SUPPLEMENTARY APPENDIX

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

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Methods

- **Cell lines**

Human *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.[1] All cell lines were cultured in RPMI-1640 media (Sigma-Aldrich, USA) supplemented with 10% foetal bovine serum (JRH Biosciences, USA), 2 mM L-glutamine (SAFC Biosciences, USA) and 100 U/mL penicillin G/streptomycin (Sigma-Aldrich) at 37°C with 5% CO_2 in a humidified incubator (Thermo Scientific, AU).

- **Primary samples from CP-CML patients or healthy donors**

Primary cells and plasma samples were collected from *de novo* CP-CML patients enrolled in the TIDEL II study[2] prior to the commencement of imatinib therapy. Peripheral blood was obtained from healthy volunteers. All peripheral blood samples were collected with informed consent in accordance with the Declaration of Helsinki. MNCs were isolated from blood using Lymphoprep (Axis Shield, Norway) density gradient centrifugation. Experiments were performed on cryopreserved cells. Plasma samples were collect using EDTA or heparin as an anticoagulant and stored at -80°C until required.

- **Drugs**

Imatinib mesylate (STI571) and ^{14}C-labelled imatinib were kindly provided by Novartis Pharmaceuticals (Switzerland). The potent OCT-1 inhibitor prazosin and PPARγ agonists GW1929, rosiglitazone, pioglitazone, antagonists GW9662 and T0070907 were all purchased from Sigma-Aldrich.

- **Lentivirus production and cell transduction**

The lentiviral plasmids expressing FLAG-tagged wild-type (WT) PPARγ1 and dominant negative (DN) PPARγ1-L466A/E469A, together with empty vector (EV), were kindly provided by A/Prof Claudine Bonder (Centre for Cancer Biology, Australia).[3] The plasmids were constructed from a previously characterized vector, pLenti4/TO-IRES EGFP.[4] Sequencing analyses verified the integrity of the inserted PPARγ1 cDNA.

The packaging cell line Hek293T and lentiviral packaging plasmid (pVSV-G and Pax2) were kindly provided by Prof Andrew Zannettino (University of Adelaide, Australia). HEK293T cells were transfected using Lipofectamine 2000 reagent (Invitrogen Life Technologies, USA)

as previously described.[5] Harvested supernatant containing infectious particles filtered through 0.45 μm Nalgene filters (Nalgene Labware, USA) was added to K562 cells at early cell passage. After 72 hours incubation at 37°C with 5% CO_2, lentivirus was removed and fresh culture media was added. GFP^+ cells were isolated using the fluorescence-activated cell sorting (FACS) and selected for subsequent experimentation.

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

The IUR assay was performed to measure the intracellular concentration of imatinib achieved and maintained in cells over a 2-hour period, in the presence or absence of OCT-1 inhibition, as previously described.[6, 7] Cell lines or primary MNCs were pre-incubated at 37°C in 5% CO_2 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. The OCT-1 activity is 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 $IC50^{imatinib}$ values**

Western blotting analyses for phosphorylated CrkL (p-CrkL) were performed and $IC50^{imatinib}$ were determined based on the *in vitro* reduction in the level of p-CrkL as previously described.[8, 9] Cells were pre-incubated with 40 μM PPARγ ligands for one hour at 37°C in 5% CO_2 prior to exposure to imatinib (ranging from 0 μM to 100 μM).

Whole cell lysates from *PPARG*-transduced cell extracts were analyzed by western blotting with anti-FLAG M2 antibodies (Sigma-Aldrich, dilution 1:5,000). Anti-PPARγ (H-100, Santa Cruz Biotechnology, USA, dilution 1:1,000) and alkaline-phosphatase conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz, dilution 1:2,000) were employed according to the manufacturer's specifications, to determine the protein level of PPARγ. Results were analyzed using ImageLab software 5.0 (Bio-Rad) with GAPDH (Cell Signaling Technology, dilution 1:1,000) as loading control to normalize the PPARγ protein.

- **Cell viability Analyses**

Cells were incubated with 10 μM PPARγ ligands for 24 hours at 37°C in 5% CO_2, prior to an additional 72-hour treatment with PPARγ ligands and varying concentration of imatinib, ranging from 0 μM to 5 μM. After incubation, cells were washed twice with culture medium and stained concurrently with Annexin V-PE and 7-AAD (both from Annexin V-PE Apoptosis

Detection Kit, BD Biosciences, USA) according to the manufacturer's instructions. Cell viability was evaluated by flow cytometry (LSR-Fortessa flow cytometer, BD Biosciences). The half-maximal concentration (ED50) that induces cell apoptosis was estimated using non-linear regression as implemented in the GraphPad™ Prism software program (ver. 7.0a; GraphPad Software, USA).

- **Examination of *PPARG* and *SLC22A1* mRNA expression in *BCR-ABL1*$^+$ CML cell lines and MNCs of *de-novo* CP-CML patients**

The expression level of *PPARG* and *SLC22A1* (encoding OCT-1) mRNA in KU812 cells were examined by RQ-PCR. RNA was extracted using TRIzol Reagent (Invitrogen, USA). The sequences for the *PPARG* primers were as follows: F, 5'-TGAAGGATGCAAGGGTTTCT-3'; R, 5'- CCAACAGCTTCTCCTTCTCG-3'. The sequences for the *SLC22A1* primers were as follows: F, 5'-CTGAGCTGTACCCCACATTCG-3'; R, 5'-CCAACACCGCAAACAAAATGA-3'. The sequences for the reference gene *hTBP* (encoding human TATA box binding protein) primers were as follows: F, 5'-CCACTCACAGACTCTCACAAC-3'; R, 5'- CTGCGGTACAATCCCAGAACT-3'. *PPARG* and *SLC22A1* mRNA expression levels in peripheral blood MNCs of CP-CML patients at diagnosis were evaluated using the Illumina Human HT-12v4 platform. The microarray was performed at the Australian Genome Research Facility (AGRF). RNA was prepared using miRNeasy Mini kit (QIAGEN, Netherlands). Raw microarray data was preprocessed using the Limma package of the Bioconductor open-source software project.[10] Raw intensities were normalized using the neqc function. Probes were filtered if not detected in any sample based on the detection p-value less than 0.05.

- **PPARγ transcriptional activity in MNCs of *de-novo* CP-CML patients**

Nuclear extracts from CP-CML patient diagnostic MNCs were prepared using the Nuclear Extraction Kit (Active Motif, USA) following the manufacturer's protocol. PPARγ transcriptional activity was then measured using the PPARγ Transcription Factor Assay Kit (Active Motif) following the manufacturer's specifications. The absorbance was read on a spectrophotometer (Bio-Tec Instruments, USA) at 450 nm with a reference wavelength of 655 nm.

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

The 15d-PGJ2 levels in plasma samples collected from CP-CML patients at diagnosis were analyzed using a 15d-PGJ2 ELISA kit (ENZO Life Sciences, USA) according to the

manufacturer's instructions. The absorbance was read on a spectrophotometer at 405 nm with a reference wavelength of 570 nm.

- **Statistical Analyses**

All statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, USA). Differences were considered to be statistically significant when the p-value was less than 0.05.

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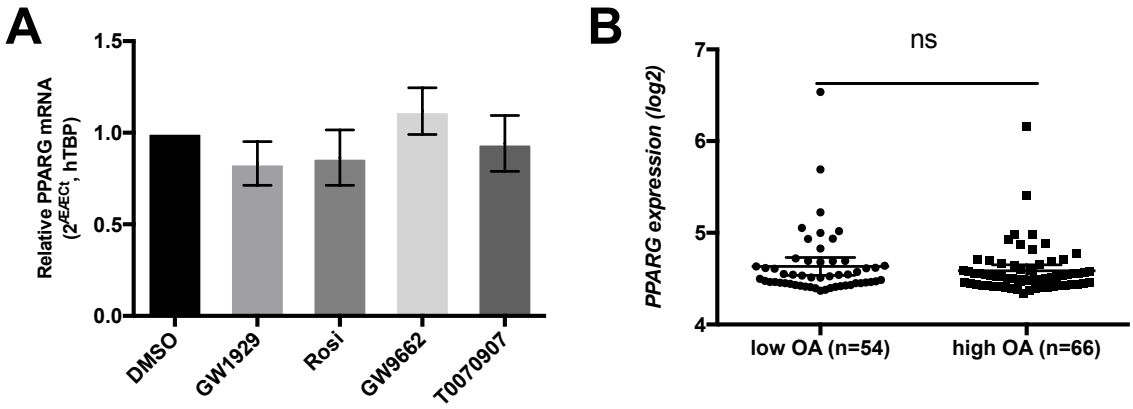
Supplementary Figure 1. Neither *PPARG* mRNA expression nor PPARγ total protein level is associated with OCT-1 activity.

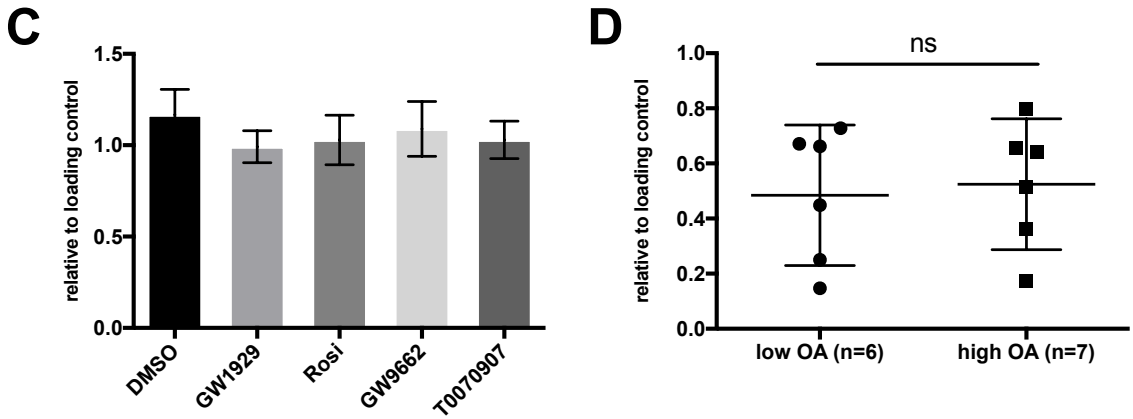
(A) *PPARG* mRNA expression levels were examined by RT-PCR in KU812 cells after 3-hour treatment with PPARγ ligands. **(B)** *PPARG* mRNA was comparable in diagnostic MNCs of CP-CML patients with low and high OA when examined using the Illumina Human HT-12v4 platform. **(C)** No difference was observed in total PPARγ protein after the treatment of PPARγ ligands in KU812 cells. **(D)** The expression of total PPARγ protein in MNCs from CP-CML patients with low and high OA are comparable. **(E)** *SLC22A1* mRNA expression remained at the same level in KU812 cells treated after 3-hour treatment with PPARγ ligands. **(F)** There was no difference in *SLC22A1* mRNA in diagnostic MNCs of CP-CML patients with low and high OA.

Data in (A), (C) and (E) are mean ± SEM for at least 3 biological replicates. The error bars in (B), (D) and (F) represent 95% confidence interval (CI) of the mean value.

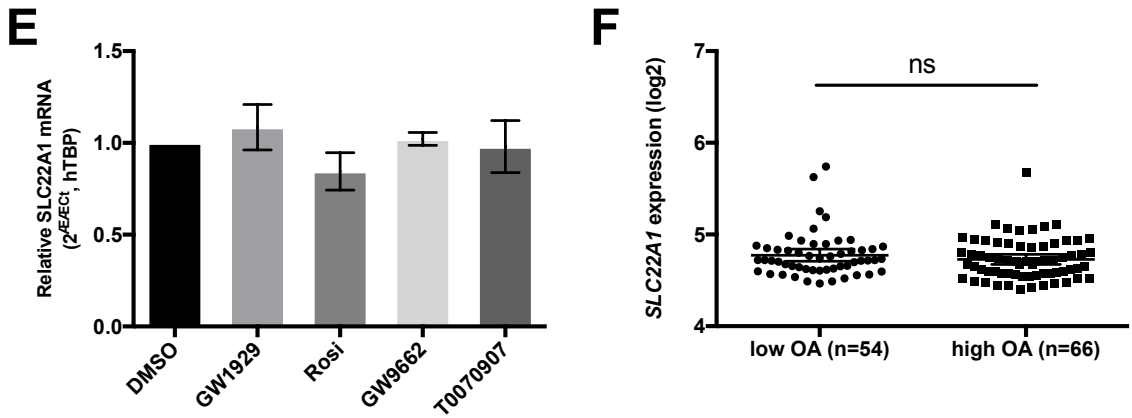
PPARG mRNA



PPARγ total protein



SLC22A1 mRNA



Supplementary Figure 2. No significant difference was observed in plasma 15d-PGJ2 between CP-CML patients with low and high OCT-1 activity or PPARγ activity.

(A) The 15d-PGJ2 plasma levels in patients with low OA and high OA were comparable. The error bars represent 95% confidence interval (CI) of the mean value. **(B)** There was no correlation between 15d-PGJ2 levels and PPARγ transcriptional activity by Pearson product-moment correlation.