity of OCT-1 (OCT-1 activity, OA) in mononuclear cells (MNC) of de novo CP-CML patients is a powerful predictor of molecular response, overall, event-free and progression-free survival.12-14 Patients with low OA demonstrate significantly inferior responses to standard imatinib therapy than those with high OA, due to low intracellular imatinib concentrations and corresponding reduced BCR-ABL kinase inhibition.15-16 Although the negative impact of low OA may be partially overcome by escalating the imatinib dose,17-18 this regimen is not tolerated by all patients and may lead to higher rates of adverse events.19,20 In a previous study, we demonstrated that the use of diclofenac, a competitive PPARγ antagonist, significantly increased OA in CML cells.21 Herein we assess the correlation between PPARγ activation and OA using primary MNC from de novo CP-CML patients and BCR-ABL1+ cell lines. Paradoxically we demonstrate that, in these cells, PPARγ agonists have an opposing effect on intracellular imatinib uptake and OA. In addition, a previous study from our laboratory has demonstrated that OA in patient MNC varies with cell lineage in the peripheral blood.22 Given the critical role of PPARγ in cell differentiation, the present study also explores the correlation between OA and the expression of the myeloid surface markers in CP-CML patient MNC at diagnosis.

Methods

Cell lines

BCR-ABL1+ KU812 and K562 cell lines were obtained from the American Type Culture Collection (ATCC, USA). BCR-ABL1-transduced HL60 cells (HL60-BCRABL) were generated as described previously.23

Primary samples from CP-CML patients or healthy donors

MNC and plasma samples were collected from de novo CP-CML patients enrolled in the TIDEL II study24 prior to the commencement of imatinib therapy. Normal MNC were obtained from healthy volunteers. All samples were collected with informed consent in accordance with the Declaration of Helsinki. Use of clinical trial patients samples were approved by the institutional review boards of the SA Pathology and the Royal Adelaide Hospital Research Ethics Committee.

Drugs

Imatinib mesylate (STI571) and 13C-labelled imatinib were kindly provided by Novartis Pharmaceuticals (Switzerland). The potent OCT-1 inhibitor prazosin and PPARγ ligands GW9662 and rosiglitazone, pioglitazone, GW9662 and T0070907 were all purchased from Sigma-Aldrich.

Lentivirus production and cell transduction

The lentiviral plasmids expressing FLAG-tagged wild-type (WT) PPARγ and dominant negative (DN) PPARγL466A/E469A,25 together with empty vector (EV), were constructed from a previously characterized vector, p lent4TO-IRES GFP.26 K562 cells were transduced as previously described,27 and GFP+ cells were isolated for subsequent experiments.

Imatinib intracellular uptake and retention (IUR) assay and OCT-1 activity (OA)

The IUR assay was performed and OA was determined as previously described.11 Cells were pre-incubated with 40 μM PPARγ ligands for one hour, and cell viability prior to the IUR assay was confirmed as greater than 98% by trypan blue exclusion assay. The assays were performed in the presence and absence of 100 μM prazosin, which is a potent inhibitor of OCT-1. OCT-1 activity was determined by calculating the difference between the IUR in the absence of prazosin and the IUR in the presence of prazosin.

Western blotting analyses and determination of IC50imatinib values

Western blotting analyses for phosphorylated CRKL (p-CRKL) were performed to IC50imatinib as previously described.28 Cells were pre-incubated with 40 μM PPARγ ligands for one hour prior to exposure to imatinib. Anti-CRKL, anti-FLAG M2, anti-PPARγ and anti-GAPDH antibodies were employed in western blotting analyses.

Cell viability Analyses

KU812 cells were incubated with 10 μM PPARγ ligands for 24 hours prior to an additional 72-hour treatment with PPARγ ligands and varying concentrations of imatinib (range: 0-5 μM). Cell viability was assessed by Annexin V/7-AAD staining and fluorescence-activated cell sorting (FACS) analysis. The half maximal effective concentration (ED50) that induces cell apoptosis was estimated using non-linear regression as implemented in the GraphPad Prism software program (version 7.0a, GraphPad Software, USA).

Examination of PPARγ and SLC22A1 mRNA expression in BCR-ABL1+ CML cell lines and MNC of de-novo CP-CML patients

The expression level of PPARγ and SLC22A1 (encoding OCT-1) mRNA in KU812 cells were examined by real-time quantitative polymerase chain reaction (RQ-PCR). PPARγ and SLC22A1 mRNA expression levels in MNC of CP-CML patients were evaluated using the Illumina HumanHT-12v4 platform.
PPARγ transcriptional activity in MNC of de-novo CP-CML patients

Nuclear extracts from CP-CML patient MNC were prepared using the Nuclear Extract Kit (Active Motif, USA). PPARγ transcriptional activity was then measured using the PPARγ Transcription Factor Assay Kit (Active Motif). Linear regression analysis was used to determine whether the PPARγ transcriptional activity level could predict OA.

Enzyme immunoassays for 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2)

The 15d-PGJ2 levels in plasma samples from CP-CML patients were analyzed using a 15d-PGJ2 ELISA kit (Enzo Life Sciences, USA).

Immunophenotyping

Cryopreserved MNC were stained with antibodies specifically targeting myeloid lineage markers (CD14-PE, CD15-FITC and CD16-PerCP-Cy5.5 antibodies, all from BD Biosciences). Neutrophils were identified as CD15+/CD14-28 with additional marker CD16 to indicate the different stages of neutrophil maturation.29

Statistical Analyses

All statistical analyses were performed using GraphPad Prism. Differences were considered to be statistically significant when the P-value was less than 0.05.

For details of the methods see the Online Supplementary Material.

Results

Treatment with PPARγ ligands significantly alters OCT-1 activity in BCR-ABL1+ CML cell lines

Treatment with the PPARγ agonist GW1929, rosiglitazone (Rosi), or pioglitazone (Pio) significantly decreased the IUR of imatinib in KU812 and BCR-ABL1-transduced HL60 cells (HL60-BCRABL, Figure 1A). An opposite effect on IUR was observed in both cell lines following treatment with PPARγ antagonists (Figure 1B).

The addition of prazosin, a potent inhibitor of OCT-1, allowed us to further evaluate the activity of the OCT-1 protein in the transport of imatinib. Treatment with the PPARγ agonist GW1929 significantly decreased OA in KU812 (from 10.8 to 7.5 ng/200,000 cells, P=0.0280) and HL60-BCRABL cells (from 11.9 to 8.9 ng/200,000 cells, P=0.0228, Figure 1C). Similar results were observed when cells were treated with the PPARγ agonist Rosi (KU812: from 10.8 to 5.5 ng/200,000 cells, P=0.0010; HL60-BCRABL: from 11.9 to 4.6 ng/200,000 cells, P=0.0001, Figure 1C).

The opposite effect on OA was also observed in both cell lines following treatment with PPARγ antagonists (Figure 1D). The presence of GW9662 significantly increased the OA (KU812: from 10.8 to 15.4 ng/200,000 cells, P=0.0011; HL60-BCRABL: from 11.9 to 15.1
Figure 2. Treatment with PPARγ ligands significantly alters OCT-1 activity in MNC from de novo CP-CML patients. OA assays were performed on thawed MNC isolated from the peripheral blood of newly diagnosed patients with CP-CML. Cells were treated with 40 µM PPARγ ligands for 1 hour prior to IUR assay. PPARγ agonists (A) GW1929, (B) rosiglitazone (Rosi) or (C) pioglitazone (Pio) significantly decreased OA in CP-CML MNC with high OA. PPARγ antagonists (D) GW9662 or (E) T0070907 significantly increased OA in CP-CML MNC with low OA. (F-J) Treatment with PPARγ ligands had no significant effect on OA in normal MNC isolated from healthy donors. The MNC samples treated with Pio (C, H) were different from those treated with GW1929 or Rosi (A, B, F, G), as indicated by different symbols. Dotted line indicates the cutoff value of OA (4 ng/200,000 cells) to define “high OA” and “low OA”. ns, P>0.05. DMSO: dimethyl sulfoxide; OCT-1: organic cation transporter-1; PPARγ: peroxisome proliferator-activated receptor γ.
Figure 3. Treatment with PPARγ ligands significantly alters IC50imatinib and ED50imatinib. (A) The in vitro reduction in the level of p-CrkI by imatinib was detected using the IC50imatinib assay. KU812 cells were incubated with 40 μM PPARγ ligands for 3 hour prior to the treatment with increasing concentrations of imatinib for 2 hours. IC50imatinib was significantly increased with PPARγ agonists GW1929, rosiglitazone (Rosi) or pioglitazone (Pio), and decreased with antagonists GW9662 or T0070907. (B) Cell viability was determined using Annexin V-PE/7-AAD staining. KU812 cells were incubated with 10 μM PPARγ ligands for 24 hours prior to an additional 72-hour treatment with PPARγ ligands and varying concentrations of imatinib, ranging from 0 μM to 5 μM. PPARγ antagonists co-administered with imatinib resulted in a significantly lower ED50imatinib. Data are mean ± SEM for at least 3 biological replicates. *P<0.05; **P<0.01; compared with DMSO control. DMSO: dimethyl sulfoxide; PPARγ: peroxisome proliferator-activated receptor γ; ED: the half maximal effective concentration.

Treatment with PPARγ ligands significantly alters OCT-1 activity in MNC from de novo CP-CML patients

Our previous studies demonstrated that CP-CML patients with low MNC OA (less than 4.0 ng/200,000 cells, lowest OA quartile) at diagnosis have the poorest response to imatinib treatment and the highest rate of transformation to accelerated phase or blast crisis. Herein we examined the effect of PPARγ ligands on OA in cryopreserved MNC isolated from CP-CML patients at diagnosis. Patient baseline MNC OA values were divided into two groups (“high OA” and “low OA”) using the cutoff as 4.0 ng/200,000 cells.

In patients with high OA, treatment with PPARγ agonists resulted in consistently reduced OA. The average OA in these samples was significantly reduced by GW1929 (from 7.9 to 5.2 ng/200,000 cells, P=0.0075, Figure 2A) or Rosi (from 7.9 to 4.7 ng/200,000 cells, P=0.0001, Figure 2B). In another 5 MNC samples with high OA, treatment with PPARγ agonist Pio resulted in a similar decrease in average OA (from 9.5 to 5.1 ng/200,000 cells, P=0.0001, Figure 2C). As a result of this decrease, OA values in 60% high OA cases (6 out of 10) were moved into the low OA group in the presence of PPARγ agonists.

Notably, treatment with PPARγ antagonists increased the OA in patients with low OA, (n=6). The average OA in these patients was increased from 2.7 to 5.1 ng/200,000 cells by GW9662 (P=0.0129, Figure 2D) and to 4.7 ng/200,000 cells by T0070907 (P=0.0155, Figure 2E). In addition, this increase in OA afforded by the PPARγ antagonists resulted in 5 out of 6 low OA samples (83.33%) moving into high OA groups.

In contrast to the results in CP-CML patient samples, no significant change was observed in OA in peripheral blood MNC isolated from healthy donors after incubation with any PPARγ ligand (P>0.05, Figures 2F-2J).

Treatment with PPARγ ligands significantly alters IC50imatinib and cell viability when co-administered with imatinib

The IC50imatinib was examined in KU812 cells to assess the observed effects of PPARγ ligands on OA translate into corresponding changes in BCR-ABL tyrosine kinase inhibition. Consistent with the results of the OA assay, a significant increase in IC50imatinib was observed in KU812 cells when treated with the PPARγ agonists GW1929 (from 4.7 to 7.2 μM, P=0.0013) or Rosi (from 4.7 to 8.5 μM, P=0.0078). In the presence of Pio, about 70% increment (from 4.7 to 6.4 μM) in IC50imatinib was observed compared to the control, although this increment was not statistically significant (P=0.1151). In contrast, treatment with PPARγ antagonists significantly reduced the IC50imatinib in KU812 cells (GW9662: from 4.2 to 2.5, P=0.0078, or T0070907: from 4.2 to 1.4 μM, P=0.0055, Figure 3A).

Annexin V-PE and 7-AAD staining was performed in KU812 cells to investigate the effects of the PPARγ ligands on cell viability when co-administered with imatinib. In the presence of varying concentrations of imatinib, co-treatment with 10 μM PPARγ antagonists significantly reduced the half-maximal effective concentration (ED50) that induces cell apoptosis (GW9662: P=0.0236, T0070907: P=0.0011) compared with vehicle control (Figure 3B). There was no significant effect on cell viability when treating KU812 cells with PPARγ agonists.
Lentiviral over-expression of PPARγ significantly decreases OCT-1 activity and increases IC50imatinib

WT and DN PPARγ (Figure 4A for construct schematics) transduced K562 cells were FAC-sorted isolated based on green fluorescent protein (GFP) intensity. Over expression of FLAG-tagged PPARγ was confirmed by western blotting with anti-FLAG M2 antibodies (Figure 4B) and RQ-PCR (Figure 4C). Compared to WT PPARγ, DN PPARγ has impaired ligands binding affinity and significantly reduced transcriptional activity. As shown in Figure 4D, compared with the empty vector (EV) control (mean OA=24.0), the OA was significantly decreased in WT PPARγ transduced K562 cells (mean OA=16.0, n=4, 0.5). There was no significant difference in OA between the EV control and DN PPARγ transduced K562 cells (mean OA=23.6, n=4, 0.5).

When examining the IC50imatinib in transduced K562 cells, a significant increase was observed in cells transduced with WT PPARγ (mean 13.3 μM) compared with EV control cells (mean 6.8 μM, n=3, 0.0074, Figure 4E). No significant change in IC50imatinib was observed in cells transduced with PPARγ-DN (mean 5.7 μM) compared to EV control cells (n=3, 0.5).

Neither PPARγ gene expression nor PPARγ protein is associated with OCT-1 activity

The effect of PPARγ ligands on OA strongly suggests the involvement of PPARγ in OA regulation. No significant change in PPARγ gene expression was observed in KU812 cells after 3-hour treatment with PPARγ ligands (P>0.5, Online Supplementary Figure S1A). The PPARγ mRNA level in diagnostic MNC of CP-CML patients was measured using the Illumina HumanHT-12v4 platform and compared between high and low OA groups to determine any association between PPARγ gene expression and OA in primary cells. As shown in the Online Supplementary Figure S1B, across 120 CP-CML patient MNC samples tested, the average PPARγ mRNA level in low OA patients was not different from that in the high OA group (mean 4.52 vs. 4.51, 0.6673). The expression of total PPARγ protein also remained unchanged in KU812 cells treated with PPARγ ligands (P>0.5, Online Supplementary Figure S1C). In whole cell lysates prepared from CP-CML patient MNC samples, no significant difference was observed in PPARγ total protein levels between patients in low OA (n=6) and those in high OA groups (n=7, 0.7732, Online Supplementary Figure S1D).
Notably, there was no significant difference in the mRNA expression level of \textit{SLC22A1} (encoding OCT-1) in KU812 cells treated with PPAR\textsubscript{γ} ligands compared with vehicle control (\(P>0.5\), Online Supplementary Figure S1E). In addition, when assessing MNC of \textit{de novo} CP-CML patients, the \textit{SLC22A1} mRNA expression levels between the two OA groups were comparable (\(P=0.3006\), Online Supplementary Figure S1F).

\textbf{PPAR\textsubscript{γ} transcriptional activity negatively correlates with OCT-1 activity in MNC of \textit{de novo} CP-CML patients}

PPAR\textsubscript{γ} plays an important role in activating the transcription of its downstream target genes that mediate multiple signaling pathways.\textsuperscript{30} However, the level of PPAR\textsubscript{γ} transcriptional activity has not previously been investigated in CP-CML, in particular its link with OA. To further evaluate this relationship, CML patients were grouped into low and high OA groups as previously defined, and the nuclear PPAR\textsubscript{γ} transcriptional activity was compared between the two groups. The result confirmed that nuclear PPAR\textsubscript{γ} transcriptional activity was significantly higher in the low OA group (average 0.1742, \(n=33\)) compared with the high OA group (average 0.0889, \(n=51\), \(P=0.0001\), Figure 5A). Additionally, a significant negative correlation was observed between the transcriptional activity of PPAR\textsubscript{γ} and the OA in individual samples (\(n=84\), \(r=-0.5677\), \(P=0.0003\)). Linear regression analysis revealed a significant relationship between PPAR\textsubscript{γ} transcriptional activity level and OA (\(P<0.0001\)), with the model described as OA=8.0-21.3×(PPAR\textsubscript{γ} activity level). Using this fitted model, we identified a PPAR\textsubscript{γ} transcriptional activity level of 0.2 or greater (rounded from 0.19 to be more conservative) to be associated with a low OA. Hence, samples with high PPAR\textsubscript{γ} activity levels (>0.2) were predicted to be low OA, whereas low PPAR\textsubscript{γ} activity levels (≤0.2) were predicted as high OA. As such, samples with high PPAR\textsubscript{γ} activity levels (\(n=11/13\), 85\%) were significantly enriched for low OA, compared to the samples of the group with low PPAR\textsubscript{γ} transcriptional activity levels (\(n=21/71\), 30\%) (OR=13.1; 95\% CI: 2.7-64.3; \(P=0.0003\), Figure 5C).

No significant difference was observed in plasma 15d-PGJ2 between CP-CML patients with low and high OCT-1 activity or PPAR\textsubscript{γ} activity

One of the major regulatory mechanisms of PPAR\textsubscript{γ} transcriptional activity is the direct binding of PPAR\textsubscript{γ} ligands, such as 15d-PGJ2,\textsuperscript{31} that result in conformational changes of PPAR\textsubscript{γ} and subsequent changes in its transcriptional activity.\textsuperscript{32} To investigate the possibility that 15d-PGJ2 plays a role in activating PPAR\textsubscript{γ} in CP-CML, the plasma levels of 15d-PGJ2 were examined in 150 CP-CML patient samples prior to imatinib treatment. No significant difference was observed in plasma 15d-PGJ2 levels between the patients in the low or high OA groups (\(P=0.2446\), Online Supplementary Figure S2A). In 59 samples with matched PPAR\textsubscript{γ} transcriptional activity results, there was no significant correlation between plasma 15d-PGJ2 levels and PPAR\textsubscript{γ} transcriptional activity (\(P=0.4112\), Online Supplementary Figure S2B).

\textbf{Cell composition of CP-CML patient MNC varies significantly between patients with low and high OA}

Our previous study reported that MNC OA varies great-
ly between cell lineages in CML and is significantly associated with the OA in isolated neutrophils. It is possible that the specific cell composition within individual patient samples may underlie their specific OA. Given that the MNC compartment in CML patients at diagnosis is predominantly comprised of immature and mature neutrophils, here the expression of the granulocytic surface markers CD15 and CD16 in the MNC population was examined and correlated with OA. As shown in Figure 6A, B, compared with high OA patients, patients with low OA had a higher percentage of CD15^brightCD16^dimCD14^- cells (44.47% vs. 20.97%, P=0.0048) and a lower percentage of CD15^-CD16^-CD14^- neutrophils (12.87% vs. 23.52%, P=0.0113) in the MNC samples. In keeping with the above findings there was a significant negative correlation between the percentage of CD15^brightCD16^-CD14^- cells and MNC OA in CML diagnosis patients. The Pearson product-moment was used to assess the correlation. (D) The mean percentage of CD15^brightCD16^-CD14^- cells in samples with high PPARγ transcriptional activity levels was significantly higher than samples with low PPARγ transcriptional activity levels. The error bars represent 95% confidence interval (CI) of the mean value. *P<0.05; **P<0.01. OA: OCT-1 activity; OCT-1: organic cation transporter-1; PPARγ: peroxisome proliferator-activated receptor γ.

Discussion

The functional OA in primary CML mononuclear cells at diagnosis is a strong and reliable predictor of both short- and long-term imatinib responses and clinical outcomes in independent clinical trials. Modulation of the OCT-1 transporter to increase the uptake of imatinib into CML cells could potentially improve the efficacy of imatinib therapy for patients with low OA. Many studies have been published investigating the SLC22A1 (encoding OCT-1) genetic variants and its link with imatinib uptake. However, as reviewed by Watkins et al., this is controversial and the mechanism regulating imatinib uptake via OCT-1 remains unclear.

Our previous data demonstrated that the treatment with PPARγ ligand diclofenac significantly increased imatinib uptake mediated by OCT-1. In the study herein, the
negative link between OA and PPARγ activation has further been elucidated by treating BCR-ABL1+ cell lines and primary MNC of CP-CML patients with various synthetic PPARγ ligands. Over-expression of WT PPARγ in K562 cells resulted in significantly decreased OA, confirming these effects as specific to PPARγ. In addition, by investigating nuclear transcriptional activity of PPARγ in CP-CML patient MNC samples, we provide evidence that activation of PPARγ negatively impacts OA and therefore reduces imatinib uptake and retention.

We have previously demonstrated that there is a significant correlation between the in vitro BCR-ABL kinase activity inhibitory concentration 50% for imatinib (IC50imatinib) and OA. These findings are substantiated herein, by demonstrating that alterations of OA by PPARγ ligands translated to corresponding changes in sensitivity to BCR-ABL kinase inhibition, as demonstrated in IC50imatinib. However, when used in combination with imatinib, significant changes in cell viability were only observed following PPARγ antagonist treatments. The change in OA and IC50imatinib did not extend to an increase in ED50imatinib in PPARγ agonist treated cells. Of note, the baseline IC50imatinib value in KUB12 cells is at a relatively high level (4.2 μM). Therefore, it is tempting to speculate that the cell viability has already reached its peak and cannot be further improved by PPARγ agonist treatments. In addition, activation of PPARγ has been recently reported to decrease STAT5 transcription in CML stem cells. It is possible that the impaired intracellular imatinib uptake by PPARγ agonists may be counterbalanced by their inhibitory effect on STAT5.

Different from the synergistic effect of pioglitazone and imatinib in CML stem cells,5 we observed an opposing effect of PPARγ and imatinib, probably due to the different target populations (MNC vs. CD34+ cells) with varying SLC22A1 mRNA expression and imatinib uptake.6 As OA in CD34+ cells has been proven to be significantly low or even below the level of detection,14 it is unlikely that OA will be decreased significantly, or measurably, within the confines of this assay, by the use of a PPARγ agonist. In addition, OA in CD34+ is not predictable for the achievement of major molecular response (MMR).15 Given that diagnostic peripheral blood MNC samples is the cell population in which the predictive value of OA was established, this cell population was investigated in the current study. While the proposed PPARγ ligand-TKI combination therapy can effectively target leukemic stem cells, the contradictory effect of PPARγ on intracellular imatinib uptake and retention observed in circulating MNC, suggests that it may not be an ideal option for de novo CP-CML patients on imatinib, as a rapid initial decline in BCR-ABL1 transcripts is critical for improved event-free survival.16 In addition, the inter-patient variation in PPARγ transcriptional activity we demonstrate herein may make this combinational therapy only applicable to those patients with low PPARγ transcriptional activity. However, patients on second- or third-generation TKIs may benefit from such therapy,17 as these TKIs are not transported via OCT-1.18,19

The role of PPARα in imatinib transport has been reported by Wang et al. using KCL22 cells and primitive CML CD34+ cells, whereby PPARα agonists upregulated imatinib uptake by increasing PPARα and SLC22A1 mRNA expression levels.20 Despite the high homology at the protein level, different or even contrary biologic functions of PPARα and PPARγ have been implicated in monocytes/macrophages21-23 or cardiomyocytes24 by several groups. In the current study, we did not observe significant changes in PPARγ or SLC22A1 mRNA expression in PPARγ ligand treated cells. Therefore, it is likely that PPARγ interacts with the OCT-1 transporter through a different mechanism from PPARα. Instead of altering the expression of SLC22A1, we speculated that the regulation of OA observed here by PPARγ is through a PPARγ direct target gene network. The downstream target genes of PPARγ, together with the overlapping and competing actions of PPAR transcription factors in CML cells, are the focus of ongoing studies.

PPARγ is known to interact with several endogenous ligands.25 Given the important role of PPARγ in inflammation regulation44 and the potential link between CML and the cyclooxygenase/prostaglandin pathway,26 we examined the plasma levels of 15d-PGJ2, the dehydration end product of prostaglandin D2. The result suggested that ligand binding by 15d-PGJ2 is not critical for the inter-patient variability in PPARγ activation, which is in agreement with the previous report that in vivo 15d-PGJ2 is insufficient to activate PPARγ.45,46

Similar to the previous findings with diclofenac,27 our results demonstrate that PPARγ regulates OA in a BCR-ABL-dependent manner, as the effects on OA by PPARγ ligands were only observed in CP-CML patient MNC but not in healthy donor MNC. It has been reported that the constitutively active tyrosine kinase BCR-ABL promotes neutrophil differentiation by downregulating c-Jun expression, while BCR-ABL inhibition by imatinib promotes monocytic differentiation in KCL22/α cells.48 Our previous work has also indicated that BCR-ABL may have an indirect effect on OA by promoting granulocytic differentiation.29 In addition, PPARγ is required for terminal maturation in the granulocytic lineage in vivo, but to a lesser extent for the early stages of hematopoietic cell development.30 Herein we examined differentiation stages of neutrophils and demonstrated that a subset of mature neutrophils (CD15−CD16+CD14-) was enriched in low OA patients. Furthermore, a higher percentage of these cells were observed in patients with high PPARγ transcriptional activity, which suggests a correlation between PPARγ activation, neutrophils maturity and OA. Based on our findings, we speculate that the variation in patient MNC OA may reflect the heterogeneous leukemia cells composition influenced by BCR-ABL and PPARγ. More research will be necessary to determine the role of PPARγ in granulocytic lineage differentiation procedures, especially in the presence of BCR-ABL.

In conclusion, the findings presented in the study herein demonstrate that treatment with PPARγ ligands significantly alters OA via a BCR-ABL-dependent mechanism. PPARγ transcriptional activity, rather than mRNA or protein expression level, has a significant correlation with OA. Furthermore, the significant enrichment of mature neutrophils in patients with low OA and high PPARγ transcriptional activity indicates the involvement of PPARγ in the granulocytic differentiation driven by BCR-ABL. These findings suggest that, while PPARγ ligand has been shown to efficiently affect CML stem cells, inter-patient variability in PPARγ plays a critical role in the heterogeneity in patients’ MNC OA at diagnosis. Personalized combination therapeutic strategy may be needed when targeting different leukemia cell populations.
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References
10. Głowacka-Mrowka E, Manda-Handzlik J. Wangviding the lentiviral plasmids and Professor Andrew Zannettino (The Centre for Cancer Biology, Australia) for kindly providing the
29. Eadie L, Hughes TP, White DL. Nilotinib does not significantly reduce imatinib OCT-
1 activity in either cell lines or primary CML cells. Leukemia. 2010;24(4):855-857.


39. Lu L, Saunders VA, Leclercq TM, Hughes TP, White DL. Ponatinib is not transported by ABCB1, ABCG2 or OCT-1 in CML cells. Leukemia. 2015;29(8):1792-1794.


