Omacetaxine may have a role in Chronic Myeloid Leukaemia eradication through down-regulation of Mcl-1 and induction of apoptosis in stem/progenitor cells

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Running title: **Omacetaxine induces stem cell apoptosis**

Abstract

Chronic Myeloid Leukaemia (CML) is maintained by a rare population of tyrosine kinase inhibitor (TKI) insensitive malignant stem cells. Our long term aim is to find a BcrAbl-independent drug that can be combined with a TKI to improve overall disease response in chronic phase CML. Omacetaxine mepesuccinate, a first in class cetaxine, has been evaluated by clinical trial in TKI-insensitive/resistant CML. Omacetaxine inhibits synthesis of anti-apoptotic proteins of the Bcl-2 family including Mcl-1, leading to cell death.

Omacetaxine effectively induced apoptosis in primary CML stem cells ($CD34^{+}38^{lo}$) by down regulation of Mcl-1 protein. In contrast to our previous findings with TKIs, omacetaxine did not accumulate undivided cells *in vitro*. Furthermore, the functionality of surviving stem cells following omacetaxine exposure was significantly reduced in a dose dependent manner as determined by colony forming cell and the more stringent long-term culture initiating cell colony assays. This stem cell directed activity was not limited to CML stem cells as both normal and non-CML $CD34^{+}$ cells were sensitive to inhibition. Thus, although, omacetaxine is not leukaemia stem cell specific, its ability to induce apoptosis of leukaemic stem cells distinguishes it from TKIs and creates the potential for a curative strategy for persistent disease.

Keywords: omacetaxine; leukaemic stem cells; CML; BcrAbl; Mcl-1

Introduction

Omacetaxine mepesuccinate (ChemGenex Pharmaceuticals), a first in class cetaxine, is a semi-synthetic alkaloid that has demonstrable activity in clinical trial in acute myeloid leukaemia (AML), myelodysplastic syndrome (MDS), as well in chronic myeloid leukaemia (CML)(1). However, with the continued success of tyrosine kinase inhibitors (TKI), such as imatinib, nilotinib and dasatinib, as first and second line treatment for CML, the role of omacetaxine has been restricted to niche indications including failure of two or more TKI or with the TKI-insensitive mutation, T315I, against which all currently licensed TKI are ineffective. Omacetaxine is a semisynthetic, subcutaneously bioavailable form of homoharringtonine (HHT). HHT was used in second line therapy for those CML patients in the pre-TKI era of the 1990s who failed interferon α therapy and were ineligible for a transplant(2) (3). Therefore this product has, for a second time, become a valuable option in the treatment of resistant disease. Moreover, the demonstration that omacetaxine can kill leukaemic stem cells in murine models(4) has allowed the drug to be considered as a therapeutic option for both persistent and resistant, disease.

HHT, from here in referred to as omacetaxine, is derived from various *Cephalotaxus* species (Chinese yew tree), and was first discovered by the Chinese to have natural anti-tumour and anti-leukaemic properties in the 1970s(5, 6). Recent work has elucidated a mechanism of action targeted to the A-site cleft of eukaryotic ribosomes (7) resulting in transient inhibition of protein synthesis of short-lived proteins such as myeloid cell leukaemia (Mcl-1) protein (a highly expressed member of the Bcl-2 family of anti-apoptotic proteins) leading to apoptosis(8). Further, it has been shown that Mcl-1 plays a critical role in the survival of leukaemia cells and its expression is up-regulated with respect to normal(9, 10). As such omacetaxine acts independently of BcrAbl kinase activity, the causative oncoprotein in CML. Taken together with a recently reported ability to kill stem cells(4), omacetaxine could therefore be an interesting therapeutic

modality in minimal residual disease in CML where the continued detection of *BcrAbl* transcripts is thought to originate from the stem cell compartment(11) and leukaemic stem cell survival may be BcrAbl independent(12).

Therefore the aim of our study was to assess the activity of omacetaxine against CML stem/progenitor cells *in vitro* in order to identify potential ways of eradicating persistent disease. Sequential clinical samples from newly diagnosed cases were selected for assay and were not subject to mutation analysis. We have previously shown CML $CD34^+$ cells to be insensitive to TKI by virtue of their quiescence and primitive phenotype. From our on-going work and that of others, it is our belief that CML stem cells may not be oncogene addicted (that is depdendent on BcrAbl for their survival), and thus any novel agents or novel indications for existing molecules with targeting potential at the (stem) cell level to complement TKI modalities is of significant interest.

Materials and methods

Reagents

Omacetaxine mepesuccinate, supplied by ChemGenex Pharmaceuticals, Inc., Menlo Park, CA, USA, was stored lyophilised at room temperature; once reconstituted in saline to 10mM, the stock solution was stored at 4°C for up to 3 months.

Cell culture

Primary CML cells were obtained with informed consent from leukapheresis samples of newly diagnosed patients with chronic phase CML or Ph^- haematological disorders (e.g. lymphoma) as controls. $CD34^+$ cells enriched by positive selection (CliniMACS, Miltenyi Biotec Ltd) according to the manufacturer's instructions to >95 % purity were cryopreserved in 10% (v/v) DMSO in 4% (w/v) ALBA (human albumin solution, Scottish National Blood Transfusion Service, Edinburgh, UK) and stored in the vapour phase of liquid

nitrogen until required. $CD34^+$ cells were cultured in Serum Free Media (SFM) comprising Iscove's modified Dulbecco's medium (IMDM) with BIT (bovine serum albumin, insulin, transferrin; Stem Cell Technologies, Vancouver, Canada), 1mmol/L glutamine, 1mmol/L streptomycin/penicillin, 40ng/mL low density lipoprotein (LDL) and 0.1mmol/L 2-mercaptoethanol (all Invitrogen Ltd, Paisley, UK) further supplemented with a 5 growth factor (5GF) cocktail as previously described(13).

The BcrAbl positive human myeloid cell lines, K562 and KCL22 were maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 10% (v/v) fetal calf serum containing 1mmol/L each of streptomycin, penicillin and glutamine ($RPMI^+$). Total cell counts and viability was assessed using the trypan blue dye exclusion method.

Flow Cytometry

Apoptosis

To assess apoptosis after drug treatment an aliquot of cell suspension containing 1 to $2x10^5$ cells was removed for staining with Annexin V-FITC and Viaprobe (BD Biosciences, Oxford, UK) as per the manufacturer's instructions and analysed by flow cytometry using a BD FACS Canto with Diva software. Cells undergoing apoptosis initially become Annexin V positive (early apoptosis) and as apoptosis progresses, become positive for both Annexin V and Viaprobe (late apoptosis).

Cell division tracking

Upon recovery from liquid nitrogen, CML $CD34^+$ cells were stained with 1µM carboxy-fluorescein diacetate succinimidyl diester (CFSE; Molecular Probes, Invitrogen Ltd, Paisley, UK) as described previously in detail(14). Cells were then cultured in SFM+5GF in the presence or absence of omacetaxine for 24 or 72h. After 3 days cells were harvested, total cell viability assessed, and an aliquot of cells from each treatment stained with anti-CD34-PE antibody (BD Pharmingen, Oxford, UK) for flow cytometry analysis. Cells cultured with 100ng/mL Colcemid

(Invitrogen), which arrests cell cycle progression, were used to identify the undivided ($CFSE^{max}$) cell population.

To measure the overall effect of the treatments on cell survival and the number of undivided cells remaining after culture with omacetaxine, the percentage recovery of $CD34^+$ cells in the undivided $CFSE^{max}$ peak was calculated with respect to the input as previously described (13) and expressed as a proportion of the No Drug Control (NDC). Briefly, the number of $CD34^+$ cells used to establish each culture was recorded. After the 3-day culture period, the total number of viable cells harvested from each culture condition was recorded, as were the percentages of viable $CD34^+$ cells found in the undivided fraction and in each division peak from the CFSE/$CD34^+$ flow cytometry plots. Percentage recovery of input cells in each peak could then be calculated by dividing the absolute number of $CD34^+$ viable cells in each peak on day 3, corrected for cell division, by the total number of input $CD34^+$ cells and multiplying by 100. Note, although the percentage in the $CFSE^{max}CD34^+$ peak may increase with omacetaxine treatment, because of a very significant reduction in total cells (undivided plus proliferating cells), the absolute number of undivided cells (product of % x total cell number) may remain unchanged.

Primitive subsets

The $CD34^+$ enriched cells were stained with anti-CD34-APC and anti-CD38-PE antibodies before cell sorting using a FACS Aria (Becton Dickinson) into three populations: $CD34^{lo}38^+$ (mature), $CD34^+38^+$ (progenitor) and $CD34^+38^{lo}$ (primitive). The $CD34^+38^{lo}$ fraction approximates the most primitive quiescent stem cell QSC pool (< 5% total $CD34^+$ cells). Cells were analysed using a BD FACS Canto instrument with Diva software.

Colony forming cell (CFC) and long term culture-initiating cell (LTC-IC)

After culture in omacetaxine for 24 or 72h, $CD34^+$ cells remaining were set up in CFC and LTC-IC assays to assess the proliferative potential of cell populations remaining after drug treatment. For CFC assays, $2.5x10^3$ cells were added to

1.5mL Methocult (Stemcell Technologies, Vancouver, BC) in duplicate 35mm culture dishes and cultured for 12 to 14 days at 37°C in a 5% CO_2 humidified incubator.

LTC-IC assays were set up as previously described(15). Briefly, M2-10B4 cells and SL/SL fibroblasts were established as feeder layers then irradiated at 80Gy. CML cells remaining in culture after drug treatment were washed 3 times and then plated in duplicate on the irradiated feeder layers in Myelocult medium supplemented with hydrocortisone at a final concentration of 1μM (Stem Cell Technologies). Cultures were maintained for 5 weeks with weekly half medium changes. After 5 weeks, cells were counted and $2.5x10^4$ cells were transferred to CFC assays and maintained in culture for a further 2 weeks in Methocult medium before the colonies were counted.

Western blotting

Following treatment with drugs, K562 and primary CML cells (2 to $5x10^6$) were pelleted by centrifugation, washed twice with ice cold PBS and then lysed in buffer containing 50mM Tris-HCl pH 7.4, 150mM NaCl, 1% Nonidet P-40, 1mM ethylene diamine tetra-acetic acid (EDTA), plus protease inhibitors (Complete mini protease inhibitor cocktail tablets; Roche Diagnostics, Burgess Hill, UK). Protein lysates were separated on 4 to 15% Tris-HCl gradient gels (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK), transferred onto nitrocellulose membrane (Bio-Rad Laboratories) and blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS)-0.1% Tween 20 (TBS-T) for 1h at room temperature (RT). Membranes were incubated with primary antibody overnight at 4ºC: Poly(ADP-ribose) polymerase (PARP), Myeloid cell leukaemia (Mcl-1), GAPDH (Cell signalling, New England Biolabs (UK) Ltd, Hertfordshire, UK) abl, Bcl-2, Bcl-2 interacting mediator of cell death (Bim) (BD Biosciences, Oxford, UK) at 1:1,000 and anti-heat shock protein 90 (HSP90) (R&D systems, Abingdon, UK) at 1:10,000 in 1% BSA/TBS-T. Membranes were then washed 5 times with TBS-T and then incubated with horse radish peroxidase-labelled

secondary antibody at 1:3,000 in 1% BSA/TBS-T for 1h at RT. Antibody detection was performed using enhanced chemiluminescence (ECL) reagent (Immun-Star™ Western C™Kit, Bio-Rad) and visualised in a ChemiDoc illuminator (Bio-Rad Laboratories) with Quantity 1 image capture software.

Results

Omacetaxine potently inhibits growth of K562, primary CML CD34$^+$ and non-CML CD34$^+$ cells *in vitro*

The drug concentration required to reduce cell numbers to 50% of the NDC (IC_{50}) was determined by viable cell counts for K562, CML CD34$^+$ and non-CML CD34$^+$ cells at 24 hourly intervals over three days (Figure 1). The mean IC_{50} values for omacetaxine treated K562 in three independent experiments were >1000, 35 and 35nM at 24, 48 and 72h, respectively (Figure 1a). Primary CML CD34$^+$ cells were more sensitive to omacetaxine treatment than K562 with IC_{50} values of 40, 4 and 2.8nM at 24, 48 and 72h, respectively (n=3; Figure 1b). The omacetaxine effect appeared to be stem/progenitor cell directed as it produced similar IC_{50} values of 20, 4.5 and 3.5nM at 24, 48 and 72h, respectively, with non-CML CD34$^+$ cells (n=2; Figure 1c).

The minimum exposure to omacetaxine required to inhibit cell growth by >50% and induce apoptosis in CD34$^+$ cells is 24h

CML CD34$^+$ cells were cultured with and without omacetaxine in SFM+5GF for various time-points from 15min to 72h (Figure 2), after which cells were washed free of drug and re-seeded in fresh SFM+5GF. Omacetaxine was used in this experiment at 10 and 100nM being in the approximate range of the calculated IC_{50} values (Figure 1) and clinically achievable concentrations (Personal communication with ChemGenex). Cells were counted after a total of 72h in culture for all samples and stained with Annexin V / Viaprobe to measure apoptosis. Total viable cells were expressed as trypan blue cell counts multiplied by the percentage cells which were negative for Annexin V / Viaprobe. Treatment of CML CD34$^+$ cells with 100nM omacetaxine for only 6h reduced

viable cell numbers at 72h to 50% of NDC. However a minimum of 24h exposure to either 10 or 100nM omacetaxine was required to significantly reduce viable cell numbers to greater than 50% with respect to 15min drug treatment (Figure 2a; $p<0.001$; n=3) (as NDC had been normalised to 100% in each experiment, there was no deviation and therefore statistics were generated with respect to the next time-point). Omacetaxine (100nM) effectively induced apoptosis in CML $CD34^+$ cells after just 24h treatment in 58% of cells as measured by the total cells staining positive for Annexin V at 72h, however prolonged 72h drug treatment at this concentration resulted in almost all cells (90%) undergoing apoptosis (Figure 2b). $CD34^+$ cells from a myeloma patient (non-CML control) that had been treated with 10nM omacetaxine for 24h, reduced in viable cell count to only 17% of the NDC (Figure 2c) and to a similar degree to CML $CD34^+$ cells, again suggesting a stem/progenitor cell directed effect of the drug. Treatment of non-CML $CD34^+$ cells induced similar levels of apoptosis as for CML (Figure 2d).

Omacetaxine does not accumulate cells in the undivided fraction

Although omacetaxine was clearly inducing apoptosis in CML $CD34^+$ cells *in vitro*, an effect not observed with TKIs(13, 16, 17), we next sought to determine if omacetaxine was anti-proliferative against the stem/progenitor cell compartment, leading to arrest of cell cycle progression or block of cell division in a proportion of cells without killing. This was done using flow cytometry with CFSE staining to facilitate analysis of cell division history(14). Although there was an increase in the percentage of cells remaining alive in the undivided fraction (e.g. 30% with 100nM omacetaxine; Fig 3a) with increasing omacetaxine concentration, at 24h there was a dose dependent significant reduction in the total number of $CD34^+$ cells recovered with respect to the NDC (Figure 3c(i), $*p<0.05$, $***p<0.001$). The fold-change in recovery of input of undivided $CD34^+$ cells is calculated as the product of the percentage in the undivided gate (which has increased) times the total $CD34^+$ cell number (which has decreased through apoptosis). There was no statistically significant change in CML $CD34^+$ cells recovered in the undivided

fraction with respect to NDC with omacetaxine at any of the concentrations tested (Figure 3d(i)). Although only assayed once in normal G-CSF mobilised donor cells, more $CD34^+$ cells were recovered in all divisions as a percentage of the NDC in comparison to CML with 10 or 50nM but not 100nM omacetaxine (Fig 3c(ii)); moreover, fewer cells were recovered in the undivided fraction in a dose dependent manner relative to NDC (Fig 3d(ii)) as compared to CML (Fig 3d(i)).

Omacetaxine and stem/progenitor cell functionality

Although, encouragingly, it appeared that omacetaxine activity *in vitro* was stem/progenitor cell directed, in that the drug reduced total $CD34^+$ cell numbers, we were interested to know the stem/progenitor cell function of any remaining apparently viable $CD34^+$ cells following treatment. This was assessed in CFC and LTC-IC assays. CFC assays demonstrated that omacetaxine significantly reduced the number of CML and non-CML CFC, in a dose dependent manner with respect to the NDCs (Figure 4a, ***$p<0.001$). We were also able to confirm in a single sample of normal G-CSF mobilised donor $CD34^+$ cells that omacetaxine at high concentration (50 and 100nM) was cytotoxic for CFC activity to a similar degree as for CML cells.

CFC assays give an indication of committed progenitor cell survival but for stem cell enumeration, LTC-IC assays are more appropriate. Mirroring the CFC result, omacetaxine reduced the LTC-IC number in CML, non-CML and normal donor $CD34^+$ cells after 24h exposure (Figure 4b).

Scheduled combination of omacetaxine with imatinib improves cell kill in Ph^+ cell lines

The Ph^+ blast crisis cell line, KCL22 was significantly more sensitive to omacetaxine than to imatinib ($p<0.05$; Figure 5a(i)). Scheduled combination of omacetaxine (20nM, approximate IC_{50}) with imatinib (0.5µM) with or without drug washout at 24h significantly reduced cell viability with respect to omacetaxine alone when omacetaxine was administered before imatinib ($p<0.05$; Figure

5a(i)). Addition of imatinib first did not achieve increased cell kill with respect to omacetaxine alone. Simultaneous treatment with omacetaxine and imatinib for 72h was no more effective than omacetaxine alone.

In order to determine any combination effect in primary $CD34^+$ cells, we selected the lowest concentration of omacetaxine (10 nM) as this had shown lesser toxicity by CFC in the normal $CD34^+$ sample tested previously [Figure 4a]. CML $CD34^+$ cells were treated with omacetaxine or imatinib (5μM) or both in combination. When administered together, all of the combinations tested were significantly more effective than treatment with either imatinib ($p<0.001$) or omacetaxine alone ($p<0.05$; Figure 5b(i)). In contrast to KCL22, treatment of primary $CD34^+$ cells with omacetaxine and imatinib for 72h was equally effective whether administered simultaneously or in a scheduled combination (Figure 5b(ii & iii). By cell count, apoptosis and colony forming potential, normal donor $CD34^+$ cells were as sensitive as CML $CD34^+$ cells to treatment with omacetaxine alone or in combination with imatinib.

Mechanism of action of omacetaxine in Ph^+ cells

Increasing concentrations of omacetaxine induced apoptosis in K562 and CML stem/progenitor cells as confirmed by PARP cleavage and loss of the anti-apoptotic protein, Mcl-1 (Figure 6a). K562 cells are known to have extremely low expression of Bcl-2(18) and indeed we did not detect this protein by Western blotting of lysates from this cell line but it was found to be unchanged in primary CML $CD34^+$ cells with omacetaxine treatment (Figure 6b). Likewise, both the pro-apoptotic Bim protein and the molecular chaperone, HSP90 were unaffected by omacetaxine treatment as determined by densitometric analysis of Western blots with respect to the loading control (GAPDH). HSP90 had previously been shown to be lost on omacetaxine treatment in Ph^+ cell lines(4) together with loss of BcrAbl protein which we did not observe.

Omacetaxine induces apoptosis of primitive CML CD34 cell subsets with reduction in Mcl-1 expression

It was of interest to determine the activity of omacetaxine in more primitive subsets of $CD34^+$ cells. To achieve this, total CML $CD34^+$ cells were sorted by flow cytometry into three cell fractions (mature $CD34^{lo}38^+$, $CD34^+38^+$ progenitors and the more primitive $CD34^+38^{lo}$ population). As can be seen in Figure 7, even the more primitive $CD34^+38^{lo}$ cells that we have previously shown to be insensitive to TKI like imatinib were not spared by omacetaxine, with levels of apoptosis in the same order as mature and progenitor cells (Figure 7a). Apoptosis was confirmed by cleavage of PARP (Figure 7b) and was mediated in the same way as in bulk $CD34^+$ by Mcl-1 down-regulation (cf Figure 6).

Discussion

Our interests in CML leukaemic stem cell survival in the face of complete BcrAbl kinase inhibition has led us to investigate other agents which exhibit activity at the stem/progenitor cell level and have a mechanism of action that is BcrAbl independent. We considered omacetaxine to be worthy of further investigation with its BcrAbl independent mechanism of action, acceptable safety in humans and potential for stem cell activity(4).

The semi-synthetic orthologue of the natural phytochemical, HHT, omacetaxine was awarded 'Fast Track' status by the Food and Drug Administration (FDA) in late 2006 for those CML patients who fail imatinib or have demonstrable T315I BcrAbl mutation in which the drug has, rather uniquely, proven activity. A number of Phase I/II clinical trials or case reports have emerged describing disappearance of T315I-BcrAbl transcripts with omacetaxine therapy(19, 20), although as might be expected cytogenetic responses were poorer in comparison to the efficacy of TKI in newly diagnosed patients(20, 21).

Omacetaxine is targeted to the A-site cleft of eukaryotic ribosomes(7). Following exposure to this translation elongation inhibitor, transient inhibition of protein synthesis of short-lived proteins, such as Mcl-1, leads to apoptosis. Leukaemia

cells, and specifically CML cells, may be more Mcl-1 dependent for survival and therefore sensitive to the activity of omacetaxine(9). We confirmed the down-regulation of Mcl-1 in K562 cells and moreover, primary CML $CD34^+$ cells in response to omacetaxine which precipitated caspase dependent apoptosis as demonstrated by PARP cleavage. Furthermore, omacetaxine induced apoptosis in primitive $CD34^+$ subsets ($CD34^+38^{lo}$), an effect not observed with TKI. In addition, primary CML cells were found to be more sensitive to omacetaxine than K562 blast crisis cells with a significantly lower IC50 in the former

Despite the evidence of efficacy from clinical trial and the known molecular mode of action of omacetaxine, it was only reported very recently(4) that the drug could eliminate >90% of leukaemia stem cells in murine models of $BcrAbl^+$ CML and B-ALL, whereas TKI (imatinib or dasatinib) achieved less than 25% kill. Interestingly, the kill was seen for both wild-type and T315I mutant-BcrAbl. This is a significant result which needs verification in primary material derived from CML patients as cellular context of BcrAbl expression (*de novo* expression in human stem cells versus retroviral transduction of bone marrow cells from 5-FU treated donor mice) may be significant given the survival mechanisms that may be invoked by stem cells programmed to stay alive.

In an *in vitro* drug combination study in CML cell lines(22) it had been suggested that cytosine arabinoside (Ara-C) or omacetaxine might act synergistically with imatinib in the case of resistance to imatinib. However, we(16) demonstrated at the stem cell level *in vitro*, that Ara-C was anti-proliferative in combination with imatinib resulting in accumulation of stem cells. In our current study we did not assay omacetaxine in combination in primary CML $CD34^+$, but uniquely demonstrated apoptosis in the primitive ($CD34^+38^{lo}$) leukaemic cell subpopulation. Thus the prevention of colony formation by omacetaxine observed by Tipping et al is most likely the result of CFC loss through death as opposed to cell cycle arrest. Of note, if omacetaxine was to be combined with imatinib then there is evidence to suggest that the sequence of exposure would

be critical. In one previous study antagonism was seen with the simultaneous combination(23) in clonogenic assays. However this could be reverted to a co-operative effect if omacetaxine was pulsed for 24h before imatinib. Indeed we have shown in a relatively less imatinib-sensitive cell line (KCL22)(24) to corroborate this latter finding suggesting that when introduced into the culture before imatinib, omacetaxine is indeed more lethal than the reverse sequence. As omacetaxine is likely to block accumulation of Bim which is critical to imatinib's induction of apoptosis(25), this may explain the antagonism seen with simultaneous addition of the two drugs(23). However the loss of the Bim negative regulator, Mcl-1, on omacetaxine treatment may sensitise the cells to the combined effect of both drugs. Both simultaneous and scheduled permutations of the combination showed parity in toxicity between CML and normal donor $CD34^+$ cells (Figure 5) at the concentrations selected to be tested. Thus, our results support the BcrAbl-independent stem cell directed effect of omacetaxine but predict toxicity *in vivo* owing to the non-selective killing of both normal and leukaemic cells which is unlikely to be abrogated through scheduled dosing regimens.

Combinations aside, it is clear from this study that omacetaxine has activity at the primary CML leukaemia stem/progenitor cell level *in vitro*. Samples were consecutive newly diagnosed cases of CML in chronic phase, in whom TKI-resistant mutations are extremely rare and never dominant in the stem cell compartment prior to evolution under TKI-selection pressure. This may mean that omacetaxine has a potential role in eradicating minimal residual disease achieved in response to TKI but maintained by persistent, as opposed, to overtly resistant leukaemia stem cells. The shortcoming for this agent is toxicity to normal stem cells which could be predicted from on-going clinical trials in which myelosuppression is a significant issue. Indeed *in vitro* toxicity of omacetaxine to Ph^- HSC is reported here. The paucity of results in normal donor stem cells is in keeping with the ethical concerns surrounding prolonging stem cell collections for the purposes of research from healthy individuals over and above the required

clinical dose, hence the decision to assay more readily available Ph^- non-CML $CD34^+$ cells from diseases in which the HSC is not implicated.

A number of reports have been made recently demonstrating stem cell activity of novel agents for use in CML (e.g. farnesyl transferase inhibitors (e.g. BMS-214662)(15), histone deacetylase inhibitors (HDACi e.g. LBH589) (26), autophagy inhibitors (e.g. chloroquine)(27), proteasome inhibitors (e.g. bortezomib)(28)). Clearly there is considerable interest in the field to uncover a suitable partner to kinase inhibition to think in terms of cure for CML. Omacetaxine however, is the most advanced in its clinical development. Omacetaxine effectively induces apoptosis in primary CML stem cells through down regulation of Mcl-1 and independent of BcrAbl. This mechanism of action could explain at least some of its clinical activity and allow omacetaxine to be considered as an option in the management of minimal residual disease in CML.

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Conflict of Interest

The authors declare that E Allan, Dr Jørgensen and Prof Holyoake received research support from ChemGenex Pharmaceuticals; Dr A Craig, an employee of ChemGenex Pharmaceuticals, originated and participated in the concept discussion that led to the experimental work.

Figure Legends

Figure 1

The inhibitory effect of omacetaxine on growth of K562, CML CD34$^+$ and non-CML CD34$^+$ *in vitro*. The IC_{50} values for omacetaxine, as indicated by the horizontal dotted lines on the line graphs, were determined by counting viable cells by trypan dye exclusion in the BcrAbl blast crisis cell line K562 (**a**), primary CML CD34$^+$ cells (**b**) and non-CML CD34$^+$ cells (**c**) at 24 hourly intervals over three days. Results represent the mean of 3 separate experiments (±SEM) using either the K562 cell line or CD34$^+$ cells from CML and non-CML patients (Ewing's Sarcoma and Multiple Myeloma). All counts were done in duplicate.

Figure 2

Determination of the minimum exposure time to omacetaxine required to inhibit cell growth and induce apoptosis in CML CD34$^+$ cells. CML CD34$^+$ cells were cultured with and without omacetaxine at 10nM (grey bars) or 100nM (white bars) in SFM+5GF for the time-points indicated, after which cells were washed free of drug, re-seeded in fresh SFM+5GF and counted at 72h. Total viable cells were determined as trypan blue cell counts multiplied by the percentage of cells which were negative for Annexin V/Viaprobe, and expressed as a percentage of the no drug control (NDC) (**a** & **c**). Percentage of cells undergoing apoptosis (sum of Annexin V$^+$ in lower right [LR] quadrant of FACS plot plus Annexin V/Viaprobe double positive in upper right [UR] quadrant) are also displayed for CML (**b**) and non-CML (Multiple Myeloma) (**d**). Results in (**a**) and (**b**) are the mean of 3 independent experiments using 3 individual CML patient samples (±SEM).

Figure 3

Effect of omacetaxine on dividing versus non-dividing cells. Representative histogram plots for CD34$^+$ cells from one CML patient (**a**) and one normal G-CSF mobilised donor (**b**) showing CFSE fluorescence in cells remaining at 72h after

treatment in the first 24h with (i) 5GF alone, (ii) 10nM omacetaxine or (iii) 100nM omacetaxine, and the overlay of all three plots in panel (iv). Dot plots below each histogram illustrate CFSE (x-axis) with $CD34^+$ (y-axis) expression in the same samples. The viable cells that are undivided ($CD34^+CFSE^{max}$) remaining in each treatment is indicated by the box. (**c**) Total recovery of $CD34^+$ cells in all divisions with respect to input cells for (i) CML (n=4) and (ii) normal (n=1) $CD34^+$ cells treated with omacetaxine for 24h and analysed by flow cytometry at 72h. Data are expressed as a percentage of NDC. Omacetaxine treatment significantly reduced the total $CD34^+$ cells recovered in a dose dependent manner with respect to the NDC (*p<0.05, ***p<0.001).(**d**) The fold change in recovery of undivided ($CD34^+CFSE^{max}$) cells after 24h treatment with omacetaxine was calculated. The calculated percentage recovery in the NDC was normalised to 1 arbitrary unit and each drug treatment scaled accordingly to give fold-change in (i) CML (presented as mean ± SEM) and (ii) normal $CD34^+$ cells treated with omacetaxine. There was no significant increase in the number of CML $CD34^+$ cells recovered in the undivided fraction following omacetaxine treatment of any concentration relative to input.

Figure 4

The clonogenic and stem cell capacity of cells remaining after omacetaxine treatment

(**a**) CFC: $CD34^+$ cells were cultured ± omacetaxine in SFM+5GF as indicated after which time cells were washed free of drug and added to semi-solid culture media to assess colony forming capacity as described in the Materials and methods. Colonies were counted and are expressed as a percentage of the NDC for (i) CML (n=4), (ii) non-CML (n=2: Multiple Myeloma and Mantle Cell Lymphoma) and (iii) normal G-CSF mobilised donor (n=1). Omacetaxine significantly reduced the colony forming potential of CML and non-CML stem cells, in a dose and time-dependent manner with respect to the NDC (***p< 0.001) and also reduced colony formation in normal stem cells. (**b**) LTC-IC: $CD34^+$ cells remaining after omacetaxine treatment as in (**a**) were cultured for 5

weeks on stroma then plated out in CFC as described in the Materials and methods. Total colonies were counted for (i) CML (n=2), (ii) non-CML (n=2), and (iii) normal $CD34^+$ (n=1).

Figure 5

Effect of scheduled combination of omacetaxine with imatinib in Ph^+ cell lines and primary CML $CD34^+$ cells

(a) The number of viable KCL22 cells remaining after culture for a total of 72h with omacetaxine (20nM) ± imatinib (0.5μM) was determined by trypan blue staining (i) KCL22 cells were significantly more sensitive to treatment with omacetaxine than to imatinib ($p<0.05$). All combinations were better than imatinib alone. When omacetaxine was administered before imatinib, cell number was significantly reduced with respect to treatment with omacetaxine alone ($p<0.05$). (ii) The percentage of cells undergoing apoptosis is also displayed for the various treatment arms. Results are the mean of 3 independent experiments (±SEM).

(b) CML $CD34^+$ cells were treated with omacetaxine (10nM) ± imatinib (5μM) or in combination as illustrated. (i) All of the combinations tested were significantly more toxic than treatment with either imatinib ($p<0.001$) or omacetaxine alone ($p<0.05$). (ii) Treated cells were stained with Annexin V to measure apoptosis or (iii) washed free of drug and added to semi-solid culture media for measurement of colony formation as described in the Materials and methods. Colonies are expressed as a percentage of the NDC. Omacetaxine + imatinib was equally effective whether administered simultaneously or with omacetaxine introduced 24h before imatinib for 48h. Results are the mean of 3 independent experiments using 3 individual CML patient samples (±SEM) except for the CFC assay where n=2.

(c) Normal donor $CD34^+$ cells (n=1) were cultured and treated with omacetaxine and/or imatinib as for CML $CD34^+$ cells. (i) Cell counts (ii) apoptosis and (iii) colony forming potential of cells remaining after treatment showed that normal $CD34^+$ cells were as sensitive to treatment with omacetaxine alone or in combination with imatinib as CML $CD34^+$ for all of the treatment arms tested.

Figure 6

Apoptotic protein expression after 24h omacetaxine treatment.

(**a**) Exposure to omacetaxine for 24h induced apoptotic signalling in (i) K562 cells and (ii) CML $CD34^+$ cells as shown by cleavage of PARP and reduction in Mcl-1 protein levels in a dose dependent manner. Levels of anti-apoptotic protein Bcl-2 remained unchanged following omacetaxine treatment of CML stem cells but were absent in the CML cell line K562. Expression levels were normalised against GAPDH loading control by densitometric analysis. (**b**) 24h treatment with omacetaxine did not change expression of HSP90, BcrAbl or Bim proteins in (i) K562 or (ii) two CML $CD34^+$ samples.

Figure 7

Apoptotic protein expression in primary CML $CD34^+$ subpopulations after omacetaxine treatment

(**a**)(i) Measurement of apoptosis by Annexin V/Viaprobe staining in sorted cell fractions ($CD34^{lo}38^+$ (white bars), $CD34^+38^+$ (grey bars) and more primitive $CD34^+38^{lo}$ (black bars)) after 24h omacetaxine uptake expressed as the total percentage of Annexin V^+ cells. (**a**)(ii) Viable cells remaining after 24h omacetaxine treatment of sorted CML cell fractions were calculated as the product of the percentage Annexin V^+ cells times the live (trypan blue negative) count and expressed as a percentage of the NDC. (**b**) Analysis of protein lysates from sorted (i) $CD34^+38^+$ and more primitive (ii) $CD34^+38^{lo}$ CML cell populations treated with omacetaxine at indicated time-points demonstrated PARP cleavage and reduced expression of Mcl-1 protein in both cell populations after 24h. GAPDH was used a loading control for all gels [(i) Mcl1:GAPDH ratio by densitometry was 0.881 and 0.248 for 0 and 24h, respectively; (ii) Mcl1:GAPDH was 0.528 and 0.272 at 0 and 24h, respectively] .

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

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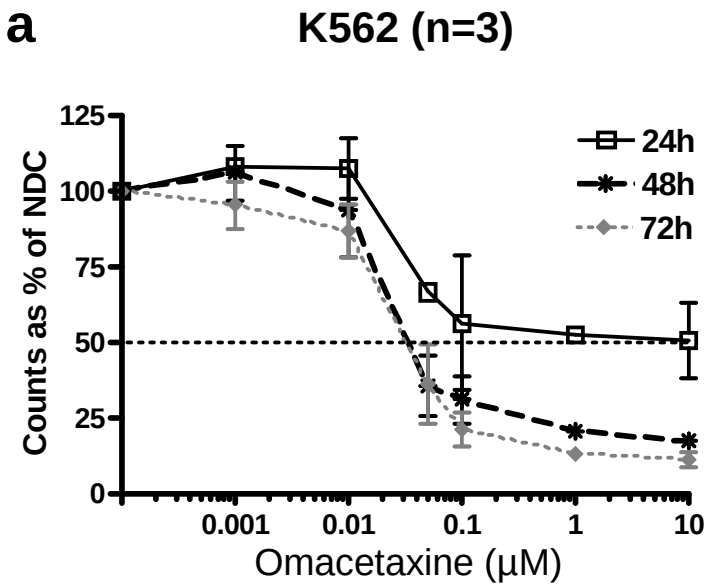
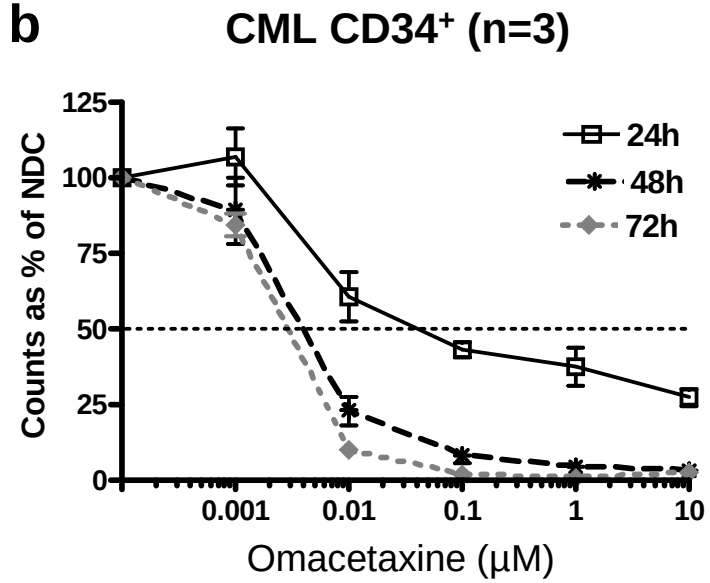
Figure 1



IC50 (nM)	24h	48h	72h
K562	>1000	34	32
CML	40	4	2.8
Non-CML	20	4.5	3.5



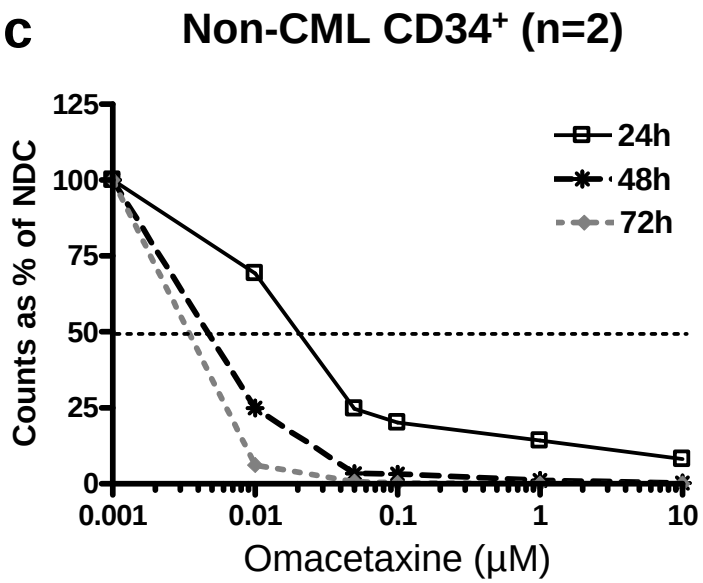


Figure 2

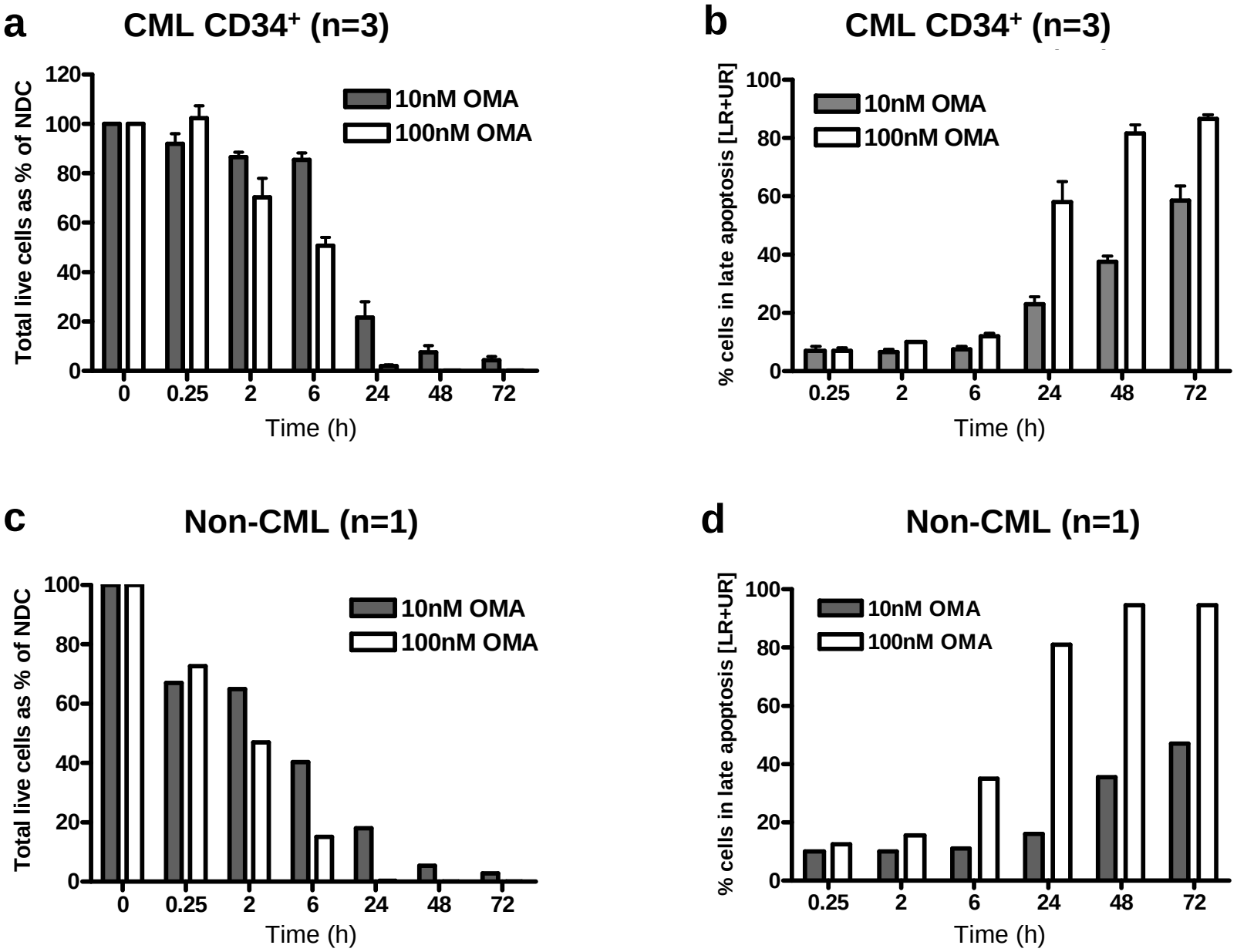

a
CML CD34+ (n=3)
Total live cells as % of NDC
120
100
80
60
40
20
0
10nM OMA
100nM OMA
0
0.25
2
6
24
48
72
Time (h)
b
CML CD34+ (n=3)
% cells in late apoptosis [LR+UR]
100
80
60
40
20
0
10nM OMA
100nM OMA
0.25
2
6
24
48
72
Time (h)
c
Non-CML (n=1)
Total live cells as % of NDC
100
80
60
40
20
0
10nM OMA
100nM OMA
0
0.25
2
6
24
48
72
Time (h)
d
Non-CML (n=1)
% cells in late apoptosis [LR+UR]
100
80
60
40
20
0
10nM OMA
100nM OMA
0.25
2
6
24
48
72
Time (h)

Figure 3

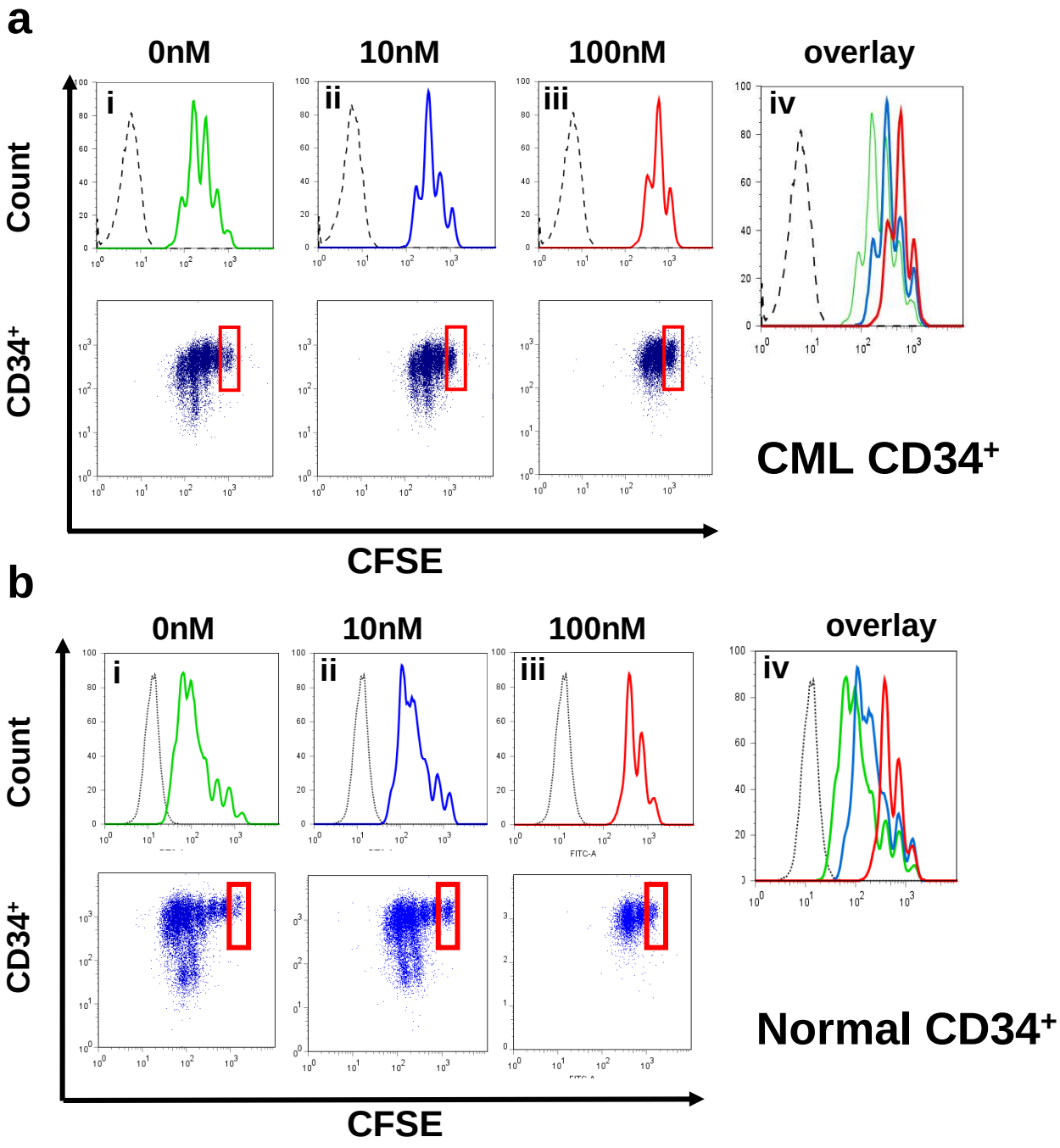
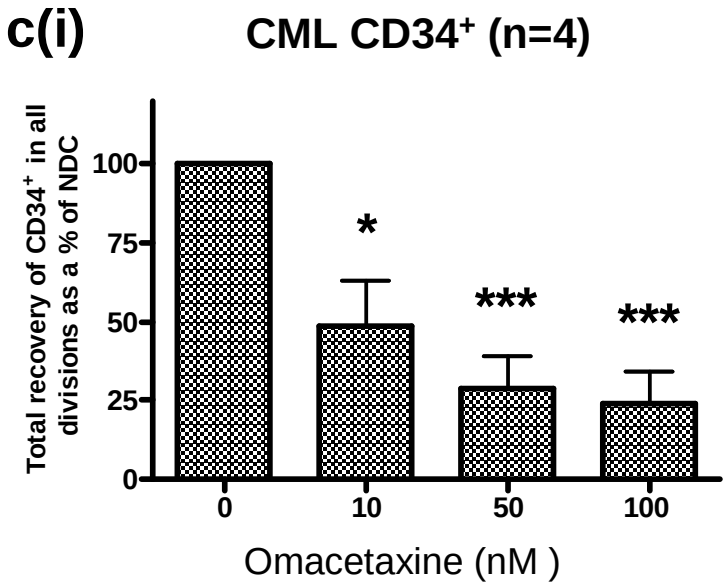
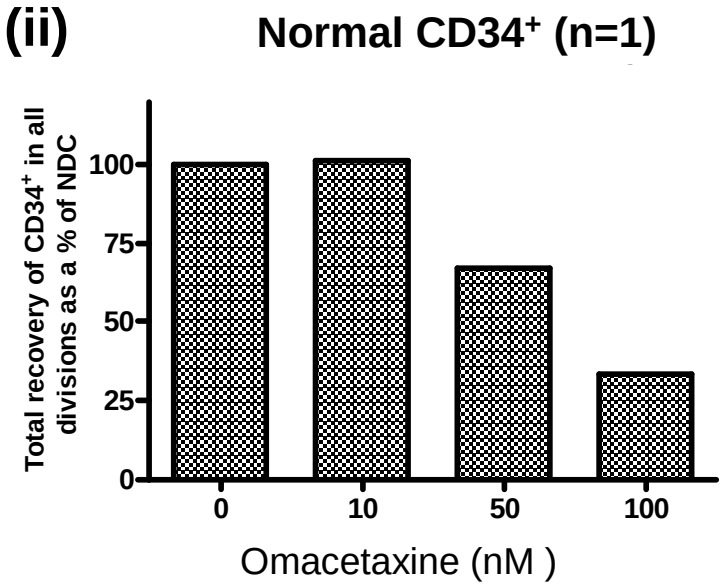
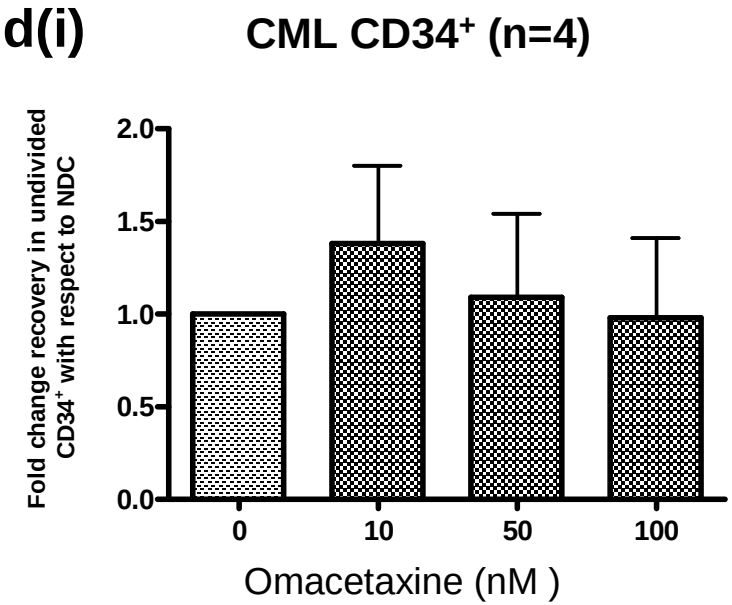

a
0nM
10nM
100nM
overlay
i
ii
iii
iv
Count
CD34+
CFSE
CML CD34+
b
0nM
10nM
100nM
overlay
i
ii
iii
iv
Count
CD34+
CFSE
Normal CD34+

Figure 3


c(i)
CML CD34+ (n=4)
Total recovery of CD34+ in all divisions as a % of NDC
100
75
50
25
0
*
***
***
0
10
50
100
Omacetaxine (nM)


(ii)
Normal CD34+ (n=1)
Total recovery of CD34+ in all divisions as a % of NDC
100
75
50
25
0
0
10
50
100
Omacetaxine (nM)


d(i)
CML CD34+ (n=4)
Fold change recovery in undivided CD34+ with respect to NDC
2.0
1.5
1.0
0.5
0.0
0
10
50
100
Omacetaxine (nM)

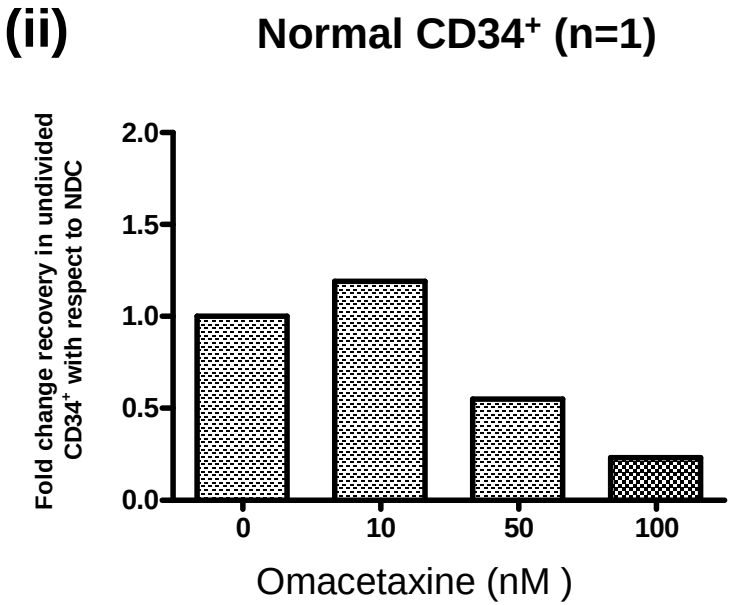
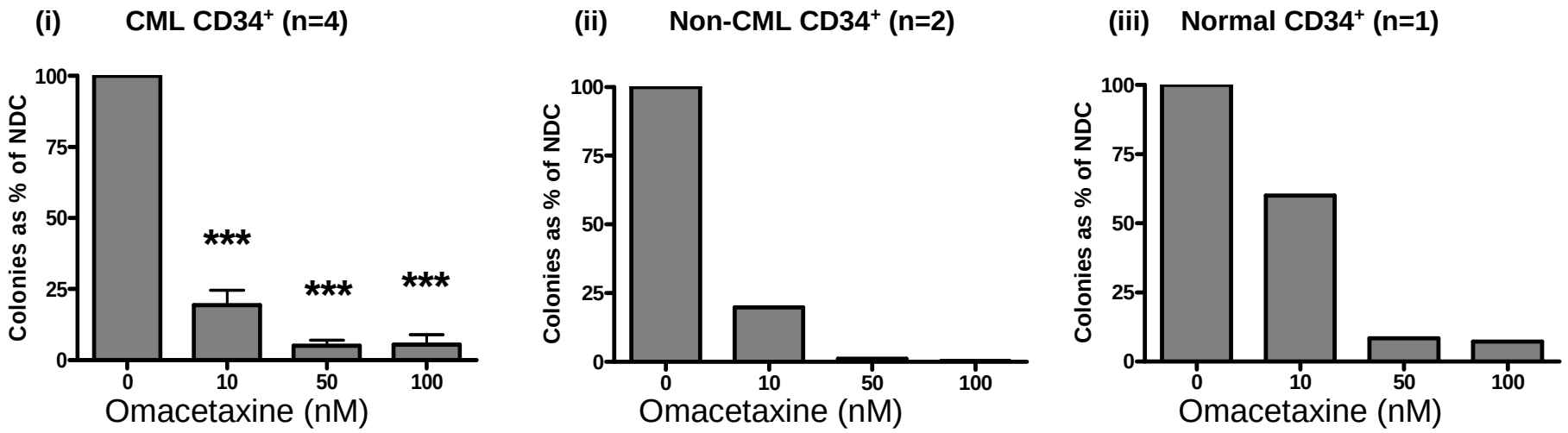

(ii)
Normal CD34+ (n=1)
Fold change recovery in undivided CD34+ with respect to NDC
2.0
1.5
1.0
0.5
0.0
0
10
50
100
Omacetaxine (nM)

Figure 4

a CFC



b LTC-IC

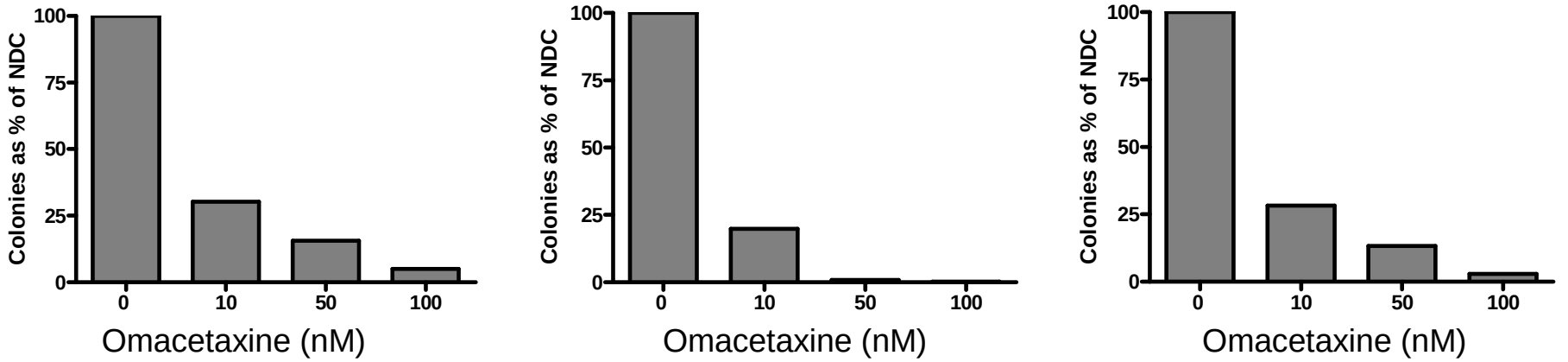
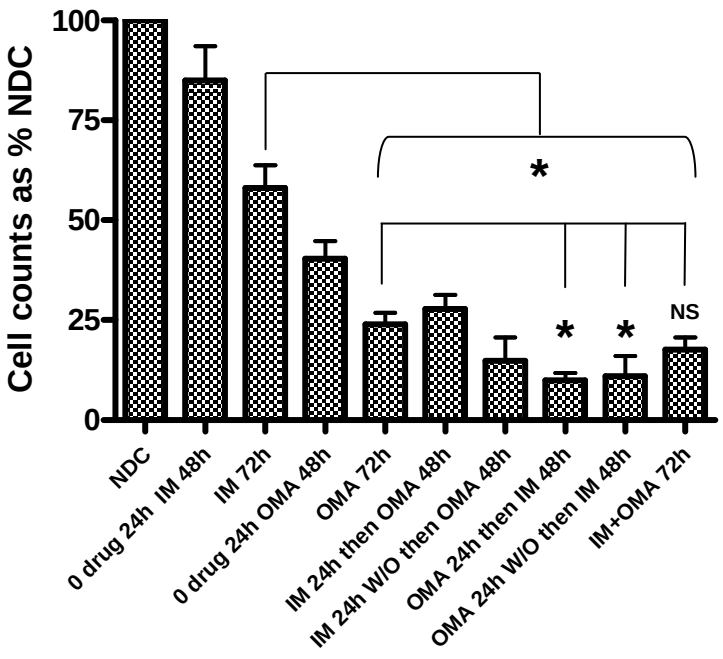


Figure 5

KCL22

a (i) Counts



(ii) Apoptosis

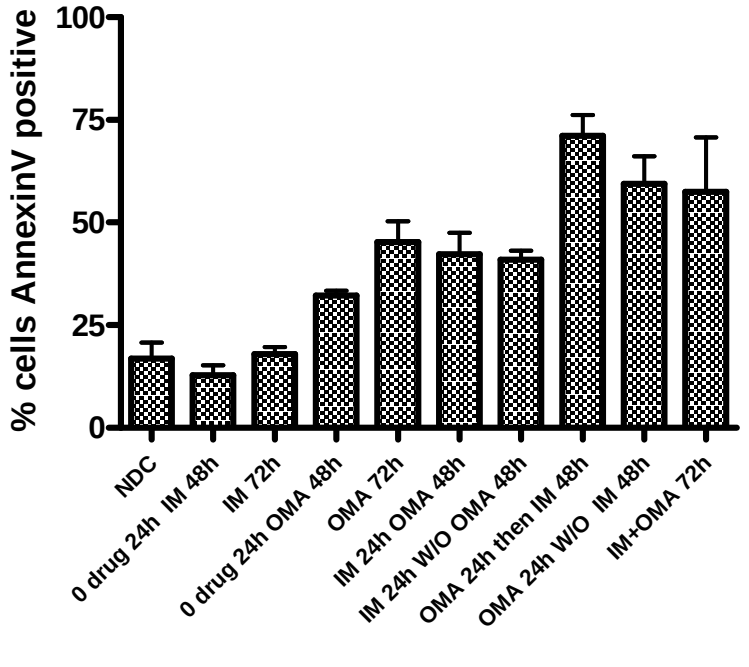
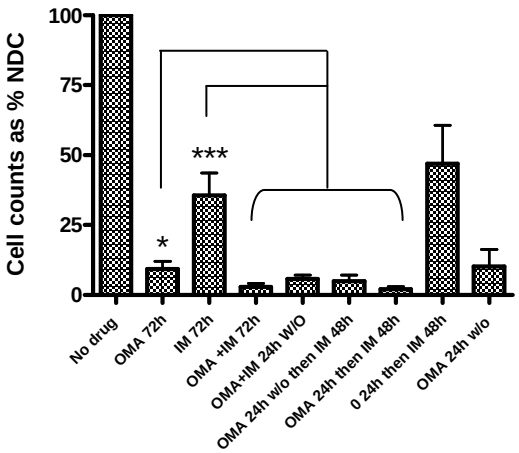


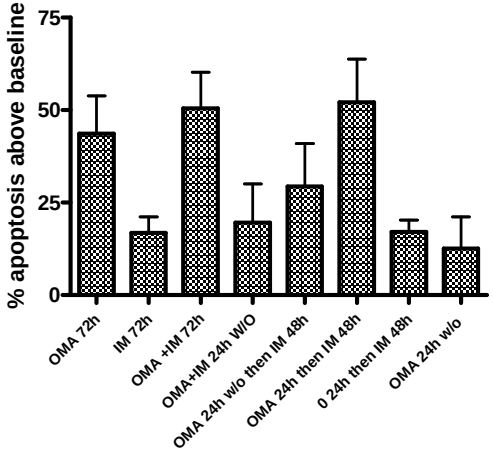
Figure 5

CML CD34+

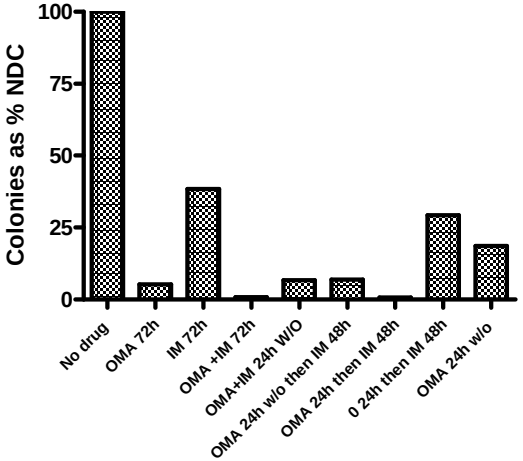
b (i)



(ii)

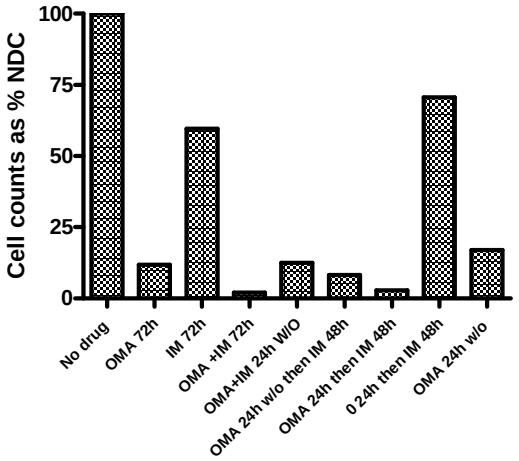


(iii)



Normal CD34+

c (i)



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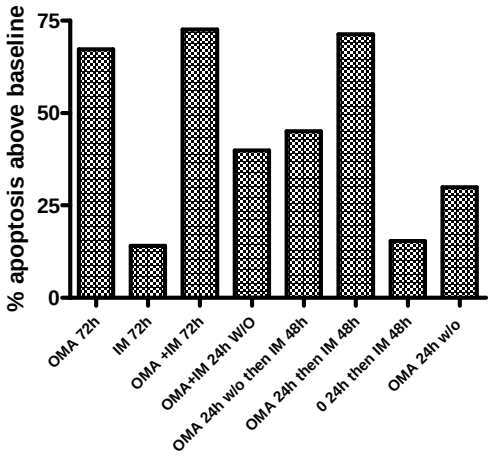


(iii)

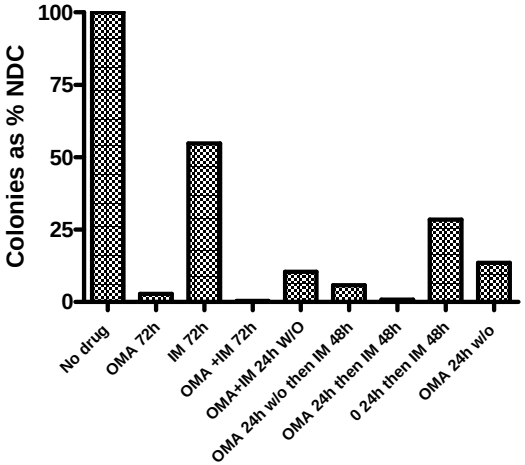
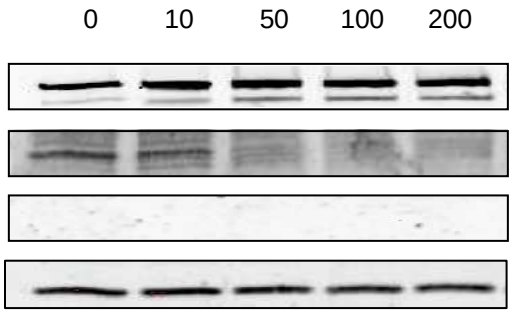


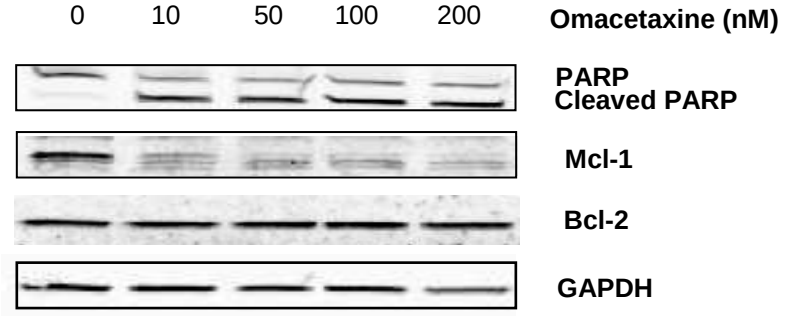
Figure 6

a (i)



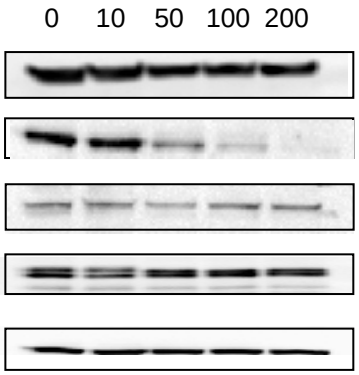
K562

(ii)



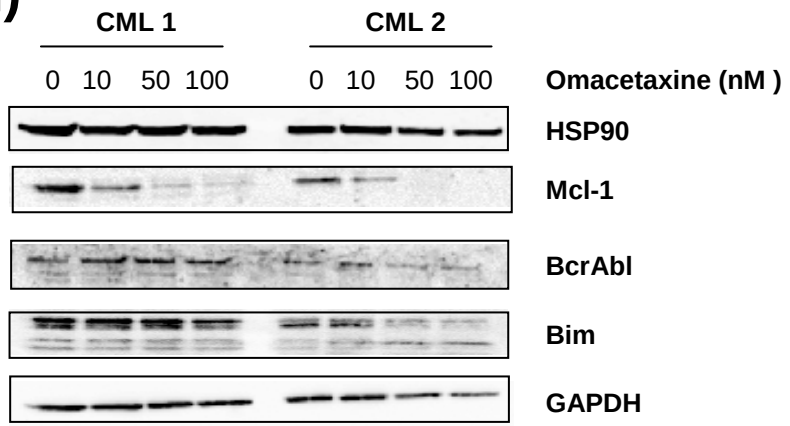
CML CD34^{+}

b (i)



K562

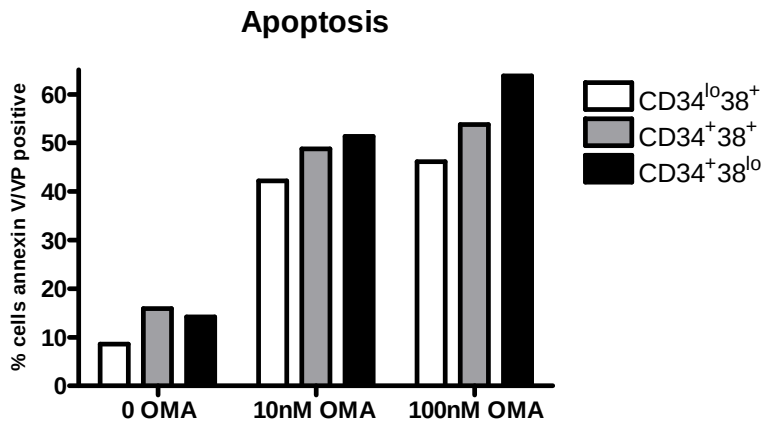
(ii)



CML CD34^{+}

Figure 7

a (i)



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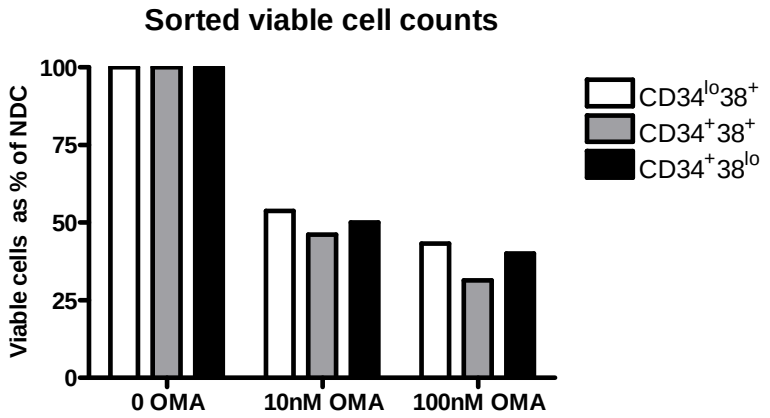
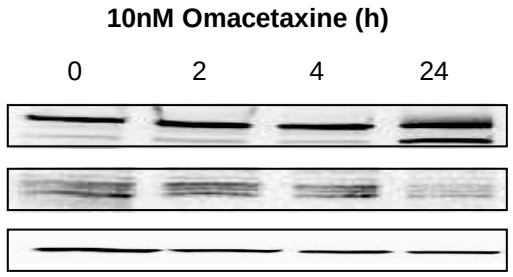
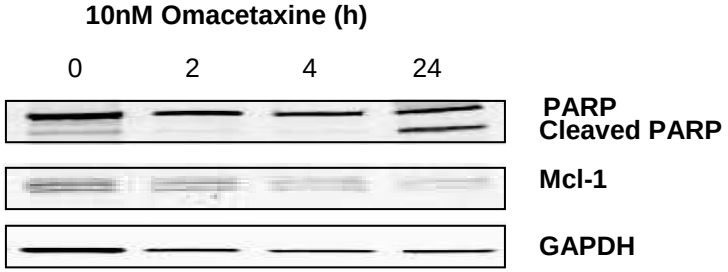


b (i)



$CD34^{+}38^{+}$

(ii)



$CD34^{+}38^{lo}$