Nilotinib concentration in cell lines and primary CD34$^+$ chronic myeloid leukemia cells is not mediated by active uptake or efflux by major drug transporters.

Running Head: Nilotinib transport in CML

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ABSTRACT

Imatinib mesylate and nilotinib are highly effective at eradicating the majority of chronic myeloid leukaemia (CML) cells, however neither agent induces apoptosis of primitive CML $CD34^+$ cells. One possible explanation is that $CD34^+$ cells do not accumulate sufficient intracellular drug due to either inadequate active uptake or increased efflux. To determine the interaction of nilotinib with major clinically implicated drug transporters we investigated interactions with MDR1 (ABCB1), MRP1 (ABCC1), ABCG2 (BCRP) and hOCT1 in CML cell lines and primitive ($CD34^+$) primary CML cells. Nilotinib is neither dependent on active import by hOCT1, nor effluxed via the ABC transporters investigated. Indeed, we found nilotinib to be an inhibitor of hOCT1, MDR1 and ABCG2. The efflux transporters MDR1, MRP1 and ABCG2 are expressed on CML $CD34^+$ cells at 13.5, 108 and 291% of control respectively although hOCT1 expression was absent; however, inhibition of efflux transporter activity did not potentiate the effect of nilotinib on apoptosis, Bcr-Abl inhibition, or CML $CD34^+$ cell proliferation. Therefore, we have found no evidence for either active uptake of nilotinib via hOCT1 or efflux via MDR1, MRP1 or ABCG2 and it is therefore unlikely that these transporters will have any effect on the clinical response to this drug.

Key words: nilotinib, CML, OCT-1, ABC efflux transporters

INTRODUCTION

In response to resistance and failure to induce molecular responses in a proportion of chronic myeloid leukaemia (CML) patients treated with imatinib (Gleevec®; Glivec®; Novartis) (1,2), new tyrosine kinase inhibitors (TKI) have been developed and both nilotinib (Tasigna®; Novartis) and dasatinib (Sprycel®; Bristol-Myers Squibb) are now in clinical use (3,4). Despite the improved potency (20-fold) of nilotinib in cell lines and on bulk primary CML cells we have found that nilotinib is no more effective than imatinib in inducing apoptosis of primitive $CD34^+$ CML cells (5). Furthermore, we have demonstrated that as with imatinib, $CD34^+$ CML cells persist after treatment and that nilotinib is anti-proliferative rather than pro-apoptotic, resulting in the accumulation of quiescent $CD34^+$ cells (5). We, and others, have proposed that this population of primitive cells which are insensitive to imatinib and nilotinib may form a pool of disease in patients under treatment and contribute to the Bcr-Abl molecular signal detected in the majority of patients (6, 7). It is therefore this population that must be specifically targeted in order to cure rather than control the disease in patients.

One possible reason for the failure of nilotinib to kill $CD34^+$ primitive CML cells is insufficient intracellular levels of the drug owing to inadequate active uptake via the Solute Carrier (SLC) transporter family, or to efflux via multidrug resistance proteins of the ATP-binding cassette (ABC) family. There has been much debate about the role of ABC transporters in mediating imatinib resistance; these include our own investigations into ABCG2 activity in CML $CD34^+$ cells (8). Imatinib may be an inhibitor or substrate of ABCG2 depending on its concentration (9-11), but in $CD34^+$ cells, at concentrations above clinical trough plasma values (1.2μM), it is an inhibitor (8). Imatinib may also be a substrate for MDR1 (ABCB1, P-gp) in cell lines (12-14) however, we have not found this in primary CML cells (15). Further, a number of studies including our own have demonstrated that imatinib is actively taken up via the human organic cation transporter 1 (hOCT1; SLC22a1) (12-17) and that reduced hOCT1 expression may correlate with a poor clinical response (17).

To date there have only been two reports that suggest nilotinib may be an ABCG2 substrate but is not actively taken up by hOCT1 (18, 19). We have now investigated the interaction of nilotinib with the major clinically implicated drug transporters MDR1, MRP1 (ABCC1), ABCG2 and hOCT1 in CML cell lines and primary $CD34^+$ cells. Additionally, we have co-treated these primitive cells with nilotinib in the presence of efflux transporter inhibitors to investigate whether this renders the drug more effective.

MATERIALS AND METHODS

Reagents. ^{14}C-labelled imatinib (specific activity, 3.386MBq/mg), nilotinib (specific activity, 3.480MBq/mg), and the MDR1 inhibitor PSC833 were kindly provided by Novartis Pharma (Basel, Switzerland). ^{14}C-labelled tetraethylammonium bromide (TEA) (specific activity, 118.4MBq/mmol) was purchased from Perkin Elmer Life Sciences (Boston, MA, USA), Gold Star scintillation cocktail from Meridian (Epsom, UK).

Cell culture. Transfection of KCL22 cells with hOCT1 was performed as previously described (20). The MDR1-transfected Type II Marin Darby canine kidney (MDCKII) cell line was a kind gift from Prof P Borst (Netherlands Cancer Institute) (16). The K562 wild type (WT) and cell lines over-expressing MDR1 or MRP1 were donated by Drs T Southgate and L Fairbairn (Paterson Institute for Cancer Research, Manchester, UK) (21). ABCG2 over-expressing OCT-AML6.2 cells and the control line OCT-AML3 were kindly provided by Dr B Sorrentino (St Jude Children's Research Hospital, Memphis, TN) (8). MDCKII were grown in Dulbecco's Modified Eagle Medium (DMEM), and all other lines in RPMI-1640 medium (Biosera, East Sussex, UK) supplemented with 2mM L-glutamine, penicillin (50U) / streptomycin (50µg) and 10% v/v foetal calf serum (Biosera, East Sussex, UK) at 37℃ with 5% CO_2.

Primary CD34$^+$ cells. Primary CML cells were obtained with written informed consent in accordance with the Declaration of Helsinki, from peripheral blood leukapheresis samples from newly diagnosed patients with chronic phase (CP) CML. Both the Royal Liverpool University Hospital and Glasgow Royal Infirmary's Local Research Ethics Committee approved the use of human tissue in this study protocol. CD34$^+$ cells enriched by positive selection (>95%) were cultured in growth factor supplemented serum free medium as previously described (8). For quantitative polymerase chain reaction (qPCR) analysis, RNA (1µg aliquots) derived from normal CD34$^+$ cells isolated from mobilized peripheral blood (mPB), was purchased from Cambrex Bioscience (Wokingham, UK) and Stem Cell Technologies UK (Vancouver, Canada).

Cellular analysis. Intracellular phospho-CrkL (p-CrkL), annexin V/propidium iodide and carboxyfluorescein succinimidyl ester (CFSE) staining were performed and analysed by flow cytometry as described previously (22, 23). Cell viability and counts were determined by dye exclusion.

Efflux studies. The efflux protocol was a modification of that previously described (24). Cells were incubated with appropriate concentrations of inhibitors/TKI as required for 15min to block pump activity before the incubation with fluorescent substrates for a further 30min. Cells were washed and incubated ± inhibitors/TKI for 1h to allow efflux before washing in ice-cold medium for analysis (8). Fluorescent substrates and inhibitors, respectively, were Rhodamine-123 (0.5µg/mL; Invitrogen, Paisley, UK) and PSC833 (5µM) for MDR1; Fluo3-AM (5µg/mL; Axxora, Nottingham, UK) and MK571 (30µM; Axxora) for MRP1; BODIPY-Prazosin (200nM; Axxora, Nottingham, UK) and fumitremorgin C (FTC, 10µM; Axxora, Nottingham, UK) for ABCG2. Retention values were calculated by designating the level of fluorescence in the presence of the known inhibitor as 100%.

Radio-labelled intracellular uptake and retention (IUR) assay using KCL22 cells. Drug transport studies of ^{14}C-radiolabelled nilotinib (4 and 1µM: spanning peak and trough plasma levels respectively of a 400mg twice daily dose) were performed using high expressing hOCT1 transfected KCL22 cells, with or without hOCT1 and MDR1 inhibitors/substrates. MDR1 inhibitors used were: PSC833 (10µM); verapamil (500µM); and tariquidar (500nM). OCT1 inhibitors/substrates used were: prazosin (100µM); amantadine (500µM) and TEA (5µM). After drug treatment cells were lysed before scintillation counting as previously published (16). Cell-associated nilotinib levels were expressed as ng per million cells.

Measurement of transepithelial drug transport by MDR1. Vectorial MDR1 efflux studies were performed on MDCKII cells as previously described (16). Cells were seeded at 1.5-2 x 10^6 cells per well onto 24mm diameter, 0.4µm pore size, polycarbonate transwell membrane inserts (Appleton Woods, UK). Transepithelial electrical resistance (TEER) was measured, after 5 to 6 days, using a Millicell-ERS Volt-ohmmeter (Millipore, UK) to determine a confluent monolayer. The blank membrane resistance was subtracted from monolayer resistance then multiplied by the surface area (4.67cm^2) to calculate TEER in Ω x cm^2. Monolayers with TEER values greater than 200 Ω x cm^2 were used. Cells were co-incubated throughout with ^{3}H-mannitol (0.05µCi/mL) (Sigma, Poole, UK) to ensure monolayer integrity.

Monolayers were washed with HBSS and allowed to equilibrate for 30min in transport medium (1% v/v HEPES in HBSS) before incubation with ^{14}C-imatinib (5μM) or ^{14}C-nilotinib (4μM) for 4h. Radioactivity was measured in medium from donor and acceptor compartments by liquid scintillation spectrometry. TKI movement was calculated as a percentage of radioactivity transported across the monolayer (25). For MDR1 inhibition studies MDCKII-MDR1 cells were pre-incubated with PSC833 (10μM) 30min prior to the addition of the radioactive TKIs, as described above.

Distribution Coefficient (logD) / Lipophilicity Studies. The lipophilicity of a compound is defined as the distribution coefficient (logD) in a biphasic system. The logD of ^{14}C-labelled nilotinib and imatinib were measured as described by Yunger and Cramer (26). In brief, equimolar quantities of the drugs (500nM) were suspended in 1ml transport medium then mixed vigorously for 15min with 1ml 1-octanol. The layers were separated by centrifugation at 2000rpm for 10min at room temperature. Radioactivity was determined by scintillation counting of 500μL aliquots from both phases. Results were derived from three experiments each consisting of four replicates. The logD distribution coefficient was calculated as below where DPM represents disintegrations per minute:

$$\text{logD} = \log\left(\frac{[\text{DPM}_{(\text{octanol})}]}{[\text{DPM}_{(\text{buffer})}]}\right)$$

RESULTS AND DISCUSSION

There has been much debate about the most suitable imatinib or nilotinib concentration for *in vitro* study that most closely represent clinically achievable levels. In our recent preliminary work (27) we have shown that plasma levels of 2.7μg/mL are achieved 10h post 400mg imatinib; those taking 600-800mg achieved imatinib plasma levels above 3μg/mL 10-16h post dose. Therefore an imatinib plasma level of 5μM (2.95μg/mL) is clinically achievable. Similarly, the use of 1 and 4μM nilotinib spans the upper and lower standard deviation range of serum steady state C_{max} levels for the typical 800mg daily dose (Novartis: data on file via personal communication with Dr P Manley, Novartis Pharma, 2008); and supported by plasma level data generated from patient pharmacokinetic (PK) profiles at the Royal Liverpool University Hospital. Based on these data we consider that our use of

these concentrations of TKI more closely mimics *in vivo* conditions than the use of low submicromolar concentrations that have been used in some other transport studies.

Nilotinib is more lipophilic than imatinib and cellular accumulation is not dependent on active import by hOCT1.

It has been demonstrated, by ourselves and others, that the response to imatinib both *in vitro* and *in vivo* is dependent on active uptake into the cells via the solute transporter hOCT1 (16, 19, 28). However, little is known about the mechanism by which nilotinib enters cells. In the only previous study by White et al, unlike the effect on imatinib transport, inhibition of hOCT1 activity by prazosin did not decrease nilotinib uptake into CML cells (19). Our data, using a stably transfected cell line model, extend this previous investigation and strongly support that hOCT1 is not involved in the import of nilotinib [Figure 1A&B]. We have also demonstrated that nilotinib uptake was not temperature dependent and occurred very rapidly reaching maximal levels by the first time point of 2min [Figure 1A], consistent with our finding that nilotinib was more lipophilic than imatinib as measured by calculation of logD (2.4 versus 0.8, respectively) and therefore more likely to be taken up into the cells without active transport.

The passive nature of this uptake was also demonstrated by data shown in Figure 1A which shows that nilotinib levels are unaffected by the level of expression of hOCT1. KCL22 cells transfected with hOCT1 were also exposed to nilotinib in the presence of the hOCT1 and 2 inhibitor amantadine or hOCT1 and 3 inhibitor prazosin; neither of which altered the uptake of nilotinib [Figure 1B]. The efficient 1 hour uptake of nilotinib in the absence of hOCT1 transport was confirmed at a functional level by measuring reduction in Bcr-Abl activity using p-CrkL as a surrogate marker (data not shown). Nilotinib did reproducibly reduce CrkL phosphorylation to a greater extent than imatinib, but in contrast to imatinib treatment, p-CrkL levels were not altered by the over-expression of hOCT1 in KCL22 cells [imatinib-treated pcDNA 78.6±12.6% v hOCT1 39.8±5.8%; P = 0.0185: nilotinib-treated pcDNA 37.4±8.0% v hOCT 49.4±14.1%; P = 0.477. n = 6, ±SEM].

Interestingly our studies show that nilotinib can reduce uptake of TEA, a known hOCT1 substrate, suggesting nilotinib disrupts hOCT1 transport function [Figure 1C]. We have also observed such activity for imatinib, however, nilotinib was more potent than imatinib in this regard. In isolation these data may suggest that nilotinib reduces TEA uptake as a competitive substrate, however as we

did not find any evidence that nilotinib is a substrate for hOCT1 [Figures 1A&B] we therefore consider that nilotinib is impeding TEA uptake by inhibiting the function of the transporter. Therefore, drugs transported by hOCT1 would be blocked when co-administered with nilotinib. This is further supported by our observation that nilotinib, at 1 and 4μM, abolished ^{14}C-imatinib uptake in KCL22-hOCT1 cells (data not shown) and similar data have been reported by White et al (28). Given the ability of nilotinib to inhibit hOCT1, one must consider the possibility of drug-drug interactions. For instance, the anti-diabetic agent metformin, is a substrate for hOCT1 transport (20); and therefore co-administration with nilotinib may inhibit metformin uptake which may have a clinically relevant impact on the control of glucose levels in diabetic CML patients taking nilotinib.

Based on the findings from this study, we conclude that nilotinib enters cells passively and can accumulate readily. Hence, unlike imatinib where modulation of uptake may improve the performance of the drug, it is unlikely that the intracellular concentration of nilotinib can be further increased by solute carrier modulation.

Nilotinib is not effluxed by clinically relevant drug transporters

In addition to examining drug uptake, there have been a number of contradictory studies investigating whether efflux transporters may limit the intracellular concentration and thus efficacy of imatinib (8-16, 29). To date the transporter that has been studied in most detail is ABCG2. There is still no consensus on whether imatinib is a substrate or inhibitor for this protein however, collation of all the data supports the proposal of a dual activity whereby at low concentration (≤0.8μM) imatinib is a substrate and at higher concentrations it is an inhibitor of ABCG2 (9-11). Our own studies have shown that ABCG2 is over-expressed on CML CD34$^+$ cells but that inhibition of efflux does not increase the effect of imatinib in this population which contains the CML stem cells (8). There have been few comprehensive studies on the interaction of imatinib and MDR1 cells in primary CML stem cells but cell line studies suggest it may be an MDR1 substrate (13, 14, 29). Our most recent work in primary CML CD34$^+$ cells suggests firstly that MDR1 is expressed at very low levels in these cells, and secondly, that as the interaction of imatinib with MDR1 is as an inhibitor, transporter blockade is unlikely to be significant in this population (15).

We found that nilotinib was not a substrate for any of the transporters tested in cell line models. In a vectorial transport assay, based on MDCKII cells engineered to over-express MDR1

apically whereby basal to apical exceeds apical to basal transport of MDR1 substrates, the transport of ^{14}C-nilotinib across the monolayer in either direction was slightly decreased compared to imatinib [Figure 1D] and was not altered by the MDR1 inhibitor PSC833. This lack of efflux by MDR1 was confirmed by the uptake and retention of nilotinib into KCL22 cells being unaffected by the MDR1 inhibitors verapamil, PSC833 or tariquidar [Figure 1E]. We also used cell lines expressing different transporters in ^{14}C-nilotinib uptake assays [Figure 2; upper panels] and in fluorescent substrate assays [Figure 2; lower panels]. Expression of MDR1, MRP1 or ABCG2 did not decrease the retention of ^{14}C-nilotinib, and furthermore inhibition of these efflux transporters with the specific inhibitors PSC833, MK571 or FTC respectively, did not cause any increase in drug retention [Figure 2; upper panels]. However, in substrate efflux assays [Figure 2; lower panels] nilotinib significantly reduced efflux via MDR1 or ABCG2 and had a small inhibitory effect on MRP1 mediated efflux. Therefore, whilst nilotinib is not a substrate for any of these transporters it can inhibit efflux via MDR1 or ABCG2.

In order to investigate whether the poor response of CML CD34$^+$ cells to nilotinib was due to low intracellular accumulation we looked at the expression of mRNA using qPCR for the three main clinically relevant transporters MDR1, MRP1 and ABCG2 that have been previously associated with drug resistance in leukaemia (30, 31). We found that all three efflux transporters are expressed in CD34$^+$ CML cells, however hOCT1 was below the level of detection in normal or CML CD34$^+$ cells. MRP1 was detected at levels similar to those in normal CD34$^+$ cells (108%), ABCG2 at higher levels (291%) as previously reported (8) and surprisingly, MDR1 was found to be expressed at a lower level in CML (13.5%) than normal CD34$^+$ cells (Supplemental Data, Table 1). MDR1 expression is often elevated in leukaemia and indeed has been found at higher levels on CML versus normal mononuclear cells (MNC) and in primary cells from patients with advanced phase CML (32), however this is not the case in the more primitive population in CP, underlining the key differences depending on cell type and disease phase.

As all three efflux transporters were expressed, we assessed the interaction of nilotinib in primary CML CD34$^+$ cells and tested the effect of inhibiting the transporters whilst these cells were treated with nilotinib. As shown in the top and middle panels of Figure 3, CML CD34$^+$ cells demonstrated moderate substrate efflux via MDR1 (56.2 ± 11.5% retention) and ABCG2 (54.4 ± 19.3% retention), but a very high level of MRP1 activity (4.89 ± 0.22% retention). These levels of activity correlate with the relative levels of mRNA for these transporters (Supplemental Data, Table 1).

When the known inhibitors were replaced with increasing doses of nilotinib [lower panel Figure 3] the TKI had no significant effect on MDR1 activity (70.6 ± 9.8% retention at 5μM); however, it was difficult to determine reproducibility due to the low control level of MDR1-mediated efflux. Similarly, efflux of BODIPY-prazosin via ABCG2 was not seen to any significant degree within this cohort of samples; the expression level of ABCG2 was also lower than we have reported for a previous cohort (8). We were therefore unable to confirm the inhibitory effect seen with nilotinib in ABCG2 over-expressing lines in CML $CD34^+$ cells. However, CML $CD34^+$ cells demonstrated a high degree of efflux of Fluo3-AM via MRP1 (4.89 ± 0.22% retention) and this efflux was slightly reduced by nilotinib (15.3 ± 6.0% retention at 5μM vs. 4.89 ± 0.22% control, not statistically significant). Additionally, $CD34^+$ CML cells were incubated in nilotinib with and without the individual inhibitors to directly assess the effect of transporter inhibition on the intracellular concentration by high pressure liquid chromatography (HPLC) analysis. The individual inhibitors had no effect on this concentration nor did the combined use of all three inhibitors (data not shown). Therefore, as seen in the cell lines, whilst nilotinib may have some inhibitory effect on MDR1 and ABCG2 (and hOCT1) it is not a substrate for these transporters and the intracellular concentration of this TKI is not controlled by any of the transporters tested.

These findings are somewhat different to those of Mahon *et al*, who have reported that nilotinib resistant cell lines have elevated MDR1 expression (33). However, those studies made use of cell lines that had developed resistance after prolonged exposure to nilotinib rather than by over-expression of specific transporters, this may be why it was not possible to fully differentiate between MDR1 mediated effects and other mechanisms of resistance such as Bcr-Abl over expression or lyn activity. Also, unlike the previous study we have directly measured drug accumulation in addition to cell death and MDR1 inhibitor effects and our finding that nilotinib is not a substrate for MDR1 is in agreement with the previous report of White and colleagues (28) who found that inhibition of MDR1 in MNC from CP CML patients did not increase the intracellular concentration of nilotinib, nor did MDR1 expression protect cell lines from nilotinib induced death.

Another previous study suggested that nilotinib is transported by ABCG2 at low concentrations (18), but the study was performed on cell lines and normal stem cells. Furthermore, the concentrations at which nilotinib was transported were in the low nanomolar range which were far below even trough levels measured in patient serum. We have used seemingly high concentrations of nilotinib throughout these experiments primarily because we have previously seen that even these

concentrations do not kill $CD34^+$ cells, and it is this insensitivity in the stem cell population that we specifically want to investigate. Additionally, the doses we used (1-5μM) are similar to the observed peak values of 3.6μM achieved by standard 400mg twice daily dosing. However, CML stem cells may reside within bone marrow niches and therefore not be exposed to serum concentrations of drug. It is not practical to measure the amount of drug within specific niches, but we have previously found that there is no significant difference in the concentration of imatinib found in bone marrow extracts and peripheral blood samples (Jorgensen & Holyoake, unpublished data). We have not made these measurements for nilotinib but given the similar pharmacokinetic data for nilotinib and imatinib we suggest that the distribution of nilotinib would not be significantly lower in bone marrow than blood but we can not exclude the possibility of highly localised depletion in the defined stem cell niche. Our finding that nilotinib is not a substrate for MDR1 is in agreement with the previous report of White and colleagues (28) who found that inhibition of MDR1 in MNC from CP CML patients did not increase the intracellular concentration of nilotinib, nor did MDR1 expression protect cell lines from nilotinib induced death.

Transporter activity does not modulate the effect of nilotinib on primary chronic myeloid leukaemia $CD34^+$ cells

The proposal that drug transport is not responsible for the insensitivity of $CD34^+$ cells to nilotinib is further supported by our findings from co-treatment experiments. Blockade of ABCG2 or MRP1, with FTC or MK571 respectively, did not potentiate the effect of nilotinib on $CD34^+$ CML cells over a period of 72h treatment [Figure 4]. P-CrkL assays at 72h showed that nilotinib significantly reduced the activity of Bcr-Abl in these cells (62.2 ± 12.21% at 72h) compared to control but this reduction was not potentiated by inhibition of efflux transporters [Figure 4A]. Similarly, when the total number of cells remaining in the cultures was assessed at 72h [Figure 4B] nilotinib treatment resulted in reduced cell numbers due to both anti-proliferative and pro-apoptotic effects as expected. Neither MRP1 nor ABCG2 inhibition further decreased cell numbers. However, the effect of MDR1 inhibition with PSC833 was less clear.

PSC833 has previously been found to have unacceptable toxicity *in vivo* (34), resulting in early termination of trials using this agent. Our preliminary experiments demonstrated that the standard concentration of 10μM used for cell line studies caused significant apoptosis and was profoundly

cytostatic in CML $CD34^+$ cells (data not shown). Therefore, we reduced the concentration to 5μM which still gave full inhibition of Rhodamine-123 efflux in cell lines but had a less detrimental effect on $CD34^+$ cells. However, we still saw an increase in cell death and some anti-proliferative effect when the CML $CD34^+$ cells were treated with PSC833 alone; this effect was maintained in the presence of nilotinib and therefore the combination resulted in a further reduction in the number of cells remaining in culture. Whilst this result initially seems to demonstrate that MDR1 inhibition enhances the effect of nilotinib we did not see an additive effect on the reduction of Bcr-Abl activity by p-CrkL assay nor did we measure any increase in the intracellular concentration of nilotinib by radio-labelled or HPLC analyses. We conclude, therefore that the effect of PSC833 is not connected to transport of nilotinib. The data may indicate that there is an endogenous substrate that accumulates when MDR1 is inhibited which is toxic to the cells, or simply that PSC833 has a non–specific toxic effect – we consider the latter to be most likely. In support of this, Mahon and colleagues also reported unexpected results when using PSC833 with CML cells (29). We also suggest that this non-specific effect may underlie some of the confusion about the effect of MDR1 inhibition in previous cell line studies of imatinib. In such studies, PSC833 appeared to increase the response to nilotinib and imatinib however, detailed analyses including determination of drug accumulation and activity, are required to deconvolute this effect and have not been performed in many of these studies.

Finally, we looked at the number of $CD34^+$ cells that remained quiescent in culture during the treatment [Figure 4C]; this population represents the putative CML stem cell population which is resistant to TKI and may be responsible for clinical persistence of the disease. As we have reported previously, nilotinib increased the number of these cells [Figure 4C] however, there was no significant effect on this population in response to transporter inhibition alone or in combination with nilotinib.

In conclusion, we have found no evidence for either active uptake of nilotinib via hOCT1 or efflux via MDR1, MRP1 or ABCG2. It is therefore unlikely that differential expression or function of these transporters will have any effect on the clinical response to this drug in either MNC or the more resistant $CD34^+$ stem cell population. However, nilotinib appears to be an inhibitor of both influx and efflux pumps, and is therefore liable to be involved in drug-drug interactions.

Author Contributions: AD, NEJ, AG, CML, SH, RJH performed experiments; AD, NEJ, AG, JCM, HGJ analysed results, made the figures, edited the manuscript; JCM, REC, TLH, MP designed and supervised the research; all authors contributed to critical review of the data and preparation of the manuscript.

TITLES AND LEGENDS TO FIGURES

Figure 1. Nilotinib is neither imported by hOCT1 nor effluxed by MDR1.

A. *Nilotinib transport is not influenced by hOCT1:* Parental (parent), hOCT1 transfected (OCT1) and empty vector transfected (pcDNA) KCL22 cells were treated with ^{14}C-nilotinib (4μM) for up to 2h. Mean data of 5 experiments ±SEM. **B.** *Nilotinib is not a substrate for hOCT1 influx:* hOCT1 and empty vector transfected KCL22 cells were treated for 30min with 1 or 4μM ^{14}C-nilotinib or co-incubated with OCT1 inhibitors: - 500μM amantadine (AMT) and 100μM prazosin (PRZ); and 5μM tetraethylammonium bromide (TEA), an OCT1 substrate. Mean data of 5 experiments; ±SEM. **C.** *Competitive uptake of TEA with TKIs:* KCL22 cells transfected with hOCT1 cells were incubated with ^{14}C-labelled TEA (5μM). TEA influx via OCT1 was competed for by imatinib (IM) (5μM) and nilotinib (NIL) (4μM). AMT (500μM) was used as a positive control for blocking TEA uptake. Mean data of 5 experiments ±SEM. **D.** *Nilotinib is not transported by MDR1 in cell lines* Efflux of ^{14}C-nilotinib (4μM) in parental (MDCKII) and MDR1-transfected (MDR1) MDCKII cells pre-incubated with PSC833 (10μM) (MDR1 + PSC). A-B = apical to basal transport; B-A = basal to apical transport. Mean data of 5 wells; ± SEM. **E.** KCL22 parental cells treated for 30min with 1 or 4μM ^{14}C-nilotinib and co-incubated with MDR1 inhibitors: - 10μM PSC833; 500nM tariquidar (TQD); 500μM verapamil (VPL). Mean data of 5 experiments; ± SEM.

Figure 2. Nilotinib is not a substrate for MDR1, MRP1 or ABCG2 in cell lines.

Top panels: *Accumulation of ^{14}C-nilotinib in cell lines.* Cell lines were incubated for 2h with 5μM ^{14}C-nilotinib. **Top left:** K562-WT or K562-MDR1 cells in the presence or absence of 5μM PSC. **Top mid**: K562-WT or K562-MRP1 cells in the presence of absence of 30 or 100μM MK571. **Top right:** AML3 or AML6.2 cells, which over express ABCG2, in the presence of absence of 10μM FTC. All data are mean ± SEM of 3 separate experiments performed in duplicate.

Lower panels: *Substrate Displacement Assay to show interaction of nilotinib with transporters.* **Lower left:** K562-MDR1 cells were loaded with 0.5μg/mL Rhodamine-123 alone or in the presence of 5μM

PSC833 or increasing concentrations of nilotinib (0.1 to 5μM). **Lower mid:** K562-WT (MRP1+) cells were loaded with 5μg/mL Fluo3-AM alone or in the presence of 30μM MK571 or increasing concentrations of nilotinib (0.1 to 5μM). **Lower right:** AML6.2 (ABCG2+) cells were loaded with 200nM BODIPY-Prazosin alone or in the presence of 10μM FTC or increasing concentrations of nilotinib (0.1 to 5μM). In all lower panels, unstained cells are denoted by black fill, with substrate by light grey fill, with substrate + inhibitor by dark grey fill, with substrate + nilotinib by black line no fill. Representative examples are shown and all experiments were performed on 3 independent samples in duplicate.

Figure 3. Nilotinib is not a substrate for MDR1, MRP1 or ABCG2 in CP CML CD34 cells.

Top panels: *Accumulation of ^{14}C-nilotinib in CD34+ CML cells.* CD34+ cells from patient in CP were incubated for 2h with 5μM ^{14}C-nilotinib ± 5μM PSC833 (**top left**), 30μM MK571 (**top mid**) or 10μM FTC (**top right**). All data are mean ± SEM of duplicate analyses of 3 separate patient samples.

Middle panels: *Substrate Displacement Assay to show efflux via transporters.* CML CD34$^+$ cells were loaded with 0.5μg/mL Rhodamine-123 alone or in the presence 5μM PSC833 **(middle left)**; 5μg/mL Fluo3-AM alone or in the presence 30μM MK571 **(middle mid)**; 200nM BODIPY-Prazosin alone or in the presence of 10μM FTC **(middle left)**.

Lower Panels: *Substrate Displacement Assay to show interaction of nilotinib with transporters.* As for middle panels but with the addition of nilotinib in place of PSC833, MK571 or FTC.

In **middle and lower panels**: unstained cells are denoted by black fill; cells with substrate alone by light grey fill, with substrate + inhibitor by dark grey fill, with substrate + nilotinib by black lines no fill. Representative examples are shown and all experiments were performed on at least 3 independent samples in duplicate.

Figure 4. Transporter inhibition does not increase the effect of nilotinib in CD34$^+$ CML cells. CML CD34$^+$ cells from patients in CP were analysed after 72h in culture with no drug or with 5μM nilotinib ± 5μM PSC833, 30μM MK571 or 10μM FTC as denoted. **A.** The percentage level of p-CrkL was determined by flow cytometry to measure the geometric mean fluorescence with no drug control cells considered to be maximally phosphorylated. Data are mean ± SEM of 5 patient samples performed in duplicate. **B.** the total number of cells was assessed by cell counting; data are mean ± SEM of between 4 and 12 patient samples performed in duplicate. **C.** The number of quiescent CD34$^+$ cells (putative CML stem cells) remaining was calculated based on the use of CFSE to track cell division and FACS analysis of CD34$^+$ at the end of the culture period. Data are mean ± SEM of 2

samples for nilotinib + MK571, and 4 to 10 samples for all other conditions, each performed in duplicate.

REFERENCES

1. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med. 2001 Apr 5;344(14):1031-7.

2. Druker BJ, Guilhot F, O'Brien SG, Gathmann I, Kantarjian H, Gattermann N, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. N Engl J Med. 2006 Dec 7;355(23):2408-17.

3. Kantarjian HM, Giles F, Gattermann N, Bhalla K, Alimena G, Palandri F, et al. Nilotinib (formerly AMN107), a highly selective BCR-ABL tyrosine kinase inhibitor, is effective in patients with Philadelphia chromosome-positive chronic myelogenous leukemia in chronic phase following imatinib resistance and intolerance. Blood. 2007 Nov 15;110(10):3540-6.

4. Ottmann O, Dombret H, Martinelli G, Simonsson B, Guilhot F, Larson RA, et al. Dasatinib induces rapid hematologic and cytogenetic responses in adult patients with Philadelphia chromosome positive acute lymphoblastic leukemia with resistance or intolerance to imatinib: interim results of a phase 2 study. Blood. 2007 Oct 1;110(7):2309-15.

5. Jorgensen HG, Allan EK, Jordanides NE, Mountford JC, Holyoake TL. Nilotinib exerts equipotent antiproliferative effects to imatinib and does not induce apoptosis in CD34+ CML cells. Blood. 2007 May 1;109(9):4016-9.

6. Elrick LJ, Jorgensen HG, Mountford JC, Holyoake TL. Punish the parent not the progeny. Blood. 2005 Mar 1;105(5):1862-6.

7. Bhatia R, Holtz M, Niu N, Gray R, Snyder DS, Sawyers CL, et al. Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment. Blood. 2003 Jun 15;101(12):4701-7.

8. 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 Aug 15;108(4):1370-3.

9. Burger H, van Tol H, Boersma AW, Brok M, Wiemer EA, Stoter G, et al. Imatinib mesylate (STI571) is a substrate for the breast cancer resistance protein (BCRP)/ABCG2 drug pump. Blood. 2004 Nov 1;104(9):2940-2.

10. Houghton PJ, Germain GS, Harwood FC, Schuetz JD, Stewart CF, Buchdunger E, 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 Apr 1;64(7):2333-7.

11. Nakanishi T, Shiozawa K, Hassel BA, Ross DD. Complex interaction of BCRP/ABCG2 and imatinib in BCR-ABL-expressing cells: BCRP-mediated resistance to imatinib is attenuated by imatinib-induced reduction of BCRP expression. Blood. 2006 Jul 15;108(2):678-84.

12. Burger H, van Tol H, Brok M, Wiemer EA, de Bruijn EA, Guetens G, et al. Chronic imatinib mesylate exposure leads to reduced intracellular drug accumulation by induction of the ABCG2 (BCRP) and ABCB1 (MDR1) drug transport pumps. Cancer Biol Ther. 2005 Jul;4(7):747-52.

13. Hamada A, Miyano H, Watanabe H, Saito H. Interaction of imatinib mesilate with human P-glycoprotein. J Pharmacol Exp Ther. 2003 Nov;307(2):824-8.

14. Illmer T, Schaich M, Platzbecker U, Freiberg-Richter J, Oelschlagel U, von Bonin M, et al. P-glycoprotein-mediated drug efflux is a resistance mechanism of chronic myelogenous leukemia cells to treatment with imatinib mesylate. Leukemia. 2004 Mar;18(3):401-8.

15. Hatziieremia S, Jordanides NE, Holyoake TL, Mountford JC, Jorgensen HG. Inhibition of MDR1 does not sensitise primitive chronic myeloid leukaemia CD34+ cell to imatinib. Exp Hematol 2009 In Press.

16. Thomas J, Wang L, Clark RE, Pirmohamed M. Active transport of imatinib into and out of cells: implications for drug resistance. Blood. 2004 Dec 1;104(12):3739-45.

17. 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 Feb;83(2):258-64.

18. Brendel C, Scharenberg C, Dohse M, Robey RW, Bates SE, Shukla S, et al. Imatinib mesylate and nilotinib (AMN107) exhibit high-affinity interaction with ABCG2 on primitive hematopoietic stem cells. Leukemia. 2007 Jun;21(6):1267-75.

19. White DL, Saunders VA, Dang P, Engler J, Zannettino AC, Cambareri AC, et al. OCT-1-mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib (AMN107):

reduced OCT-1 activity is the cause of low in vitro sensitivity to imatinib. Blood. 2006 Jul 15;108(2):697-704.

20. Wang DS, Jonker JW, Kato Y, Kusuhara H, Schinkel AH, Sugiyama Y. Involvement of organic cation transporter 1 in hepatic and intestinal distribution of metformin. J Pharmacol Exp Ther. 2002 Aug;302(2):510-5.

21. Southgate TD, Garside E, Margison GP, Fairbairn LJ. Dual agent chemoprotection by retroviral co-expression of either MDR1 or MRP1 with the P140K mutant of O6-methylguanine-DNA-methyl transferase. J Gene Med. 2006 Aug;8(8):972-9.

22. Graham SM, Jorgensen 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 Jan 1;99(1):319-25.

23. Hamilton A, Elrick L, Myssina S, Copland M, Jorgensen 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 Jun;20(6):1035-9.

24. 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 Jun 6;1512(2):171-82.

25. 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 Mar;304(3):1085-92.

26. Yunger LM, Cramer RD, 3rd. Measurement of correlation of partition coefficients of polar amino acids. Mol Pharmacol. 1981 Nov;20(3):602-8.

27. Davies *et al*, Haematologica 2008: 93(s1):52 Abs.0131

28. 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 Apr 15;109(8):3609-10.

29. Mahon FX, Belloc F, Lagarde V, Chollet C, Moreau-Gaudry F, Reiffers J, et al. MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models. Blood. 2003 Mar 15;101(6):2368-73.

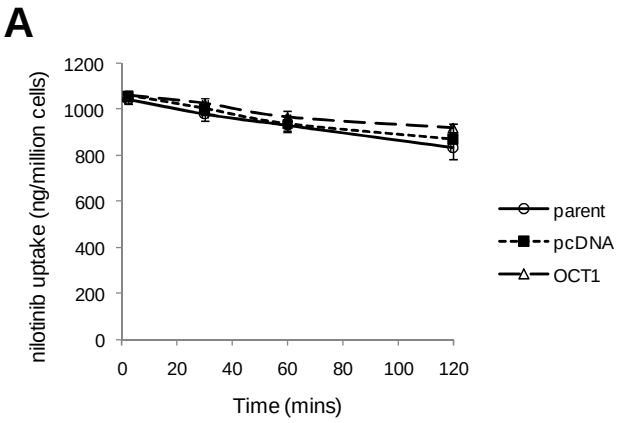
30. Kock K, Grube M, Jedlitschky G, Oevermann L, Siegmund W, Ritter CA, et al. Expression of adenosine triphosphate-binding cassette (ABC) drug transporters in peripheral blood cells: relevance for physiology and pharmacotherapy. Clin Pharmacokinet. 2007;46(6):449-70.

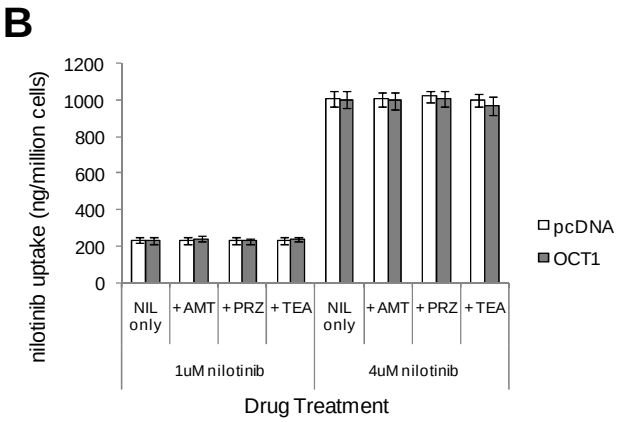
31. Norgaard JM, Olesen LH, Hokland P. Changing picture of cellular drug resistance in human leukemia. Crit Rev Oncol Hematol. 2004 Apr;50(1):39-49.

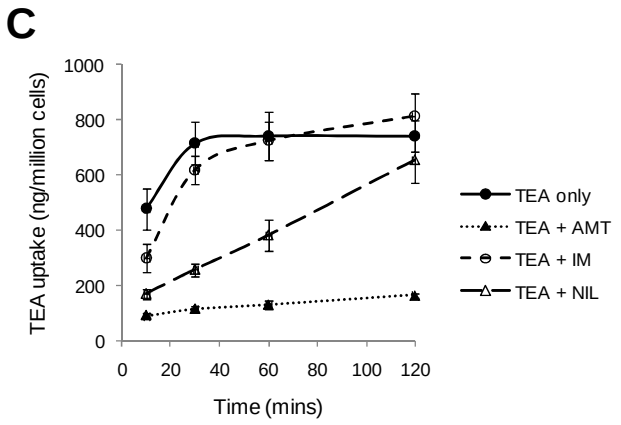
32. Galimberti S, Cervetti G, Guerrini F, Testi R, Pacini S, Fazzi R, et al. Quantitative molecular monitoring of BCR-ABL and MDR1 transcripts in patients with chronic myeloid leukemia during Imatinib treatment. Cancer Genet Cytogenet. 2005 Oct 1;162(1):57-62.

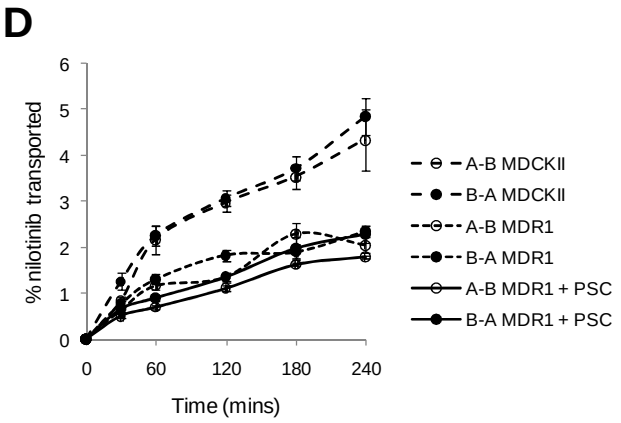
33. Mahon F-X, Hayette S, Lagarde V, Belloc F, Turcq B, Nicolini F, et al. Evidence that resistance to nilotinib may be due to BCR-ABL, Pgp, or Src kinase overexpression. Cancer Res. 2008 Dec 1;68(23):9809-16

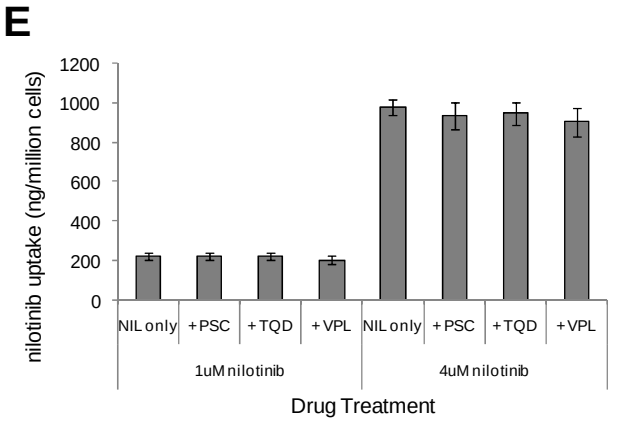
34. Baer MR, George SL, Dodge RK, O'Loughlin KL, Minderman H, Caligiuri MA, et al. Phase 3 study of the multidrug resistance modulator PSC-833 in previously untreated patients 60 years of age and older with acute myeloid leukemia: Cancer and Leukemia Group B Study 9720. Blood. 2002 Aug 15;100(4):1224-32.


A
nilotinib uptake (ng/million cells)
1200
1000
800
600
400
200
0
0
20
40
60
80
100
120
Time (mins)
parent
pcDNA
OCT1


B
nilotinib uptake (ng/million cells)
1200
1000
800
600
400
200
0
NIL only
+ AMT
+ PRZ
+ TEA
NIL only
+ AMT
+ PRZ
+ TEA
1uM nilotinib
4uM nilotinib
Drug Treatment
pcDNA
OCT1


C
TEA uptake (ng/million cells)
1000
800
600
400
200
0
0
20
40
60
80
100
120
Time (mins)
TEA only
TEA + AMT
TEA + IM
TEA + NIL


D
% nilotinib transported
6
5
4
3
2
1
0
0
60
120
180
240
Time (mins)
A-B MDCKII
B-A MDCKII
A-B MDR1
B-A MDR1
A-B MDR1 + PSC
B-A MDR1 + PSC


E
nilotinib uptake (ng/million cells)
1200
1000
800
600
400
200
0
NIL only
+ PSC
+ TQD
+ VPL
NIL only
+ PSC
+ TQD
+ VPL
1uM nilotinib
4uM nilotinib
Drug Treatment

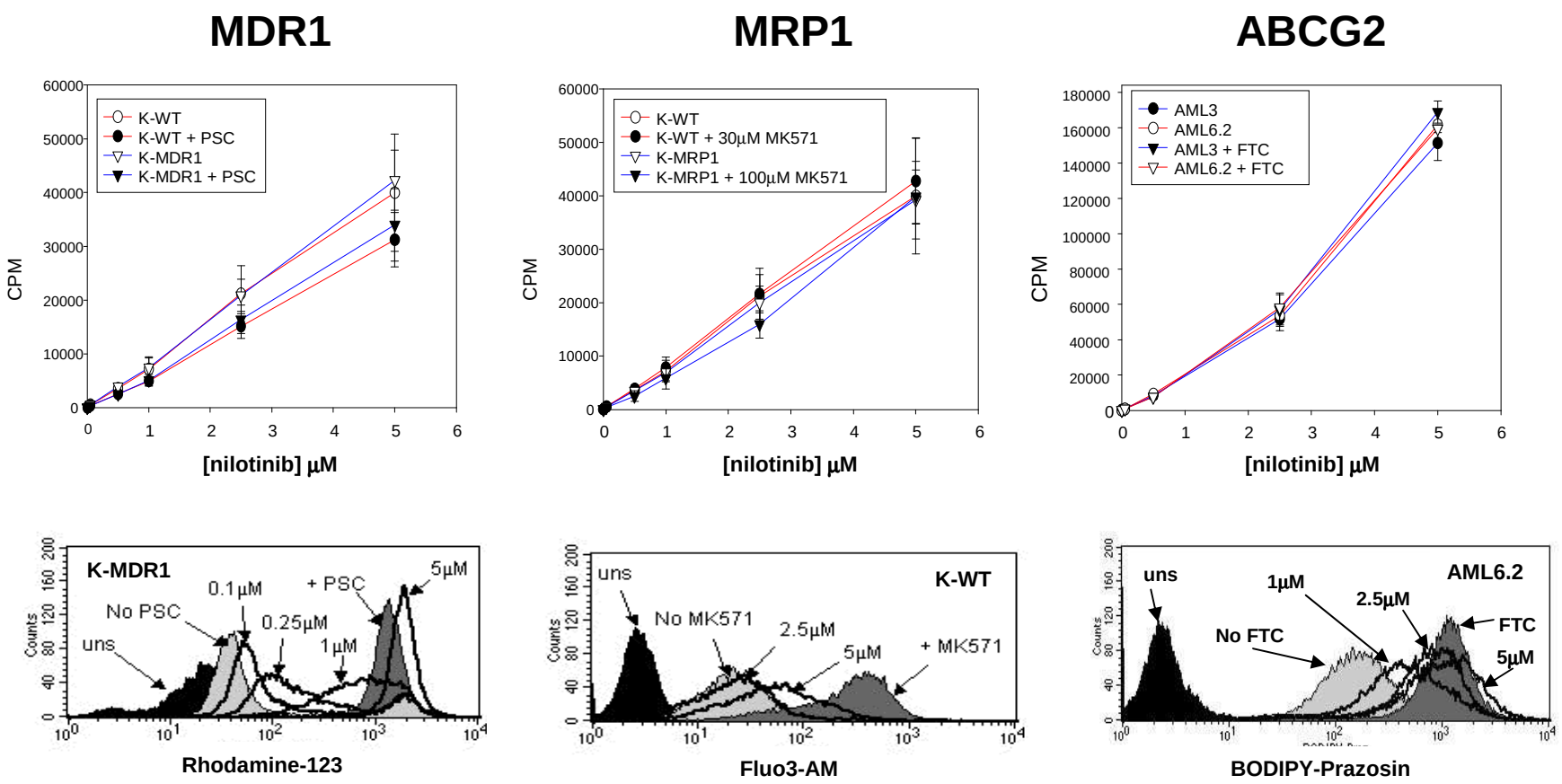


Figure 2. Nilotinib is not a substrate for MDR1, MRP1 or ABCG2 in cell lines.

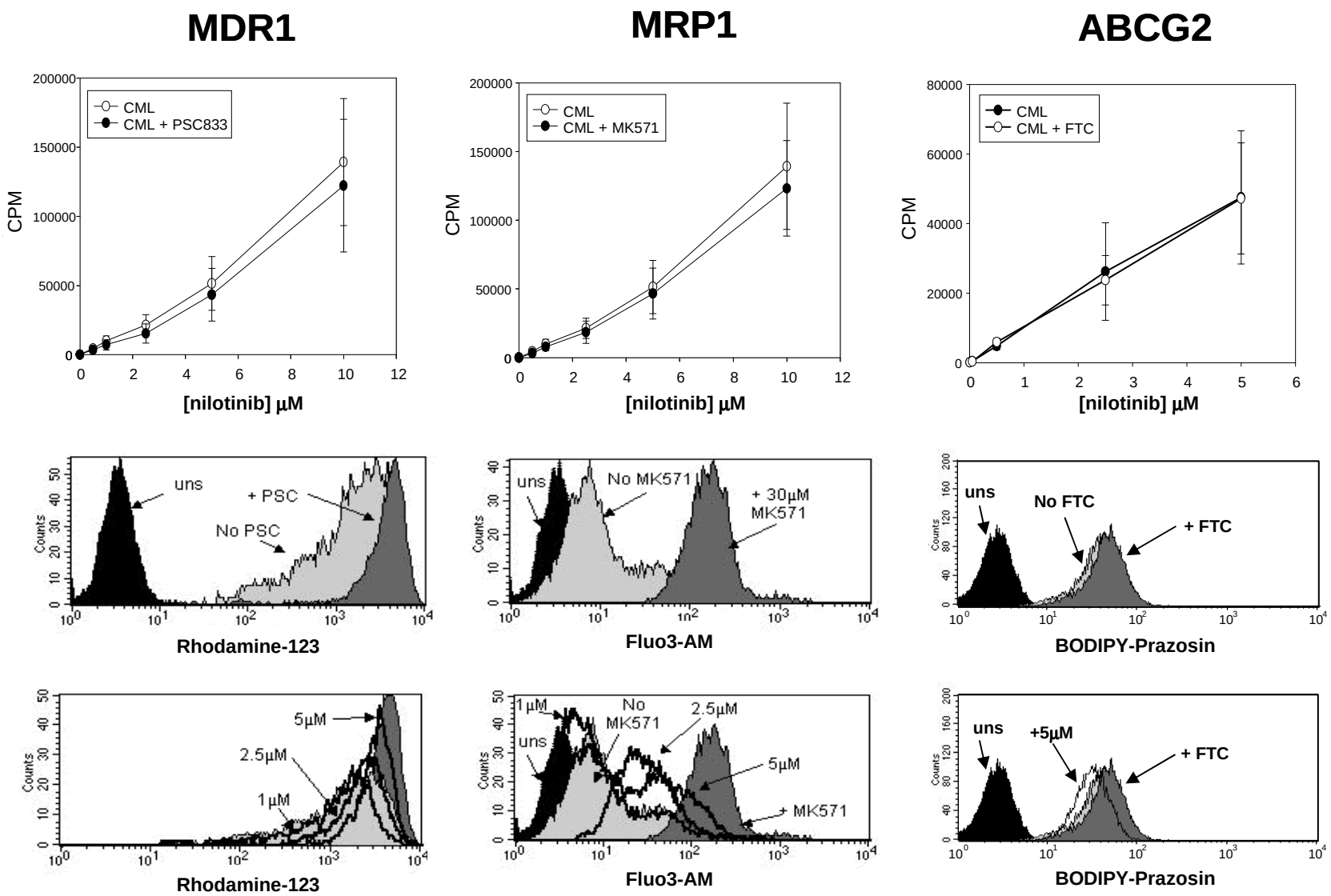
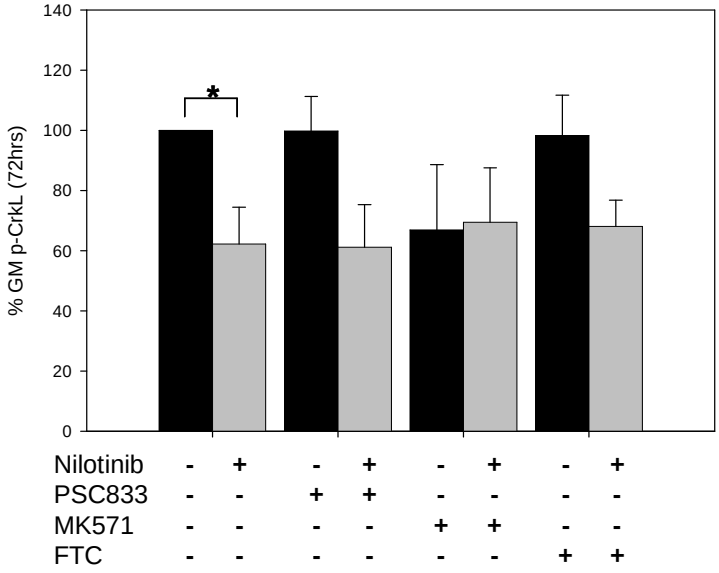
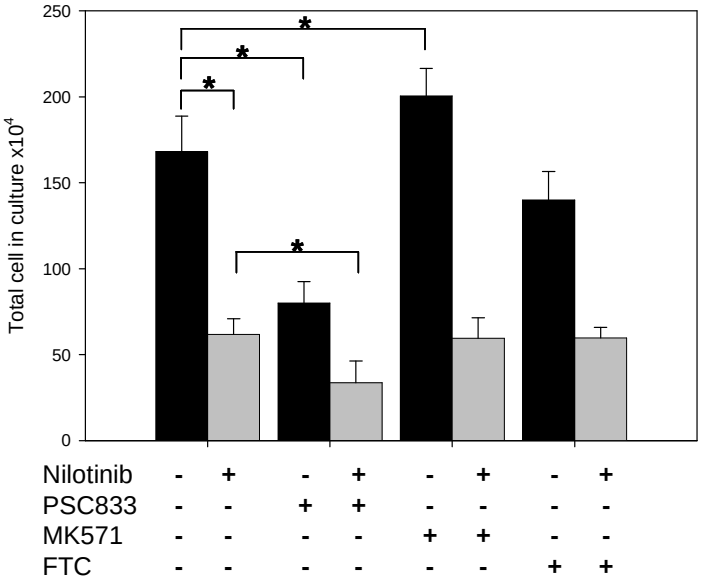


Figure 3. Nilotinib is not a substrate for MDR1, MRP1 or ABCG2 in CML CD34 cells from CP patients.

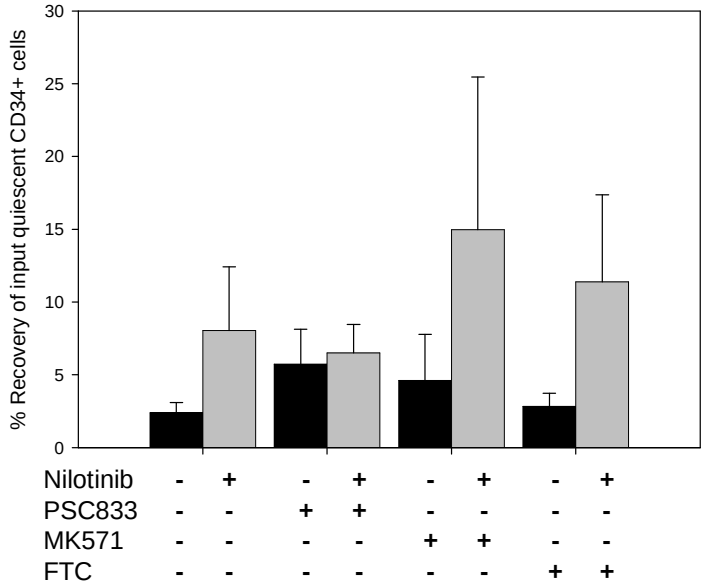
4A


% GM p-CrkL (72hrs)
140
120
100
80
60
40
20
0
*
Nilotinib - + - + - + - +
PSC833 - - + + - - - -
MK571 - - - - + + - -
FTC - - - - - - + +

4B


Total cell in culture x10^4
250
200
150
100
50
0
*
*
*
*
Nilotinib - + - + - + - +
PSC833 - - + + - - - -
MK571 - - - - + + - -
FTC - - - - - - + +

4C


% Recovery of input quiescent CD34+ cells
30
25
20
15
10
5
0
Nilotinib - + - + - + - +
PSC833 - - + + - - - -
MK571 - - - - + + - -
FTC - - - - - - + +