Editorial Manager(tm) for Experimental Hematology
Manuscript Draft

Manuscript Number:

Title: Inhibition of MDR1 does not sensitise primitive chronic myeloid leukaemia CD34+ cells to imatinib

Article Type: Regular Submission

Keywords: BCR-ABL; chronic myeloid leukaemia; imatinib mesylate; ATP-binding cassette (ABC) efflux transporters; MDR1.

Corresponding Author: Dr Heather Graham Jorgensen, PhD

Corresponding Author's Institution: University of Glasgow

First Author: Sophia Hatziieremia, BSc, PhD

Order of Authors: Sophia Hatziieremia, BSc, PhD; Niove E Jordanides, BSc, PhD; Tessa L Holyaoke, MD, PhD; Joanne C Mountford, BSc, PhD; Heather Graham Jorgensen, PhD

Abstract: Objective
To investigate the interaction of imatinib mesylate (IM) with the clinically relevant ATP-binding cassette (ABC) efflux transporter MDR1 (ABCB1) in cells from patients with chronic myeloid leukaemia (CML), to explore whether inhibition of this transporter would improve IM's efficacy in the elimination of CML CD34+ cells by increasing cell associated drug accumulation.
Methods
Cells from newly diagnosed chronic phase CML patients were harvested by leukapheresis and enriched to >95% CD34+. Expression of the transporter gene MDR1 was performed by qRT-PCR. Interaction of IM with MDR1 was analysed by substrate (rhodamine 123) displacement assay. Cell associated levels of IM in CML CD34+ cells were measured by HPLC. Intracellular phospho-CrkL (p-CrkL) levels, apoptosis in total CML CD34+ cells and high resolution tracking of cell division were assayed by flow cytometry.
Results
Measurements of cell associated IM uptake showed significantly lower drug levels in CD34+ cells, particularly the CD38- sub-population, as compared to IM sensitive K562 cells. MDR1 was expressed at low level and dye efflux studies demonstrated very little MDR1 activity in CML CD34+ cells. Furthermore, combination treatment of primitive CML cells, with IM and the MDR1 inhibitor PSC833, did not result in elevated cell associated IM levels. Although we observed slightly enhanced cytostasis with IM when combined with PSC833, this was independent of BCR-ABL inhibition as no associated decrease in p-CrkL was observed.
Conclusions
Our findings demonstrate that inhibition of MDR1 neither enhances the effect of IM against BCR-ABL activity, nor significantly potentiates IM's efficiency in eliminating primitive CML cells.

Inhibition of MDR1 does not sensitise primitive chronic myeloid leukaemia CD34$^+$ cells to imatinib

Sophia Hatziieremia*, Niove E Jordanides*, Tessa L Holyoake, Joanne C Mountford and Heather G Jørgensen.

Section of Experimental Haematology, Division of Cancer Sciences and Molecular Pathology, Paul O'Gorman Leukaemia Research Centre, Gartnavel General Hospital, Faculty of Medicine, University of Glasgow, 1053 Great Western Road, Glasgow, G12 0YN

Running title: Imatinib transport in CML

*** SH and NEJ contributed equally in the performance of the experiments, analysis of the results, figures and writing of this manuscript.**

Correspondence: Dr Heather G Jorgensen BSc, PhD, Section of Experimental Haematology, Division of Cancer Sciences and Molecular Pathology, Paul O'Gorman Leukaemia Research Centre, Gartnavel General Hospital, Faculty of Medicine, University of Glasgow, 1053 Great Western Road, Glasgow, G12 0YN, United Kingdom.

Tel: +44 (0)141 301 7880; Fax: +44 (0)141 301 7898; e-mail: hgj1b@clinmed.gla.ac.uk

Table of Contents Category: Haematological Malignancies

Word count: 4100

Objective

To investigate the interaction of imatinib mesylate (IM) with the clinically relevant ATP-binding cassette (ABC) efflux transporter MDR1 (ABCB1) in cells from patients with chronic myeloid leukaemia (CML), to explore whether inhibition of this transporter would improve IM's efficacy in the elimination of CML $CD34^+$ cells by increasing cell associated drug accumulation.

Methods

Cells from newly diagnosed chronic phase CML patients were harvested by leukapheresis and enriched to >95% $CD34^+$. Expression of the transporter gene *MDR1* was performed by qRT-PCR. Interaction of IM with MDR1 was analysed by substrate (rhodamine 123) displacement assay. Cell associated levels of IM in CML $CD34^+$ cells were measured by HPLC. Intracellular phospho-CrkL (p-CrkL) levels, apoptosis in total CML $CD34^+$ cells and high resolution tracking of cell division were assayed by flow cytometry.

Results

Measurements of cell associated IM uptake showed significantly lower drug levels in $CD34^+$ cells, particularly the $CD38^-$ sub-population, as compared to IM sensitive K562 cells. *MDR1* was expressed at low level and dye efflux studies demonstrated very little MDR1 activity in CML $CD34^+$ cells. Furthermore, combination treatment of primitive CML cells, with IM and the MDR1 inhibitor PSC833, did not result in elevated cell associated IM levels. Although we observed slightly enhanced cytostasis with IM when combined with PSC833, this was independent of BCR-ABL inhibition as no associated decrease in p-CrkL was observed.

Conclusions

Our findings demonstrate that inhibition of MDR1 neither enhances the effect of IM against BCR-ABL activity, nor significantly potentiates IM's efficiency in eliminating primitive CML cells.

Keywords: BCR-ABL; chronic myeloid leukaemia (CML); imatinib mesylate (IM); ATP-binding cassette (ABC) efflux transporters; MDR1.

Introduction

Imatinib mesylate (IM; Glivec®/Gleevec®, Novartis Pharma AG, Basel, Switzerland) is a drug targeted against the Philadelphia (Ph) chromosome product, BCR-ABL tyrosine kinase, that has proven to be remarkably effective in the treatment of patients with chronic myeloid leukaemia (CML) in early chronic phase (CP).[1] However, even with IM treatment most patients with CML fail to achieve complete molecular responses and still have detectable levels of minimal residual disease (MRD), as demonstrated by positivity for *BCR-ABL* transcripts.[2]

Several different mechanisms for IM-resistance have previously been proposed, including *BCR-ABL* gene amplification, clonal evolution and over-expression of Src-related kinases.[3-8] However, point mutations within the kinase domain of *BCR-ABL* account for over 50% of cases of IM-resistance.[9,10] In many instances, with the major exception of the T315I mutant, these mutations are only partially resistant to IM and either dose escalation [11] or treatment with higher potency new tyrosine kinase inhibitors (TKI), such as dasatinib (Sprycel™; BMS-354825; Bristol-Myers Squibb, New York, USA) and nilotinib (Tasigna™; AMN107; Novartis, Basel, Switzerland) can overcome resistance. [12,13]

There is accumulating evidence to suggest that variable expression of multidrug transporters in CML patients' cells may play a critical role in the clinical response to IM treatment. [14] These transporters are transmembrane proteins functioning either as efflux or influx pumps for a wide range of compounds. Recent studies on IM influx have demonstrated an active uptake mechanism by cells via the organic cation transporter OCT-1 (slc22a1), with IM being a substrate for OCT-1.[15] However, the interaction of IM with efflux transporters has proven rather controversial. MDR1 (P-glycoprotein, Pgp; ABCB1), is the archetypal member of the ATP-binding cassette (ABC) transporter protein family and has been studied extensively as a factor modulating chemotherapeutic drug levels. Recent reports provide biochemical evidence that IM may interact with MDR1 as a substrate [16-18] and suggest that

MDR1 expression can be a limiting factor in IM distribution in the body.[19] Furthermore, several *in vitro* studies suggest that MDR1 activity confers resistance to IM in CML cell lines that have been modified to aberrantly express MDR1. This has been demonstrated by modulation of MDR1 function using specific inhibitors that abrogate the MDR1-dependent reduction of cell associated IM and restore sensitivity to IM.[20-22] Similar results have been obtained from Ph^+ IM-resistant cell lines using a stable knockdown of MDR1 by RNAi silencing. [23, 24] Although together these data suggest that MDR1 mediates resistance to IM, contradictory evidence for the role of MDR1 exists. Ferrao *et al*, demonstrated that MDR1 does not confer IM-resistance *in vitro,* as growth of K562 cells engineered to over-express MDR1, in the presence of IM, did not differ from control K562 cells.[25] Additionally, studies from Zong *et al* on the *in vivo* contribution of MDR1 to IM-resistance, performed in a murine model of CML, showed no difference in haemopoietic response to IM upon expression of MDR1.[26] However, despite the controversy regarding the role for MDR1 in mediating IM-resistance in cells lines and murine models, there has as yet been no data for primitive CML cells derived from patients in CP.

Previously we and others have identified a rare population of primitive ($CD34^+38^-$), leukaemic, quiescent stem cells (qSC), that comprise <5% of total $CD34^+$ cells and can regenerate CML populations in immunodeficient mice.[27-29] In addition, we have shown that $CD34^+$ and $CD34^+38^-$ cells express significantly increased levels (>10-fold) of *BCR-ABL* at the mRNA and protein levels, with respect to mature mononuclear cells.[30] Our recent findings demonstrate that primitive $CD34^+$ and $CD34^+38^-$ cells are both insensitive to IM *in vitro*, even at concentrations [31] that exceed the approximate *in vivo* peak steady state plasma level.[32] We have previously shown that ABCG2 over-expression is not associated with IM-resistance in primitive CML cells, as at therapeutic concentrations IM was shown to be an inhibitor of ABCG2 rather than a substrate.[33] The present study was designed to investigate the role of MDR1 in the resistance of CML $CD34^+$ cells to IM and to determine the

type of interaction (substrate/inhibitor) IM exhibits with the MDR1 transporter in these primitive cells.

We report here direct measurements of cell associated levels of IM in cell lines, $CD34^+$ CML and non-CML cells, using an analytical approach based on high pressure liquid chromatography (HPLC). Additionally, in CML patient derived $CD34^+$ cells, we investigated the level of expression of the *MDR1* gene and whether modulation of the function of MDR1 protein would result in increased cell associated levels of IM, thereby enhancing inhibition of BCR-ABL activity and cellular proliferation, thus sensitising the primitive population to IM treatment.

Materials and Methods

Cell lines and primary samples

K562-wild type (K-WT) and the transduced cell line K-MDR-GFP (K-MDR), which over-expresses the *MDR1* gene, were donated by Dr T. Southgate and the late Dr L. Fairbairn (Paterson Institute for Cancer Research, Manchester, UK). These were cultured in RPMI supplemented with 10% v/v FCS, 1mmol/L glutamine and 1mmol/L streptomycin/penicillin. Cells from newly diagnosed patients with CP CML and from patients with Ph^- haematological disorders that do not involve haemopoietic stem cells (non-CML) were harvested by leukapheresis and enriched to >95% $CD34^+$ using CliniMACS (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions and cryopreserved in 10% (v/v) dimethyl sulfoxide in ALBA (4% [w/v] Human Albumin Solution, Scottish National Blood Transfusion Service). Further selection of $CD34^+38^-$ cells was achieved by fluorescence activated cell sorting (FACS; BD FACSAria). Primary cells were cultured in IMDM with BIT, 1mmol/L glutamine, 1mmol/L streptomycin/penicillin, 40*n*g/mL LDL and 10^{-4}mol/L 2-mercaptoethanol, with or without 5 growth factors (GF) where indicated, as previously described.[31]

RNA synthesis

Cells were washed twice in PBS and total RNA was isolated from pellets using the RNeasy Mini Kit (Qiagen, Crawley, UK). The resulting RNA was quantitated using a Nanodrop spectrophotometer Nd-1000 (Labtech International, East Sussex, UK) and the integrity of the RNA was determined using the Agilent 2100 Bioanalyser (Agilent Technologies, Wokingham, UK). RNA was synthesised to cDNA by the High Capacity cDNA Archive kit (Applied Biosystems, Warrington, UK) and the cDNA samples were stored at -80ºC until further use. RNA (1μg aliquots) derived from normal $CD34^+$ cells isolated from mobilised peripheral blood (mPB) was purchased from StemCell Technologies UK (London, UK).

Semi-quantitative RT-PCR

The expression of transporter gene *MDR1* [31,34-36] was measured using custom Taqman® Low Density Array (TLDA) cards (Applied Biosystems, Warrington, UK). The formula used for the relative quantification of *MDR1* was based on the comparative crossing threshold method. This is calculated by initially subtracting the endogenous control Ct (cycle number at which the curve intersects the threshold) from the target gene Ct to give a ΔCt. The comparison between the target CML sample with regard to the normal calibrator sample is then calculated by subtracting the ΔCt value of the normal sample from the CML sample to give a ΔΔCt, which is then used as the number in the power calculation $2^{-\Delta\Delta Ct}$ (i.e. $2^{-(\Delta Ct[\text{target sample}]-\Delta Ct[\text{calibrator sample}])}$), providing the fold difference in CML compared to the normal sample.

Cell associated IM levels and the effect of efflux transporter inhibition

For a single experiment, cells were plated at $5x10^4$ cells/mL/well for cell lines and $1x10^5$ cells/mL/well for patient cells and left to equilibrate for 2h at 37ºC and 5% CO_2, prior to addition of different concentrations of IM for 2h. To test the effect of MDR1 inhibition on cell associated levels of IM, cells were incubated with 5μM PSC833 (a specific inhibitor of MDR1; Dr R. Clark, University of Liverpool, UK) for

30 mins, prior to addition of IM (5, 10 μM) for 2h. PSC833 was maintained in the cell culture medium throughout IM incubation.

Following drug treatment, cells were centrifuged at 276g for 10 mins at 4ºC and the resulting pellet was washed three times with 1mL ice-cold PBS, collecting the supernatant each time for analysis of extracellular IM levels to assure that the number of washes was sufficient in eliminating any extracellular IM. For cell associated concentrations, cells were lysed with 100μL 0.2M NaOH/1% SDS, vortexed and sonicated for 2 mins. For extracellular IM levels, 100μL of supernatant was used from washing steps. An internal standard (IS), clozapine was added at 100μg/mL to each sample prior to extraction. Extraction of IM from lysed cells and supernatants was performed by protein precipitation with 150μL of ice-cold acetonitrile, followed by brief vigorous mixing and centrifuged at 432*g* for 15 mins at 4ºC.

The cell associated IM content of drug-treated cells was determined by a modified HPLC isocratic elution method. Analysis was performed on a Dionex Ultimate 3000 LC with a UV detector (260nM) and temperature controlled column compartment (35ºC). A 150x2mm, 5μm Gemini Phenomenex reversed phase column was used at a flow rate of 0.450mL/min. The mobile phase, used for LC analysis, consisted of 65:35 methanol:20mM NH_4Ac buffer, pH10. Intracellular IM levels as well as extracellular levels were analysed by injecting 20μL of extracted lysed cells or supernatant/washing solution onto the HPLC column.

Cell volumes

Cell volumes (V_{cell}) were calculated based on the equation: $V_{cell}={}^4/_3\ \pi\ r^3$. The cell radius was measured by taking images of live cells (~30 cells) in suspension with an Olympus CKX41 microscope and using Cell^B imaging software.

Efflux studies

The MDR1 efflux protocol was a modification of the protocol previously described for ABCG2 efflux transporter function.[33] Briefly, cells were incubated with either PSC833 (5μM) and/or IM (1-5μM) to block pump activity before the addition of rhodamine 123 (0.5μg/mL) (MDR1 substrate). Cells were then washed in ice-cold medium and analysed by flow cytometry.

Cellular analysis

Measurement of intracellular levels of phosphorylated CrkL (p-CrkL) , assessment of apoptotic cells, viability counts and high resolution tracking of cell division by CFSE (carboxyfluorescein succinimidyl ester) staining, were performed on CML $CD34^+$ cells, treated either with IM (5μM) or PSC833 (5μM), alone or in combination, for 72h. Intracellular p-CrkL and CFSE staining were performed and analysed by flow cytometry as described previously. [28-30, 39] For analysis of apoptosis, cells were incubated with 5μl Annexin V-FITC and 10µl Viaprobe in 100μl Annexin binding buffer for 15 mins in the dark. Finally, 400μl Annexin buffer were added to the cells and fluorescence was read by flow cytometry, within the hour, to identify apoptotic (Annexin V + Viaprobe positive) cells. Viability of CML $CD34^+$ cells was measured by trypan blue dye exclusion assay.

Statistical analysis

Results are shown as the mean ± standard deviation (SD) values obtained in repeated independent experiments. Differences between treatments were assessed using the paired Student's *t*-test.

Results

Efficiency of HPLC methodology for the measurement of cell associated IM levels

The lower limit of quantification (LLOQ) of this assay was assigned as 0.1μM (58.97ng/mL) IM, with signal to noise ratio >5. The analytical method was validated for accuracy and precision, based on 5 different concentrations, using quality controls (QCs) at 0.5 (294.8ng/mL), 1 (589.7ng/mL), 5 (2948ng/mL), 10 (5897ng/mL) and

50μM (29480ng/mL), for cell lysates performed in 5 replicates per day, over a 3 day period. Cell lysates were derived from K-WT cells. For all the QCs performed with cell lysates, accuracy was within 11% of the nominal value, while precision ranged from 3.8-7.5% as measured by the coefficient of variance. Recovery of IM by acetonitrile precipitation was determined at 2 concentrations (1 and 10μM) and gave 92% recovery of IM from cell lysates. Results were expressed as pg/cell, taking into account the number of cells per experiment, the volume of lysis buffer and extraction solvent and the injection volume.

Uptake of IM in cell lines and in CML and non-CML CD34$^+$ cells

HPLC analysis was used to determine the cell associated levels of IM achieved *in vitro* at concentrations of IM approximating the steady state plasma level in patients taking 400mg per day (1 and 5μM) [32] and at a higher concentration of 10μM. Although incubation of the Ph$^-$ cell line HL60 with 1μM IM lead to similar cell associated IM levels as for the Ph$^+$ cell line K-WT (HL60: 1.60±0.69pg/cell, n=3; K-WT: 1.83±0.75pg/cell, n=7; p=0.77), incubation with 5 (p=0.02) or 10μM (p=0.05) IM revealed significantly lower levels of IM in HL60 versus K-WT (Figure 1a). Lower levels of drug uptake were observed in CP CML CD34$^+$ cells (5μM: 1.36±0.36pg/cell, n=6; 10μM: 2.39±0.36pg/cell, n=6) and non-CML CD34$^+$ samples (5μM: 0.79±0.25pg/cell, n=3; 10μM: 1.77±0.24pg/cell, n=6) (Figure 1b). Analysis of IM accumulation in the CD34$^+$38$^-$ subset of CML samples, which approximates the qSC population *in vivo*, revealed a lower accumulation at both concentrations (5μM: 0.47 ± 0.09pg/cell, n=6; 10μM: 0.97 ± 0.09pg/cell, n=6) compared to the total CML CD34$^+$ population (p=0.04 and 0.009) (Figure 1b). However, measurement of the cell volumes demonstrated that there were significant cell volume differences between cell lines (HL60: 1498μm^3; K-WT: 2635μm^3) and patient cells (CML CD34$^+$: 800μm^3; CML CD34$^+$38$^-$: 641.1μm^3; non-CML CD34$^+$: 588.7 μm^3), which may explain the higher cell associated levels observed in cell lines (Table 1).

MDR1 efflux transporter expression and functionality in CML CD34$^+$ cells

To determine whether MDR1 activity contributed to cell associated levels of IM, we investigated *MDR1* expression in CML and normal CD34$^+$ cells and determined whether the MDR1 transporter was functional in these cells.

MDR1 mRNA expression was measured in 7 CP CML and 11 normal CD34$^+$ samples using real time qPCR. Using *GAPDH* as the endogenous control, CML samples consistently expressed *MDR1* mRNA at approximately 7-fold less than their normal counterparts (CML: ΔCt=8.33±0.47, normal: ΔCt=5.44±0.24 (p=0.0003)). The protein function of MDR1 was measured by substrate displacement studies using the specific MDR1 fluorescent substrate rhodamine 123 and the specific MDR1 inhibitor PSC833. The assay was initially validated in K-WT and K-MDR cells (Figures 2a & b). K-MDR efficiently effluxed 50ng/mL of rhodamine 123 and this efflux was maximally inhibited by 5μM PSC833 (Figure 2b), confirming that *MDR1* was functional in the cell line K-MDR. In CML samples (n=3), minimal efflux was observed in the absence of PSC833 compared to the presence of this specific inhibitor (Figure 2c), suggesting very low levels of functional MDR1 protein, in keeping with the mRNA data. Although there was a slight increase in rhodamine 123 retention in the presence of PSC833, the difference observed did not reach statistical significance.

Effect of IM on MDR1 efflux transporter function in CML CD34$^+$ cells

To determine whether IM interacts with the MDR1 efflux transporter, the substrate displacement assay was repeated by replacing the known inhibitor PSC833 with IM. In K-MDR cells, the addition of IM resulted in a concentration-dependent decrease in the efflux of rhodamine 123, although even 5μM IM did not fully reverse rhodamine 123 efflux as shown for PSC833 (Figure 3a). When this assay was repeated in primary CML CD34$^+$ cells, no increase in rhodamine 123 fluorescence was observed (Figure 3b).

Effect of inhibition of MDR1 on IM uptake by CML CD34$^+$ cells

To further confirm the results of the displacement assays, cell associated levels of IM were measured by HPLC for cells cultured in the presence and absence of PSC833. There were no significant differences in the concentration of IM, in the presence or absence of PSC833, for K-WT, CML or non-CML $CD34^+$ cells (Figures 4a-c), supporting the earlier observation that MDR1 is expressed at too low a level to be of functional significance in these cells (CML $CD34^+$ cells 5μM IM: 1.13±0.41pg/cell, *vs* 5μM IM + 5μM PSC833: 0.59±0.26pg/cell, *n=4*, *p*=0.17).

Effect of inhibition of MDR1 on primitive CML $CD34^+$ cell proliferation and viability

To confirm that MDR1 activity does not limit the action of IM, the effect of MDR1 inhibition on the proliferation of CML $CD34^+$ cells was evaluated. Cells were cultured with 5GF, in the presence or absence of either 5μM PSC833, 5μM IM or both, for 72h. Incubation with PSC833 resulted in a significant reduction in total viable CML $CD34^+$ cells, as compared to untreated control, similar to that observed with IM treatment (untreated, 177±21.9x10^4 cells *vs* PSC833 treated, 80±12x10^4 cells, *p*=0.002; or *vs* IM treated 90.03±10.74x10^4 cells, *p*=0.002; all n=4), suggesting that PSC833 exerts an inhibitory effect on CML $CD34^+$ cells. In addition, the combination of PSC833 and IM further decreased the total number of viable $CD34^+$ cells compared to IM alone (PSC833 5μM + IM 5μM, 30.2±7.1x10^4 cells, *n*=4, *p*=0.002 compared to untreated), although this was not significant when compared to either IM or PSC833 alone (Figure 5a). The effect of PSC833 on the undivided $CD34^+$ population remaining following 72h of culture, as defined by $CFSE^{max}$, was also examined (Figure 5b). As demonstrated previously, IM resulted in an accumulation of undivided $CD34^+$ cells through its anti-proliferative effect,[31] however, PSC833 alone or in combination with IM had no effect on this critical qSC population.

Effect of inhibition of MDR1 on CrkL phosphorylation and apoptosis in CML $CD34^+$ cells

The previous results suggested that the combination of IM and PSC833 had an additive effect when total cell numbers were used as a measure of drug action. In

addition, further analysis demonstrated a slight increase in apoptosis with either IM or PSC833 alone, or in combination (Figure 5d). However, this combined effect may be due to either MDR1 inhibition or non-specific toxicity. To further define this inhibition, we analysed the effect on BCR-ABL activity by measuring the phosphorylation of the downstream substrate CrkL, which is used as a surrogate marker for establishing the level of BCR-ABL activity. As expected, following 72h treatment, IM-treated CML $CD34^+$ cells showed reduced p-CrkL activity, however PSC833 alone had no effect. Furthermore, the combination of PSC833 and IM did not further reduce p-CrkL levels in comparison to IM alone (Figure 5c). Therefore, PSC833 did not potentiate the inhibition of BCR-ABL by IM strongly suggesting that the decreased cell numbers seen with PSC833 or PSC833 with IM occurred through a BCR-ABL independent mechanism.

Discussion

With the introduction of TKIs such as IM, the majority of CML patients now expect to achieve cytogenetic and even molecular responses.[1] However, few patients achieve sustained molecular remission and a significant proportion develop IM-resistance. Although BCR-ABL domain mutations are a recognisable cause of IM-resistance, other contributory factors have been suggested including the expression of proteins that facilitate the transport of IM in and out of cells. In particular, many studies have suggested that MDR1 and ABCG2 play an important role in IM-resistance. We have previously demonstrated that although ABCG2 is over-expressed in CML $CD34^+$ in comparison to normal $CD34^+$ cells, IM acts as an inhibitor for this transporter and thus the increased expression of ABCG2 does not affect cell associated IM levels nor mediate resistance in this population.[33]

In this study we extended our work on efflux transportation of IM by focusing on the significance of MDR1 in modulating the cellular accumulation of IM in $CD34^+$ CP CML cells, a cell population that includes persistent qSCs that we have proposed to be responsible for the persistence of disease after treatment. Here, we have shown that

transcript levels for *MDR1* are in fact lower in CML $CD34^+$ cells in comparison to the normal $CD34^+$ population. Jiang *et al* have also compared the expression of *MDR1* in leukaemic and normal $CD34^+$ cells and reported an elevated expression of *MDR1* in the leukaemic population.[40] Although we do not fully understand this disparity in results it may relate to factors that would render valid comparison of these two studies impossible. Jiang *et al* examined only specimens in which long-term culture initiating cells (LTC-ICs) were predominately Ph^+/BCR-ABL^+ (only 10-15% of new diagnosis CML in CP). This may have led to preferential selection of more advanced phase CML samples in which *MDR1* expression might be expected to be elevated. In contrast, the cohort analysed in this work was unselected in terms of LTC-IC genotype. We therefore consider that our data will better reflect the baseline expression in standard CML CP $CD34^+$ cells. Furthermore, Thomas *et al* also measured expression of *MDR1* in patient samples and found that although *MDR1* gene transcripts were detectable in peripheral blood leukocytes from patients in both CP and accelerated phase, they were expressed at low levels, which is in concordance with our finding that primary $CD34^+$ CML cells express low levels of MDR1 mRNA and demonstrate only weak MDR1 function.[41] These results are supported by previous studies that measured MDR1 functional activity in mononuclear cells (MNC) from peripheral blood of patients in CP or accelerated phase CML and similarly found no significant MDR1 activity.[42,43]

We also found that treatment of CML $CD34^+$ cells with IM, in combination with PSC833, does not enhance cell associated IM levels, indicating that MDR1 does not mediate energy-dependent exclusion of IM from these cells. Furthermore, in the cell line engineered to over-express MDR1, K-MDR, we observed a concentration-dependent increase in the accumulation of the known MDR1 substrate rhodamine 123 by IM, demonstrating that IM interacts with MDR1 as an inhibitor rather than substrate. Our data is consistent with the data of White *et al*, suggesting that IM is not effluxed by MDR1, but instead may actually inhibit MDR1-mediated efflux of known substrates.[44] Furthermore, inhibition of MDR1 with PSC833, in combination with

IM, did not enhance the efficacy of IM alone against bulk $CD34^+$ cells or the qSC fraction, as assessed by viable cell number (qSC), apoptosis and BCR-ABL inhibition. Previously, it has been speculated by Shukla *et al* that there may be a narrow concentration range in which ABC transporters can transport IM. [17] This was derived from the contradictory results on the interaction of IM with ABCG2. Firstly, Houghton *et al* [45] reported that 1μM ^{14}C-IM accumulation was similar in cell lines expressing functional and non-functional ABCG2. However, Burger *et al* [46] found that at 10 fold lower concentrations intracellular levels of ^{14}C-IM were significantly reduced in cell lines that over-expressed ABCG2. Although the behaviour of IM in relation to its interaction with transporter proteins might change in low and high concentrations, in our study we have used concentrations that relate to the steady state trough plasma levels (<5μM) previously reported in patient clinical trials. [32, 47]

Finally, we have previously reported that the inhibitory concentrations (IC_{50}) for IM, in proliferation and cell viability assays, is significantly lower for K-WT than for primary $CD34^+$ CML cells (0.6, and 5μM respectively).[31] It was therefore interesting to observe a differential accumulation of IM, which may in part explain the variation in sensitivity to IM treatment between cell lines and primary $CD34^+$ cells. However, cell associated IM levels may be simply related to cell volume. By direct measurements of the cell diameter, we were able to calculate cell volumes and found that there were differences in cell volume between cell lines and patient cells, which may explain the higher cell associated levels observed in the larger cell lines. However, if a direct relationship between cell volume and IM accumulation is assumed, the amount we measured in the CML $CD34^+38^-$ cell population is lower than that expected when compared to total CML $CD34^+$ cells (Table 1). This may imply that cell volume is only one parameter influencing cell associated IM levels and as we have shown that efflux transportation is not a strong determinant of IM level, influx may play a critical role. OCT1has been shown to be important for IM uptake, and Jiang *et al* [40] have previously reported that CML stem cells ($CD34^+38^-$) have particularly low expression of this influx transporter. Similarly, we have found that

OCT-1 mRNA is undetectable in CD34+ CML cells (unpublished data). Thus, the lack of OCT-1 may be in part responsible for the low cell associated IM levels in primitive CML that we report here. In general, OCT-1 influx transport is proving to be an important clinical determinant to IM-response in patients. [14,15,48] White *et al* showed that patients with high OCT-1 activity achieved significantly greater molecular responses over 24 months of IM treatment than patients with low OCT-1 activity [14]. Thus, it seems that in general in CML cells influx rather than efflux is a strong determinant of cell associated IM levels and response to treatment, however the very low or undetectable levels of OCT-1 in the most primitive cells implies that there maybe another as yet unidentified mechanism involved.

Taken together, our results demonstrate conclusively that MDR1, although present at low levels in primary $CD34^+$ CML cells, is unlikely to mediate IM-resistance in this primitive population. Even when expressed at higher levels, such as in advanced phase CML, MDR1 is unlikely to mediate IM-resistance since IM appears to inhibit MDR1, rather than interact with the transporter as a substrate. Whether alternative members of the ABC drug efflux transporter family [49] are expressed on $CD34^+$ CML cells and capable of mediating IM-resistance in this cellular context is as yet unknown.

Acknowledgements: SH was funded by the Chief Scientist Office; NEJ was funded by Leukaemia Research UK; HGJ is a William Thyne and Dr Rhona Reid Charitable Trust Fellow.

References

1. Druker BJ, Guilhot F, O'Brien SG, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. N Engl J Med. 2006;355:2408-2417.

2. Hughes T, Deininger M, Hochhaus A, et al. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. Blood. 2006;108:28-37.

3. Roumiantsev S, Shah NP, Gorre ME, et al. Clinical resistance to the kinase inhibitor STI-571 in chronic myeloid leukemia by mutation of Tyr-253 in the Abl kinase domain P-loop. Proc Natl Acad Sci USA. 2002;99:10700-10705.

4. Gorre ME, Mohammed M, Ellwood K, et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science. 2001;293:876-880.

5. Swords R, Quinn J, Fay M, O'Donnell R, Goldman J, Murphy PT. CML clonal evolution with resistance to single agent imatinib therapy. Clin Lab Haematol. 2005;27:347-349.

6. Willis SG, Lange T, Demehri S, et al. High-sensitivity detection of BCR-ABL kinase domain mutations in imatinib-naive patients: correlation with clonal cytogenetic evolution but not response to therapy. Blood. 2005;106:2128-2137.

7. Quintás-Cardama A, Kantarjian H, Cortes J. Targeting ABL and SRC kinases in chronic myeloid leukemia: experience with dasatinib. Future Oncol. 2006;2:655-665.

8. Melo JV, Chuah C. Resistance to imatinib mesylate in chronic myeloid leukaemia. Cancer Lett. 2007;249:121-132.

9. Azam M, Latek RR, Daley GQ. Mechanisms of autoinhibition and STI571/imatinib resistance revealed by mutagenesis of BCR–ABL. Cel.l 2003;112:831–843.

10. Corbin AS, La Rosee P, Stoffregen EP, Druker BJ, Deininger MW. Several Bcr-Abl kinase domain mutants associated with imatinib mesylate resistance remain sensitive to imatinib. Blood. 2003;101:4611–4614.

11. Kantarjian H, Talpaz M, O'Brien S, Garcia-Manero G, Verstovsek S, Giles F et al. High-dose imatinib mesylate therapy in newly diagnosed Philadelphia chromosome-positive chronic phase chronic myeloid leukemia. Blood. 2004;103:2873-2878.

12. Talpaz M, Shah NP, Kantarjian H, et al. Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. N Engl J Med 2006;354:2531–2541.

13. Kantarjian H, Giles F, Wunderle L, et al. Nilotinib in imatinib-resistant CML and Philadelphia chromosome-positive ALL. N Engl J Med 2006;354:2542–2551.

14. White DL, Saunders VA, Dang P, 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;110:4064-4072.

15. White DL, Saunders VA, Dang P, 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;108:697-704.

16. Hegedus T, Orfi L, Seprodi A, Varadi A, Sarkadi B & Keri G. Interaction of tyrosine kinase inhibitors with the human multidrug transporter proteins, MDR1 and MRP1. Biochim Biophys Acta. 2002;1587:318−325.

17. Shukla S, Sauna ZE, Ambudkar SV. Evidence for the interaction of imatinib at the transport-substrate sites of the multidrug-resistance linked ABC drug transporters ABCB1 (P-glycoprotein) and ABCG2. Leukemia. 2008;22:445-447.

18. Hamada A, Miyano H, Watanabe H, Saito H. Interaction of imatinib mesilate with human P-glycoprotein. J Pharmacol Exp Ther. 2003;307:824-828.

19. Dai H, Marbach P, Lemaire M, Hayes M & Elmquist WF. Distribution of STI-571 to the brain is limited by P-glycoprotein-mediated efflux. J Pharmacol Exp Ther. 2003;304:1085−1092.

20. Hirayama C, Watanabe H, Nakashima R, Nanbu T, Hamada A, Kuniyasu A et al. Constitutive overexpression of P-glycoprotein, rather than breast cancer resistance protein or organic cation transporter 1, contributes to acquisition of imatinib-resistance in K562 Cells. Pharm Res. 2008;25:827-835.

21. Illmer T, Schaich M, Platzbecker U, et al. P-glycoprotein-mediated drug efflux is a resistance mechanism of chronic myelogenous leukemia cells to treatment with imatinib mesylate. Leukemia. 2004;18:401-408.

22. Mahon FX, Belloc F, Lagarde V, et al. MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models. Blood. 2003;101:2368-2373.

23. Widmer N, Rumpold H, Untergasser G, Fayet A, Buclin T, Decosterd. Resistant reversal by RNAi silencing of MDR1 in CML cells associated with increase in imatinib intracellular levels. Leukemia. 2007;21:1561-1562.

24. Rumpold H, Wolf AM, Gruenewald K, Gastl G, Gunsilius E, Wolf D. RNAi-mediated knockdown of P-glycoprotein using a transposon-based vector system durably restores imatinib sensitivity in imatinib-resistant CML cell lines. Exp Hematol. 2005;33:767-775.

25. Ferrao PT, Frost MJ, Siah SP, Ashman LK. Overexpression of P-glycoprotein in K562 cells does not confer resistance to the growth inhibitory effects of imatinib (STI571) in vitro. Blood. 2003;102:4499-4503.

26. Zong Y, Zhou S, Sorrentino BP. Loss of P-glycoprotein expression in hematopoietic stem cells does not improve responses to imatinib in a murine model of chronic myelogenous leukemia. Leukemia. 2005;19:1590-1596.

27. Udomsakdi C, Eaves CJ, Swolin B, Reid DS, Barnett MJ, Eaves AC. Rapid decline of chronic myeloid leukemic cells in long-term culture due to a defect at the leukemic stem cell level. Proc Natl Acad Sci USA. 1990;89:6192–6196.

28. Holyoake T, Jiang X, Eaves C, Eaves A. Isolation of a highly quiescent subpopulation of primitive leukemic cells in chronic myeloid leukemia. Blood. 1999;94:2056-2064.

29. Holyoake TL, Jiang X, Jorgensen HG, Graham S, Alcorn MJ, Laird C, et al. Primitive quiescent leukemic cells from patients with chronic myeloid leukemia spontaneously initiate factor-independent growth in vitro in association with up-regulation of expression of interleukin-3. Blood. 2001;97:720-728.

30. Copland M, Hamilton A, Elrick LJ, Baird JW, Allan EK, Jordanides N, et al. Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML but does not eliminate the quiescent fraction. Blood. 2006;107:4532-4539.

31. Graham SM, Jørgensen HG, Allan E, Pearson C, Alcorn MJ, Richmond L, et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. Blood. 2002;99:319-325.

32. Larson RA, Druker BJ, Guilhot F, et al. Imatinib pharmacokinetics and its correlation with response and safety in chronic-phase chronic myeloid leukemia: a subanalysis of the IRIS study. Blood. 2008;111:4022-4028.

33. Jordanides NE, Jorgensen HG, Holyoake TL, Mountford JC. Functional ABCG2 is overexpressed on primary CML CD34+ cells and is inhibited by imatinib mesylate. Blood. 2006;108:1370-1373.

34. Noonan KE, Beck C, Holzmayer TA, Chin JE, Wunder JS, Andrulis IL, Gazdar AF et al. Quantitative analysis of MDR1 (multidrug resistance) gene expression in

human tumors by polymerase chain reaction. Proc Natl Acad Sci USA. 1990;87:7160-7164. do you use this?

35. Galimberti S, Guerrini F, Palumbo GA, Consoli U, Fazzi R, Morabito F et al. Evaluation of BCRP and MDR-1 co-expression by quantitative molecular assessment in AML patients. Leuk Res. 2004;28:367–372 do you use this?

36. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001;25:402–408. do you use this?

37. Guetens G, Prenen H, De Boeck G, van Oosterom A, Schöffski P, Highley M, et al. Simultaneous determination of AMN107 and Imatinib (Gleevec, Glivec, STI571) in cultured tumour cells using an isocratic high-performance liquid chromatography procedure with UV detection. J Chromatogr B Analyt Technol Biomed Life Sci 2007: 846;341-345.

38. Robey RW, Honjo Y, van de Laar A, Miyake K, Regis JT, Litman T. et al. A functional assay for detection of the mitoxantrone resistance protein, MXR (ABCG2). Biochim Biophys Acta. 2001;1512:171-182.

39. Hamilton A, Elrick L, Myssina S, Copland M, Jørgensen H, Melo JV, et al. BCR-ABL activity and its response to drugs can be determined in CD34+ CML stem cells by CrkL phosphorylation status using flow cytometry. Leukemia. 2006;20:1035-1039.

40. 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;21:926-935.

41. Thomas J, Wang L, Clark RE, Pirmohamed M. Active transport of imatinib into and out of cells: implications for drug resistance. Blood. 2004;104:3739-3745.

42. Stavrovskaya A, Turkina A, Sedyakhina N, et al. Prognostic value of p-glycoprotein and leukocyte differentiation antigens in chronic myeloid leukemia. Leuk Lymph. 1998;28:469-482.

43. Carter A, Dann EJ, Katz T, et al. Cells from chronic myelogenous leukaemia patients at presentation exhibit multidrug resistance not mediated by either MDR1 or MRP1. Br J Haematol. 2001;114:581-590.

44. White DL, Saunders VA, Quinn SR, Manley PW, Hughes TP. Imatinib increases the intracellular concentration of nilotinib, which may explain the observed synergy between these drugs. Blood. 2007;109:3609-3610.

45. Houghton PJ, Germain GS, Harwood FC, et al. Imatinib mesylate is a potent inhibitor of the ABCG2 (BCRP) transporter and reverses resistance to topotecan and SN-38 in vitro. Cancer Res. 2004;64:2333-2337.

46. Burger H, van Tol H, Boersma AW, et al. Imatinib mesylate (STI571) is a substrate for the breast cancer resistance protein (BCRP)/ABCG2 drug pump. Blood. 2004;104:2940-2942.

47. Peng B, Hayes M, Resta D, et al. Pharmacokinetics and pharmacodynamics of imatinib in a phase I trial with chronic myeloid leukemia patients. J Clin Oncol. 2004;22: 935-942.

48. 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;83:258-264.

49. Szakács G, Váradi A, Ozvegy-Laczka C, Sarkadi B. The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox). Drug Discov Today. 2008;13:379-393.

Titles and Legends to Figures:

Figure 1: Cell associated levels of IM. (a) Levels of IM in K-WT (*n*=7) and HL60 (*n*=3) cell lines. (b) Levels of IM in CML (n=6) and non-CML (*n*=3) $CD34^+$ cells and the CML $CD34^+38^-$ subpopulation (*n*=6). Cell associated levels were measured in $5x10^4$ cells for cell lines and $1x10^5$ cells for primary cells as described in Materials & Methods. Statistical analysis was performed between groups treated with the same concentration of IM, *p* values are reported in graphs only when statistically significant ($p<0.05$).

Figure 2: The function of MDR1 in Ph^+ cell lines and CML $CD34^+$ cells. The effect of the specific MDR1 inhibitor PSC833 to inhibit substrate rhodamine 123 efflux was determined in (a) K-WT, (b) K-MDR cells and (c) primary CML $CD34^+$ cells. MDR1 function was measured by substrate displacement assays using flow cytometry analysis as described in Materials & Methods. Unstained cells (black fill), cells treated with rhodamine 123 in the absence (light grey fill) and presence of PSC833 (dark grey fill). The plots are representative of 3 independent experiments.

Figure 3: IM interaction with MDR1 protein. Increasing concentrations of IM were added prior to addition of the specific MDR1 substrate rhodamine 123 and compared with the effect of the known inhibitor, PSC833 in (a) K-MDR cells and (b) primary CML $CD34^+$ cells. MDR1 function was measured by substrate displacement assays by flow cytometry as described in Materials & Methods. Representation of different treatments is assigned on FACS plots. The plots are representative of 3 independent experiments.

Figure 4: Effect of the MDR1 specific inhibitor PSC833 on the cell associated levels of IM. Levels of IM in K-WT cell line (*n*=3) (a), CML (n=4) (b) and non-CML $CD34^+$ (*n*=3) cells (c). Cell associated levels of IM (5 and 10μM) were measured in the presence or absence of 5μM PSC833 in $5x10^4$cells for K-WT and $1x10^5$ cells for primary cells by HPLC as described in Materials & Methods. No statistical significance was observed in cell associated levels of IM in cells treated with the same amount of IM in the presence or absence of PSC833 ($p>0.05$).

Figure 5: The effect of inhibition of MDR1 function with PSC833 on efficacy of IM in CML $CD34^+$ cells. Effect of PSC833 on (a) the viability of total $CD34^+$ cells, (b) the undivided CML $CD34^+$ population, (c) p-CrkL and (d) $CD34^+$ cells that undergo apoptosis. $CD34^+$ cells were seeded at $1x10^5$ cells/mL and treated with either IM or PSC833 alone or in combination for 72h and measured as described in Materials and Methods. Results are expressed as the mean ± SD for three independent experiments; $^*P<0.05$, $^{**}P<0.01$ significantly different from no-drug control (ND).

Table 1: Predictions and comparison of calculated cell associated IM levels based on cell size and measured IM levels by HPLC. Predicted IM levels were based on values normalised against non-CML $CD34^+$ cell size for primary cells and HL60 for cell lines.

Table 1:

Cell type	Actual volume (μm^3)	Relative volume (μm^3)	Assumed IM cell associated concentrations measured (pg/cell)	IM cell associated concentrations (pg/cell ± SD)
Non-CML $CD34^+$	589	1.00	0.79	0.79±0.25
CML $CD34^+38^-$	641	1.09	0.86	0.47±0.09
CML $CD34^+$	800	1.36	1.07	1.36±0.36
HL60	1498	1.00	3.51	3.51±0.97
K562	2635	1.76	6.18	9.01±2.74

Figure 1

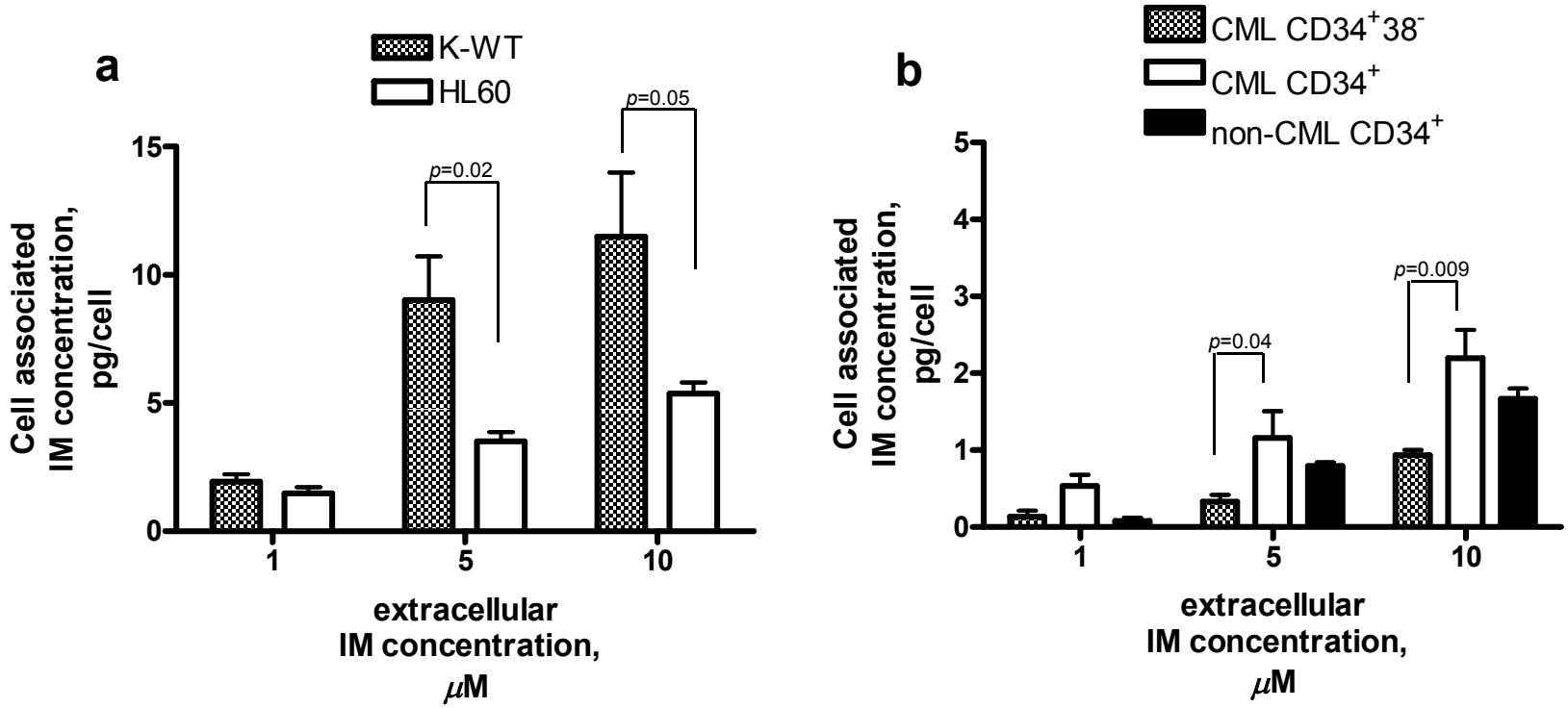

a
K-WT
HL60
p=0.02
p=0.05
Cell associated IM concentration, pg/cell
15
10
5
0
1
5
10
extracellular IM concentration, μM
b
CML CD34+38-
CML CD34+
non-CML CD34+
p=0.04
p=0.009
Cell associated IM concentration, pg/cell
5
4
3
2
1
0
1
5
10
extracellular IM concentration, μM

Figure 2

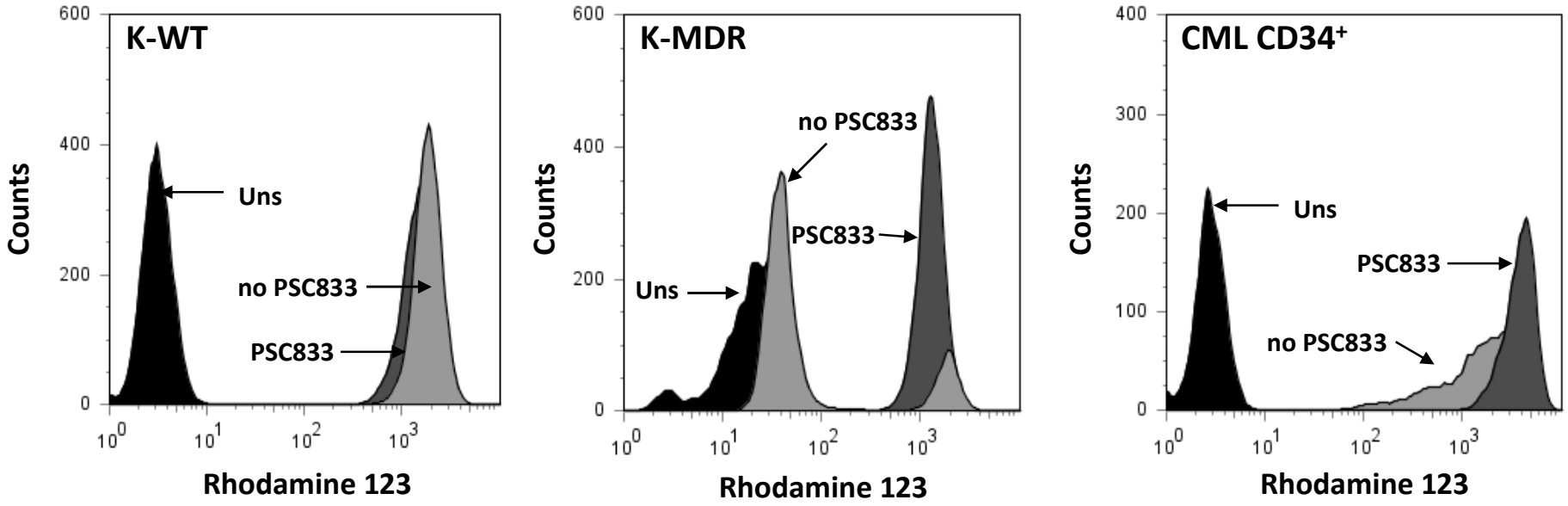

K-WT
Counts
Uns
no PSC833
PSC833
Rhodamine 123
K-MDR
Counts
no PSC833
PSC833
Uns
Rhodamine 123
CML CD34+
Counts
Uns
PSC833
no PSC833
Rhodamine 123

Figure 3

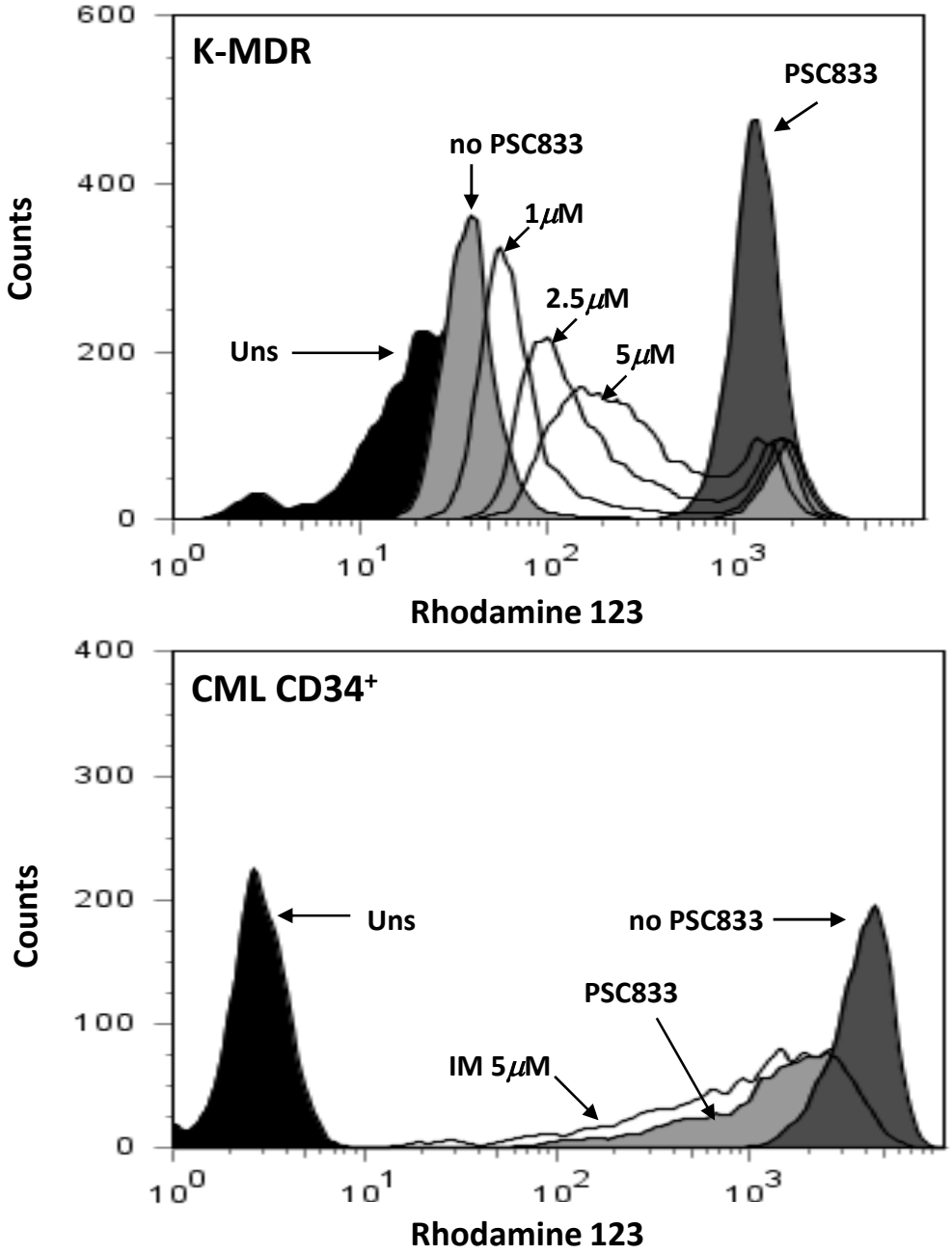

K-MDR
PSC833
no PSC833
1μM
2.5μM
5μM
Uns
Counts
600
400
200
0
10^0
10^1
10^2
10^3
Rhodamine 123
CML CD34+
Uns
no PSC833
PSC833
IM 5μM
Counts
400
300
200
100
0
10^0
10^1
10^2
10^3
Rhodamine 123

Figure 4

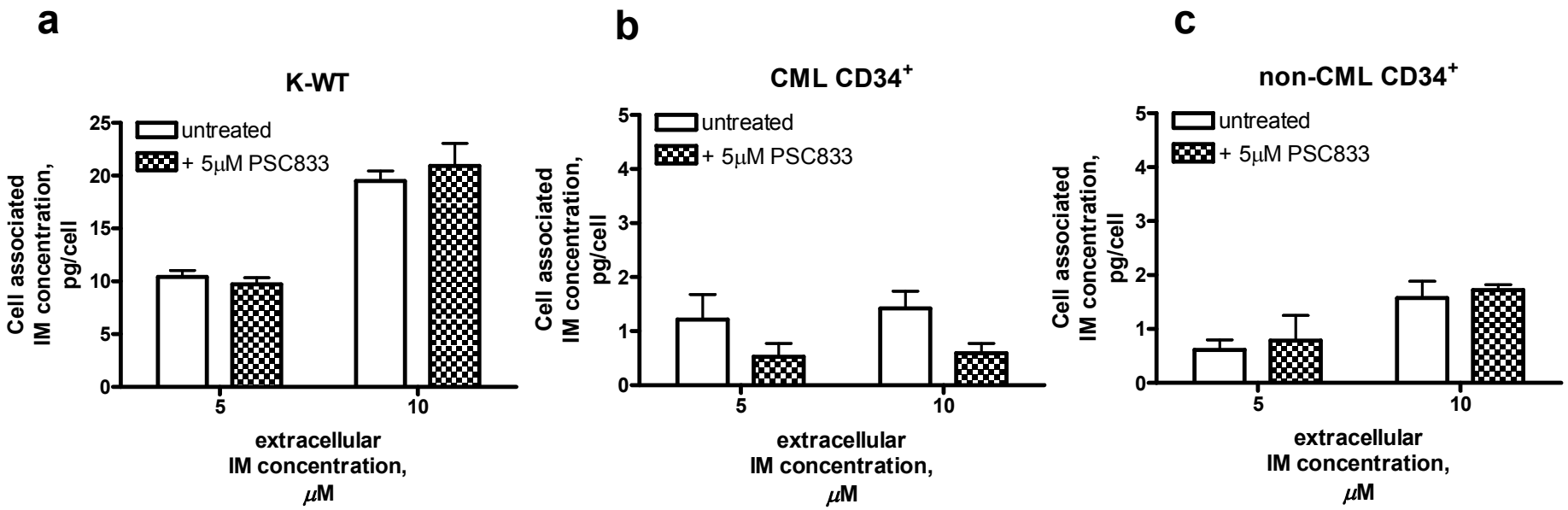
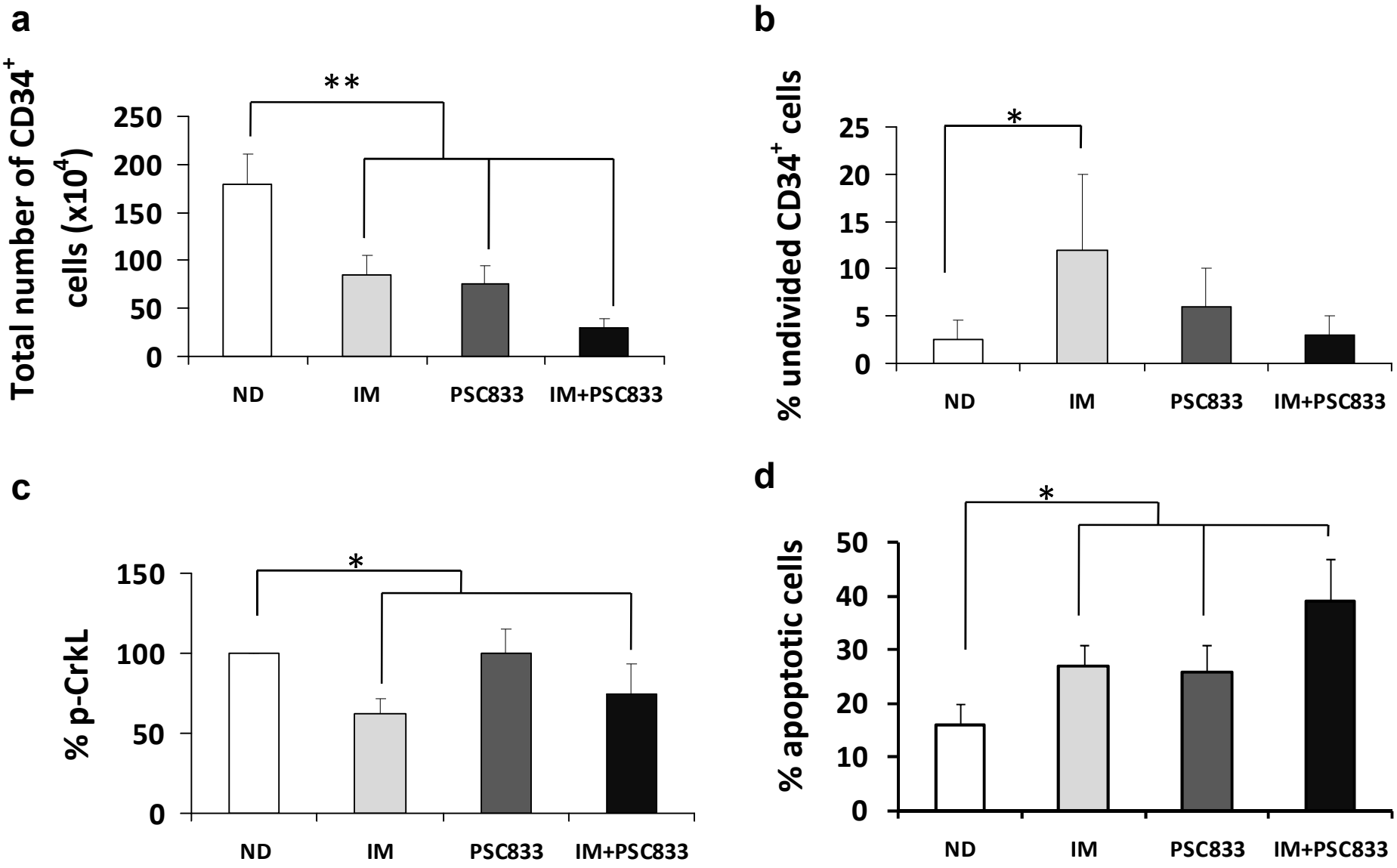

a
K-WT
untreated
+ 5μM PSC833
Cell associated IM concentration, pg/cell
0
5
10
15
20
25
5
10
extracellular IM concentration, μM
b
CML CD34+
untreated
+ 5μM PSC833
Cell associated IM concentration, pg/cell
0
1
2
3
4
5
5
10
extracellular IM concentration, μM
c
non-CML CD34+
untreated
+ 5μM PSC833
Cell associated IM concentration, pg/cell
0
1
2
3
4
5
5
10
extracellular IM concentration, μM

Figure 5


a
Total number of CD34+ cells (x10^4)
250
200
150
100
50
0
**
ND
IM
PSC833
IM+PSC833
b
% undivided CD34+ cells
25
20
15
10
5
0
*
ND
IM
PSC833
IM+PSC833
c
% p-CrkL
150
100
50
0
*
ND
IM
PSC833
IM+PSC833
d
% apoptotic cells
50
40
30
20
10
0
*
ND
IM
PSC833
IM+PSC833

*Letter of Submission

University of Glasgow

Professor Tessa L Holyoake PhD FRCP FRCPath FRSE

23rd December 2008

Dear Sir

Inhibition of MDR1 does not sensitise primitive chronic myeloid leukaemia CD34$^+$ cells to imatinib.

S Hatziieremia et al

I am pleased to submit this article for consideration for publication in Experimental Hematology. In this work, we confirm that efflux by MDR1 is not a mechanism for resistance of CML stem cells to imatinib. We uniquely combine HPLC quantification of cell associated drug concentrations with flow cytometry based functional assays for transporter activity in the primitive (CD34+ CD38-) subpopulation of CML stem / progenitor cells.

I look forward to hearing your decision.

Heather G Jorgensen PhD

Paul O'Gorman Leukaemia Research Centre,
Section of Experimental Haematology, Faculty of Medicine, University of Glasgow,
Gartnavel General Hospital, 1053 Great Western Road, Glasgow G12 0YN.
Professor Tessa L Holyoake, Dr Mhairi Copland, Dr Alison M Michie, Dr Heather G Jorgensen.
Email; tlh1g@clinmed.gla.ac.uk; mc196d@clinmed.gla.ac.uk; a.michie@clinmed.gla.ac.uk; hgj1b@clinmed.gla.ac.uk;
Secretaries: Mrs Diane Verrecchia (Email; dmi3c@clinmed.gla.ac.uk)
Mrs Angela Newlands (Email; aln1u@clinmed.gla.ac.uk)
Telephone +44(0) 141 301 7880 Fax +44(0) 141 301 7898

***Statement of Authorship**

This piece of the submission is being sent via mail.