BMS-214662 potently induces apoptosis of Chronic Myeloid Leukemia stem and progenitor cells and synergises with tyrosine kinase inhibitors

Running title: BMS-214662 induces apoptosis of CML stem cells

Authors:
Mhairi Copland\textsuperscript{1}, Francesca Pellicano\textsuperscript{1}, Linda Richmond\textsuperscript{2}, Elaine K. Allan\textsuperscript{1}, Ashley Hamilton\textsuperscript{1}, Francis Y. Lee\textsuperscript{3}, Roberto Weinmann\textsuperscript{3}, and Tessa L. Holyoake\textsuperscript{1}

\textsuperscript{1}Section of Experimental Haematology and Haemopoietic Stem Cells, Division of Cancer Sciences and Molecular Pathology, University of Glasgow, Glasgow, UK, \textsuperscript{2}Department of Haematology, Glasgow Royal Infirmary, Glasgow, UK, \textsuperscript{3}Bristol-Myers Squibb Oncology, Princeton, NJ 08543, USA

Corresponding Author:
Professor Tessa L Holyoake, Section of Experimental Haematology, Division of Cancer Sciences and Molecular Pathology, 3\textsuperscript{rd} Floor, Queen Elizabeth Building, Glasgow Royal Infirmary, 10 Alexandra Parade, Glasgow G31 2ER, UK. Tel: 0141 211 1202/4676 Fax: 0141 211 0414 E-mail: tlh1g@clinmed.gla.ac.uk
Abstract

Chronic myeloid leukemia (CML), a hematopoietic stem cell disorder, cannot be eradicated by conventional chemotherapy or the tyrosine kinase inhibitor (TKI) imatinib mesylate (IM). To target CML stem/progenitor cells we investigated BMS-214662, a cytotoxic farnesyltransferase inhibitor, previously reported to kill non-proliferating tumor cells. IM or dasatinib alone reversibly arrested proliferation of CML stem/progenitor cells without inducing apoptosis. In contrast, BMS-214662, alone or in combination with IM or dasatinib, potently induced apoptosis of both proliferating and quiescent CML stem/progenitor cells with <1% recovery of Philadelphia positive LTC-IC. Normal stem/progenitor cells were relatively spared by BMS-214662, suggesting selectivity for leukemic stem/progenitor cells. The ability to induce selective apoptosis of leukemic stem/progenitor cells was unique to BMS-214662 and not seen with a structurally similar agent BMS-225975. BMS-214662 was cytotoxic against CML blast crisis stem/progenitor cells, particularly in combination with a TKI and equally effective in cell lines harbouring wild-type versus mutant BCR-ABL, including the T315I mutation. This is the first report of an agent with activity in resistant and blast crisis CML that selectively kills CML stem/progenitor cells through apoptosis and offers potential for eradication of chronic phase CML.
Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder originating in a hematopoietic stem cell. It is characterised by the Philadelphia (Ph) chromosome\(^1\), arising from a reciprocal translocation between the long arms of chromosomes 9 and 22, resulting in fusion of the $BCR$ gene on chromosome 22 with the $ABL$ oncogene on chromosome 9, with expression of its fusion gene product, BCR-ABL, a constitutively active tyrosine kinase\(^2\). The deregulated BCR-ABL tyrosine kinase activity is essential for its transforming ability\(^3\), resulting in phosphorylation of cellular substrates and activation of signal transduction pathways including the RAS-ERK cascade, JAK-STAT, PI3-kinase, c-Myc, c-CBL and CrKL, affecting cell growth, stromal interaction and apoptosis\(^4\)\(^-\)\(^6\).

Imatinib mesylate (IM; Novartis Pharma) is a tyrosine kinase inhibitor (TKI) active against ABL, c-KIT and PDGFR, acting by competitive inhibition of ATP binding to the tyrosine kinase\(^7\). Despite an impressive rate of durable complete cytogenetic response in chronic phase CML patients treated with IM\(^8\), only a minority of patients achieve complete molecular remission\(^9\). At least two mechanisms of resistance appear to account for residual disease in these patients; the innate insensitivity of primitive quiescent CML stem/progenitor cells, which greatly over-express BCR-ABL, to IM\(^10\)\(^,\)\(^11\) and BCR-ABL kinase mutations\(^12\) which are present prior to IM therapy in a sub-group of patients and result in IM resistance\(^13\)\(^,\)\(^14\). However, there is currently no direct evidence that these resistance mechanisms, observed in vitro, are responsible for residual disease in IM-treated patients.
Two main strategies to overcome IM resistance have emerged. These are the development of second generation TKIs \(^{15,16}\) and the use of IM in drug combinations \(^{17}\). Dasatinib (Sprycel\textsuperscript{TM}, formerly BMS-354825; Bristol-Myers Squibb) is an oral, multi-targeted inhibitor of BCR-ABL and SRC kinases, with greatly improved potency against wild-type BCR-ABL, capable of binding all known BCR-ABL kinase mutants resistant to IM except T315I \(^{15}\). In our previous work, although dasatinib induced durable inhibition of BCR-ABL in primitive progenitor cells from CML patients as compared to either IM or nilotinib which were ineffective \(^{11,18}\), none of these TKIs targeted the most primitive, quiescent CML stem/progenitor cells. Of a wide range of rational drug combinations \(^{17}\), the only agent to synergise with IM against these cells was the cytostatic farnesyltransferase inhibitor (FTI) lonafarnib (Schering-Plough) \(^{17}\).

FTIs inhibit oncogenic RAS and have entered clinical trials in solid tumors and acute leukemias \(^{19-21}\). However, the observed anti-tumor effects of FTIs are not solely due to RAS inhibition \(^{22,23}\); they may also act by inhibiting farnesylation of other proteins \(^{24,25}\). BMS-214662 is a cytotoxic FTI \(^{26}\) that produces potent tumor regression and curative responses in human tumor xenografts and transgenic tumor models and differs from other, cytostatic FTIs, including lonafarnib and tipifarnib, which have non-curative activity \(^{22,27}\). In addition, BMS-214662 has been shown to preferentially kill non-proliferating cells \(^{28}\) and has anti-leukemic activity in acute myeloid leukemia (AML) \(^{20}\).
We hypothesised that BMS-214662 might therefore target quiescent CML stem/progenitor cells and synergise with TKIs which induce potent antiproliferative effects on CML stem/progenitor cells. We show that BMS-214662 selectively induces apoptosis of CML stem/progenitor cells, both in vitro and in vivo, and synergises with IM or dasatinib to kill stem/progenitor cells reversibly arrested in G1.

Materials and Methods

Reagents

BMS-214662, BMS-225975 and dasatinib were obtained from Bristol-Myers Squibb. Stock solutions of 10mg/mL in dimethyl sulphoxide (DMSO; Sigma-Aldrich Company Ltd) were stored at -20°C. IM was obtained from Novartis Pharma. A 100mM stock solution in sterile distilled water was stored at 4°C. Dilutions of these drugs in PBS were freshly prepared for each experiment.

Patient samples and isolation of CD34\(^+\) and CD34\(^+\)38\(^-\) cells

Fresh leukapheresis or peripheral blood samples were obtained with written informed consent and approval off North Glasgow University Hospital Division of NHS Greater Glasgo Institution Review Board from patients with newly diagnosed chronic phase CML (n=11), blast crisis CML (n=4) and normal donors of peripheral blood stem cells (n=6). Samples were enriched for CD34\(^+\) cells using CliniMACS (Miltenyi Biotec Ltd) according to the manufacturer’s instructions and cryopreserved in 10% (v/v) DMSO in ALBA (4% [w/v] Human Albumin Solution, Scottish National Blood Transfusion Service). CD34\(^+\) CML and normal cells were simultaneously stained with
CD34-APC and CD38-FITC (BD Biosciences) and sorted to isolate the CD34^+38^- sub-population using a FACSAria (BD Biosciences).

**Primary cell culture**

CD34^+ cells were cultured in serum free medium (SFM) consisting of Iscove’s Modified Dulbecco’s Medium (IMDM, Sigma-Aldrich) containing a serum substitute (bovine serum albumin [BSA], insulin, transferrin [BIT]; StemCell Technologies), 0.1μM 2-mercaptoethanol (Sigma-Aldrich), ± a high concentration five growth factor (5GF) cocktail comprising 100ng/mL Flt3-ligand, 100ng/mL stem cell factor, and 20ng/mL each of interleukin (IL)-3, IL-6 (all from StemCell Technologies) and granulocyte-colony stimulating factor (G-CSF; Chugai Pharma Europe). Drug synergy for BMS-214662 with IM or dasatinib was assessed after 48 hours culture using total viable cell counts and the median-effect method of Chou and Talaleý and analysed using CalcuSyn software (Biosoft).

**Tracking of cell division using carboxyfluorescein diacetate succinimidyl diester (CFSE) staining**

CD34^+ cells were stained with 1μM CFSE (Molecular Probes) as described previously. CFSE is an intracellular fluorescent stain, the intensity of which halves with each cell division. The CFSE^+ CD34^+ cells were cultured in the presence of different drug combinations. Total cell viability was assessed using trypan blue dye exclusion. Cells cultured in Colcemid® (Invitrogen) 100ng/mL, which arrests cell cycle progression, were used to establish the CFSE\textsuperscript{max} quiescent cell population at all time points studied. Flow cytometric
analysis was performed using the FACSCaliber (BD Biosciences). CFSE\textsuperscript{+} cells from each test condition were stained with anti-CD34-PE (BD Pharmingen) and propidium iodide (PI; Sigma-Aldrich), which allows exclusion of dead cells. To assess the efficacy of each drug alone or in combination against primitive CML progenitor cells, the number of quiescent cells remaining at each time point was determined by measuring the percentage recovery of the starting number of viable (PI\textsuperscript{-}) CD34\textsuperscript{+} cells in the CFSE\textsuperscript{max} quiescent sub-population as described previously \textsuperscript{10}.

**Long-term culture-initiating cell assay (LTC-IC)**

After culture for 72 hours in the presence of different drug treatments, CD34\textsuperscript{+} cells were set up in LTC-IC assays as previously described \textsuperscript{33}. M2-10B4 cells and S1/S1 fibroblasts were established as feeder layers and then irradiated at 80Gy. The cells remaining after culture were washed three times to remove all drug and then plated in duplicate on the irradiated feeder layers in Myelocult medium (StemCell Technologies). Cultures were maintained for 5 weeks with weekly half medium changes. After 5 weeks, cell counts were performed and 2.5x10\textsuperscript{4} cells were transferred to colony forming cell (CFC) assays and maintained in culture for a further 2 weeks in Methocult medium (StemCell Technologies) before the colonies were counted.

**Fluorescence in situ hybridization (FISH)**

FISH was performed with the LS1 BCR-ABL Dual Colour, Dual Fusion translocation probe according to the manufacturer’s instructions (Abbott
Diagnostics) to determine the percentage of Ph^+ cells before and after drug treatment

**Assessment of apoptosis using caspase-3, TUNEL and TMRE assays**

To assess apoptosis, after 24, 48, 72 and 96 hours in each drug condition, an aliquot (2-3x10^5) of cells was removed for assessment of caspase-3 activity as described in 18. Cells were cultured in SFM ± 5GF as indicated. For the TUNEL assay, after 24 hours treatment with BMS-214662, 50μl of reaction mixture (Cell Death Detection Kit, Roche) was added and the cells incubated at room temperature for 60 minutes. The cells were then washed twice in PBS and analysed by FACS. Loss of membrane potential was measured after 24 hours drug treatment. The cells were incubated with 50μl of TMRE (Molecular Probes) for 15 minutes and then analysed by FACS.

**Culture of Ba/F3 cell lines and cell proliferation assays**

Parental Ba/F3 cells and Ba/F3 cells containing wild-type (WT) or kinase domain mutated BCR-ABL were maintained in RPMI (Sigma-Aldrich) containing 2% FCS, 1% glutamine (100mM), and 1% penicillin-streptomycin (100mM; all Invitrogen), and supplemented with 10% conditioned medium from WEHI-3B cells. Five replicates of 2x10^4 Ba/F3 cells containing wild type or kinase domain mutated BCR-ABL were set up for each concentration of BMS-214662 and incubated for 48 hours. After 42 hours culture, 0.2μCi \(^3\)H-thymidine (Amersham Pharmacia Biotech Ltd) was added to each well. The cells were then harvested for beta counting using the MicroBeta TriLux (Perkin Elmer) according to the manufacturer’s instructions.
Western blotting

2.5 x 10^6 cells/well were plated in 6-well plates. After 16 and 72 hours drug treatment, the cells were lysed in Laemmli 2X sample buffer (Bio-Rad) and analysed on SDS-PAGE. The proteins transferred to Hybond ECL™ nitrocellulose membranes (Amersham) were blocked with 10% BSA in TBS-T (20mM Tris, pH7.6, 13.7mM NaCl, 0.1% Tween 20) for 2 hours. Incubation at 1:1000 dilution of primary antibodies (anti-rabbit actin, Bcl-2, Mcl-1, and Bim, [all Cell Signalling]) was carried out at 4°C overnight, and with secondary antibody (1:3000 anti-rabbit HRP, Cell Signalling) for 1 hour at room temperature and then developed with the ECL plus™ kit according to the manufacturers’ instructions.

Statistical analysis

Statistical analyses were performed using the Student’s T-test. A level of P≤0.05 was taken to be statistically significant.

Results

BMS-214662 is more effective against Ph+ leukemic cells as compared to normal cells, including primary CD34+ cells and synergises with TKIs

Primary human CD34+ normal and CML cells, parental Ba/F3 and Ba/F3 cells expressing WT p210BCR-ABL were cultured for 48 hours with increasing concentrations of BMS-214662. The IC_{50} for BMS-214662 against CD34+ CML cells was approximately 62.5nM (Figure 1A; assessed by total viable cell counts), which compared favourably with the IC_{50} against normal CD34+ cells
which was approaching 250nM (Figure 1A). BMS-214662 at a concentration of 62.5nM had minimal effect on normal CD34\(^+\) cells. In the murine Ba/F3 WT p210\(^{BCR-ABL}\) cell line, BMS-214662 had an IC\(_{50}\) of approximately 125nM (Figure 1B), whereas, in parental Ba/F3 cells, the IC\(_{50}\) was not reached at a concentration of 1000nM.

Synergy experiments were performed with BMS-214662 in combination with IM or dasatinib against CD34\(^+\) normal versus CML cells to determine whether there were synergistic or additive effects between BMS-214662 and the TKIs. CD34\(^+\) cells derived from CML patients and normal donors were treated with combinations of BMS-214662 and IM or dasatinib for 72 hours at the concentrations indicated in Supplementary Figure 1. When BMS-214662 was combined with the TKIs, in CD34\(^+\) CML cells, we observed an increased concentration-dependent lethality, indicating synergistic activity between BMS-214662 and TKIs. Figure 1C shows results with BMS-214662 62.5nM in combination with varying concentrations of either IM or dasatinib in both normal and CML CD34\(^+\) cells. The combination of BMS-214662 (62.5nM) with IM (2.5-20\(\mu\)M) had a minimal effect on normal CD34\(^+\) cells (survivals all >60%). Similar results were obtained with dasatinib (37.5-150nM). To confirm that BMS-214662 in combination with TKIs had a synergistic effect on CML CD34\(^+\) cells, the interaction between the drugs was analyzed using the CalcuSyn software program. Based on the Chou-Talalay method to calculate the Combination Index (CI) \(^{29}\), we generated an algebraic estimate and a conservative isobologram of varying concentrations BMS-214662 and IM at the ratio 40:1 (drugs concentrations at the IC\(_{50}\), IC\(_{75}\) and IC\(_{90}\); Figure 1D, E).
At doses ranging from 62.5 to 1000nM of BMS-214662 and from 2.5 to 40μM of IM, CI ranged from 0.8 to 0.2 (Figure 1D), indicating that this combination was highly synergistic. In the conservative isobologram, data points below the curve also indicated synergism (Figure 1E). Similar analyses were carried out for the combination of BMS-214662 and dasatinib, but synergism was only detected at higher drug concentrations. This may be because dasatinib is a stronger TKI than IM and, therefore, higher concentrations of BMS-214662 are required to show synergism.

BMS-214662 enhances the cytotoxic effect of IM or dasatinib against the total CD34+ CML cell population

To assess the cytotoxic effect of BMS-214662 alone and in combination with IM or dasatinib, primary CD34+ CML cells were cultured in SFM supplemented with 5GF as follows: no drug control; BMS-214662; IM; BMS-214662+IM; dasatinib and BMS-214662+dasatinib. The concentration of BMS-214662 (250nM) was chosen to be clinically achievable \(^{21}\), and the concentrations of IM (5μM) and dasatinib (150nM) have been used in previous in vitro studies employing a high concentration 5GF cocktail \(^{10, 11}\), and are also clinically achievable at higher drug dose levels \(^{34, 35}\). After 3 and 6 days there were significant reductions in total viable cells in the treatment arms compared to the no drug control (3 days P=0.04 [data not shown], and 6 days p=0.001, Figure 2A). Importantly, by 6 days the combinations of BMS-214662+IM and BMS-214662+dasatinib showed increased cytotoxicity over either IM or dasatinib alone (P=0.024 and P=0.034, respectively).
BMS-214662 is highly effective against primitive quiescent CML stem/progenitor cells, whereas IM or dasatinib induce cytostasis

CML stem/progenitor cells share properties with their normal counterparts being primitive, transplantable and quiescent \(^3,^31\). To follow these cells in culture we combined CD34 as a primitive marker, with CFSE to enable high resolution tracking of cell division \(^10,^30\). A high concentration 5GF cocktail was used to stimulate cell division \(^10\). Previous studies demonstrated that either IM or dasatinib led to increased numbers of undivided (CFSE\(^{\text{max}}\)) CD34\(^+\) CML cells remaining after culture \(^10,^11,^36\), in part due to failure to induce apoptosis and as a result of potent anti-proliferative effects leading to reversible G\(_1\) arrest. Here, BMS-214662 was assessed alone and in combination with IM or dasatinib. By 6 days, the IM and dasatinib arms showed significant accumulation of CFSE\(^{\text{max}}\) CD34\(^+\) CML cells over control (P=0.04 and P=0.023, respectively), whereas the arms containing BMS-214662 with either IM or dasatinib showed a significant reduction in these primitive cells to <50% of control (Figure 2B; P=0.023 and P=0.005 respectively). In addition, the reduction in CFSE\(^{\text{max}}\) CD34\(^+\) CML cells was significantly lower when either IM or dasatinib alone was compared to the combination with BMS-214662 (P=0.01 and P=0.043, respectively). There were no significant differences in residual CFSE\(^{\text{max}}\) CD34\(^+\) CML cells between the BMS-214662-containing arms (Figure 2B). IM and dasatinib exerted marked anti-proliferative effects as demonstrated in Figures 2C-E, whereas BMS-214662 had minimal anti-proliferative effect (Figures 2C-E) and did not overcome the anti-proliferative effect of either IM or dasatinib (Figure 2E), indicating that BMS-214662 is able to exert its cytotoxic effect on quiescent cells. Critically, these are the first
drug combinations we have tested, using the CFSE assay, which show a significant reduction in quiescent stem/progenitor cells.

**BMS-214662 selectively targets Ph+ LTC-IC**

LTC-IC is recognised as the most stringent assay to detect very primitive human hematopoietic stem cells *in vitro*. For successful read-out, stem cells must be fully functional, survive for 5 weeks in the presence of a stromal layer and then give rise to colonies after a further 2 weeks in CFC assay. We performed LTC-IC assays with both chronic phase CML and normal CD34+ cells after treatment for 72 hours under the same conditions as above. Compared to control, LTC-IC recoveries were increased in the IM and dasatinib arms (191 and 175% respectively; P=0.033; Figure 3A), indicating that these drugs exert a protective effect on CML stem/progenitor cells in these culture conditions via their anti-proliferative effects. It is possible that the marked anti-proliferative and hence protective effect of TKIs on LTC-IC in these experiments is related to the high growth factor concentrations used in the initial 72 hour culture period in the presence of drug. This differs from conditions used by other groups which have used low growth factor concentration culture conditions and a slightly different LTC-IC method. The use of high concentration growth factors means that, in the no drug control, a large number of stem/progenitor cells enter cell division and proceed towards terminal differentiation. However, in the TKI-containing arms, this proliferative effect is overcome, resulting in increased numbers of cells remaining capable of colony formation after LTC-IC.
The addition of BMS-214662 significantly reduced LTC-IC compared to either IM or dasatinib alone (P=0.032 and P=0.027, respectively). Furthermore, BMS-214662 alone significantly reduced LTC-IC compared to either IM or dasatinib alone (P=0.032 and P=0.028, respectively). All three BMS-214662 containing arms showed a virtual elimination of colonies to <1% of control (P=0.033), with no significant difference between the arms. For normal Ph- CD34+ cells (n=3) there was an effect against LTC-IC in the BMS-214662-containing arms compared to control that did not reach statistical significance after 3 independent experiments (Figure 3B; P=0.079-0.29, lack of significance thought to be related to small sample size). However, LTC-IC survival was significantly higher for normal compared to Ph+ CD34+ cells (P=0.001), with a >20 fold reduction in LTC-IC from Ph+ CD34+ cells compared to normal CD34+ cells. Some effect against normal LTC-IC is not unexpected as, in clinical trials, myelosuppression has been a recognised but manageable side effect of treatment with BMS-214662. These results confirm that BMS-214662 is targeting primitive CML stem/progenitor cells and provide further evidence of selectivity for CML over normal cells.

To further evaluate the Ph status of LTC-IC after treatment, FISH was performed at baseline and on the colonies produced following LTC-IC experiments. All samples were >90% Ph+ at baseline (Table I). However, after LTC-IC, only 1 of 3 patient samples (CML 166) remained Ph+ in residual colonies in the BMS-214662-containing arms. This case illustrates the 10-15% of chronic phase CML patients at diagnosis in whom LTC-IC are already exclusively Ph+ (i.e. no residual normal hematopoiesis). In the other samples
(CML 189 and 215), following treatment with BMS-214662, more than 90% of colonies were Ph−, indicating that these patients had Ph− stem/progenitor cell reserves which selectively survived exposure to BMS-214662 as compared to their Ph+ counterparts which were eradicated. This further illustrates the degree of Ph+ versus Ph− selectivity for BMS-214662.

**BMS-214662 is cytotoxic by inducing apoptosis in CD34+ CML stem/progenitor cells**

To determine the mechanism of CML stem/progenitor cell elimination under BMS-214662 treatment, we assessed active caspase-3, an irreversible apoptosis step, at 48, 72 and 96 hours culture of CD34+ CML cells. The greatest number of cells with caspase activation was seen at 72 hours in the BMS-214662- containing arms (BMS-214662 14.6%, BMS-214662+IM 18.4% and BMS-214662+dasatinib 12.4%) compared to control (5.5%), IM or dasatinib (both 7.9%; Figure 4A). At 48 and 72 hours there was an increase in caspase-3+ cells in the quiescent CFSEmax population. With IM or dasatinib alone, a modest increase in apoptosis was seen (10.9 and 12.2%, respectively) compared to control (8.3%); however, the apoptotic effect was significantly increased by the addition of BMS-214662 (Figure 4B) with 22.0, 30.8 and 24.0% apoptotic cells in the BMS-214662, BMS-214662+IM and BMS-214662+dasatinib arms, respectively (P=0.045 for BMS-214662-containing versus non-containing arms). To further confirm induction of apoptosis by BMS-214662 in CD34+ CML cells cultured in SFM only, TUNEL assay and assessment of TMRE were also performed. Exposure to 250nM
BMS-214662 for 24 hours doubled the amount of DNA fragmentation (TUNEL positive cells; Figure 4C) and decreased the membrane potential (Figure 4D).

To illustrate the effect of BMS-214662 on both mature and primitive CML progenitor cells, total CD34+ cells were sorted into CD34+38+ and the more primitive CD34+38− (<5% of total CD34+ cells; Figure 4E) fractions and were analysed for caspase-3 activity after culture in SFM only. BMS-214662 increased caspase-3 activity in both CD34+38+ and CD34+38− fractions (31.5% and 27.7%, respectively, after 48 hours of treatment), highlighting the activity of this drug in both quiescent and cycling cell populations. Interestingly, caspase-3 activity was increased earlier in the more mature CD34+38+ progenitors compared to the CD34+38− fraction (36.6% versus 7.2%, respectively, after 24 hours of treatment). In normal CD34+38− cells, after 48 hours drug exposure, there was only a modest increase in caspase-3 activity to 9.7% by 48 hours, (Figure 4F) indicating the specificity of the drug for leukemia stem/progenitor cells. Importantly, since CD34+38− cells are almost exclusively quiescent, these results highlight the effectiveness of BMS-214662 in the more quiescent leukemia stem/progenitor cell population, which has proved resistant to therapy in the past.

The effect of BMS-214662 on CML stem/progenitor cells is novel and does not occur with other FTIs

Our group has previously shown that the FTI lonafarnib did not significantly reduce survival of quiescent CML stem/progenitor cells. BMS-225975 is another FTI, which is structurally very similar to BMS-214662, the only
difference being π N methylation on the imidazole ring. These compounds have similar inhibitory effects on FT with IC₅₀s of 0.7nM and 0.8nM on the purified enzyme and in cells, for BMS-214662 and BMS-225975, respectively, but differ dramatically in their apoptotic and xenograft anti-tumor activity. To determine if the apoptotic effect of BMS-214662 in CML was due to more potent inhibition of FT in primary CML, we directly compared its activity with that of BMS-225975. The treatment conditions were: no drug control; BMS-214662; BMS-225975 250nM; dasatinib; BMS-214662+dasatinib; BMS-225975+dasatinib. By 6 days, BMS-214662 had significantly reduced the number of quiescent CFSEmax CD34+ CML cells compared to control (P=0.018) and to BMS-225975 containing arms (P=0.024). There was no significant difference between control and BMS-225975 arms (Figure 5A).

BMS-225975 did not increase caspase-3 activity in either the BMS-225975 or BMS-225975+dasatinib arms. To determine whether 5GF protected primitive CML cells from apoptosis in response to FTIs, CD34+ cells (Figure 5B) were cultured in the absence of growth factors. Upon BMS-214662 treatment, a 3-5-fold increase over control in caspase-3 activation was observed at 24 hours, similar to 5GF-containing cultures. Increased caspase-3 activation did not occur with BMS-225975.

Previous investigations of BMS-214662 in apoptosis induction in B-cell chronic lymphocytic leukemia and myeloma have identified inhibition of Mcl-1 and Bax or Bak activation. For CML cell lines and primary CD34+ cells Bcl-2 family member expression levels were unchanged after treatment with BMS-214662 (data not shown).
BMS-214662 is active in blast crisis CML when combined with a TKI and in cells expressing BCR-ABL kinase domain mutations

In preliminary experiments we next assessed BMS-214662 alone and in combination with IM or dasatinib in blast crisis CML. BMS-214662 alone showed a trend towards reducing total viable cells (Figure 6A) compared to no drug control. In combination with either IM or dasatinib it significantly reduced total viable cells compared to either agent alone (P=0.04 for both). Not all blast crisis samples assessed were CD34+, however, in those that were, BMS-214662 alone showed a trend towards reducing the number of CFSE$_{\text{max}}$ CD34+ CML cells (Figure 6B). Interestingly, BMS-214662 was much more effective against the CFSE$_{\text{max}}$ CD34+ CML population when combined with either IM or dasatinib, although results failed to reach statistical significance due to the small sample size. This suggests that although BMS-214662 alone may be sufficient to eliminate CML stem/progenitor cells in chronic phase CML, the combination of BMS-214662 with a TKI is likely to be superior in blast crisis CML.

None of the currently available BCR-ABL-specific TKIs inhibit the BCR-ABL kinase mutation T315I. Furthermore, CML stem/progenitor cells may express a variety of BCR-ABL mutations at low levels $^{42}$. We therefore determined the efficacy of BMS-214662 in Ba/F3 cells expressing p210 WT BCR-ABL and kinase mutations (WT BCR-ABL, T315I, M351T and H396P). BMS-214662 was equipotent against WT BCR-ABL and mutant BCR-ABL kinase expressing cells including T315I (Figure 6C).
Discussion

CML was the first malignancy recognised to be caused by a single genetic abnormality, leading to intensive investigation of its molecular pathogenesis. The concept that CML arises in a pluripotent stem cell was first developed in 1951 \(^4\) with later studies proving clonal expansion in multiple lineages, self-renewal and transplantation capability \(^5\). Our efforts to characterise this population have focused on identification of CML cells that are primitive, quiescent and remain cytokine non-responsive for several days in culture, thereby exhibiting critical stem cell characteristics \(^6\). The consistent presence of these candidate stem cells in chronic phase patients provides a possible explanation for the historic failure of intensive chemotherapy to eradicate CML and for the occasional late relapse post-transplant. CML is also an excellent paradigm for hematological malignancies and solid tumors in which cancer stem cells have been identified and isolated, including breast cancer and tumors of the nervous system \(^7\).

There have been major advances in the treatment of CML in recent years with the development of IM \(^7\), dasatinib \(^8\) and nilotinib \(^9\). The newer compounds target IM-resistant mutations and, in the case of dasatinib, show enhanced efficacy against primitive cells \(^10\). However, despite moderate inhibition of BCR-ABL, quiescent CML stem/progenitor cells remain insensitive to these compounds.
Here we showed that BMS-214662 was selectively cytotoxic by inducing apoptosis in progenitor (CD34+38−) and more primitive (CD34+38−) CML cells, including the quiescent fraction, in chronic phase CML. Synergism was demonstrated with TKIs, which represent the most likely combination agents for clinical trials with BMS-214662. Although BMS-214662 exhibited little or no anti-proliferative activity as a single agent, it was capable of killing cells arrested in a non-proliferating state by TKIs. This was best demonstrated in Figure 2E in which the BMS-214662 profile matched that of the PBS control across all divisions, whereas all treatment arms that included a TKI showed equivalent anti-proliferative effects. The effect of BMS-214662 was shown to include very primitive stem/progenitor cells, such as GF-resistant, quiescent, CD34+CFSEmax cells, and LTC-IC. BMS-214662 dramatically reduced survival and propagation (to <1%) of the key stem cell population that is maintained in long-term culture. BMS-214662 also demonstrated preferential cytotoxicity for leukemic stem/progenitor cells as evidenced by superior recovery of normal versus CML LTC-IC and enrichment of Ph− hematopoiesis during LTC-IC assay in 2 of 3 CML samples. In the third sample, in which colonies were BCR-ABL positive at the end of LTC-IC, the patient required growth factor support and a reduced dose of IM to manage the cytopenias resulting from IM therapy consistent with inadequate normal hematopoietic stem cell reserve.

These results demonstrate that BMS-214662 has novel and potent activity in eliminating quiescent CML stem/progenitor cells which separates it from cytostatic FTIs such as lonafarnib and BMS-225975. BMS-214662 was
equally effective at inducing apoptosis in the presence or absence of added GFs. The concentration of BMS-214662 used in these experiments (250nM) is clinically achievable. Our preliminary experiments show that, in vitro, intermittent exposure to BMS-214662, in combination with either IM or dasatinib, enhances the efficacy of these TKIs in CML (data not shown). Although BMS-214662 has yet to enter clinical trial in CML, phase 1 trials in AML have shown promising activity. In a future clinical trial under development with Bristol-Myers Squibb and the New Agents Committee Cancer Research UK, we propose that BMS-214662 be given intermittently in combination with continuous oral dasatinib. In clinical trials in advanced solid tumors, intermittent infusions of BMS-214662 have been successfully combined with cisplatin, carboplatin and paclitaxel.

Whereas BMS-214662 had single agent activity against chronic phase CML CD34+ populations, it appeared most effective when used in combination with a TKI for more advanced phase, as demonstrated by the blast crisis data. The activity of BMS-214662 in blast crisis CML and cell lines expressing BCR-ABL kinase mutations is also important. Patients with blast crisis CML initially respond to IM but then relapse. A proportion of these IM-resistant patients will respond to dasatinib or nilotinib but nearly all relapse within 6 months. Therefore, BMS-214662 in combination with a TKI may increase the number of patients who respond and the length of remission. For those patients with BCR-ABL kinase mutations, the majority will respond to either dasatinib or nilotinib, however, patients with the T315I mutation are resistant to these drugs. These patients may benefit from therapy with BMS-
214662, which appears equipotent in wild type and mutant BCR-ABL expressing cell lines, including T315I. Since BCR-ABL mutations have been detected in CD34+ cells from IM treated cases of CML it is reassuring to find BMS-214662 not only induces apoptosis of CML stem/progenitor cells but is equally effective in cells harboring mutations.

The effect of BMS-214662 on non-proliferating cells was first identified in solid tumors. However, despite extensive investigation, to date, the exact mode(s) of action of BMS-214662 has remained elusive. Recent in vitro studies of BMS-214662 in B-cell chronic lymphocytic leukemia and myeloma have identified inhibition of Mcl-1 and Bax or Bak activation in association with apoptosis, however this was not confirmed here for CML. What was very clear was that BMS-214662 caused cytotoxicity through apoptosis – this was confirmed using 3 alternative assays, expression of active caspase-3, TUNEL and TMRE staining.

The importance of cancer stem cells is a rapidly emerging area of research, and the ability of BMS-214662 to selectively target quiescent leukemia stem/progenitor cells is a very unusual and important property which is shared by very few anti-cancer agents. Studies are ongoing to determine if this property extends to other leukemias and malignancies in which quiescent stem/progenitor cells appear to sustain the disease.
Since BMS-214662 appears to induce selective apoptosis of leukemic stem/progenitor cells, it should prove a useful tool for developing novel approaches for targeting cancer stem cells and in future drug discovery.

Acknowledgements

We would like to thank Dr Elisabeth Buchdunger (Novartis Pharma, Basel, Switzerland) for providing imatinib and Professor Junia Melo (Hammersmith Hospital, London) and Dr Brian Druker (Portland Oregon) for kindly providing the Ba/F3 cell lines. We are grateful to Dr Graham Templeton for CD34+ cell selection and also Dr Heather Jorgensen for assistance with FACS sorting. We are indebted to normal donors and to CML patients and to the UK hematologists who provided leukapheresis samples. This work was supported by grants from the Medical Research Council, UK (G84/6317; M.C.), the Leukaemia Research Fund UK (03/20; A.H), the Leukaemia Research Trust for Scotland (M.C., F.P.), the Scottish National Blood Transfusion Service (E.K.A.) and the Richard Rockefeller Foundation. FYL and RW are employees of Bristol-Myers Squibb.

Author contributions

M.C., F.Y.L., R.W. and T.L.H. participated in designing the research; M.C., F.P., L.R., A.H. and E.K.A. performed the research; M.C. and F.P. analysed the data; M.C., F.P. and T.L.H. wrote the paper and all authors checked the final version of the manuscript.

Conflict of Interest Disclosure: F.Y.L and R.W are employees of Bristol-Myers Squibb. M.C. and T.L.H have undertaken consultancy work for Bristol-Myers Squibb and received honoraria. T.L.H. has received limited research funding from Bristol-Myers Squibb. All other authors declare no competing financial interests.
References


Figure Legends

Figure 1. (A) Concentration response curve for BMS-214662 against CD34+ CML (n=3) and normal cells (n=3). The IC_{50} was approximately 62.5nM in CD34+ CML cells and 250nM in normal CD34+ cells as assessed by total viable cell counts. (B) Concentration response curve for BMS-214662 against parental and wild-type (WT) p210^{BCR-ABL} Ba/F3 cell lines as assessed by total viable cell counts. The IC_{50} was approximately 125nM against WT p210^{BCR-ABL} Ba/F3 cells and not reached at a concentration of 1000nM in parental Ba/F3 cells. Results represent the mean of 3 independent experiments. (C) Line graphs showing the results of synergism experiments with BMS-214662 62.5nM in combination with IM (dose range 0-20μM) or dasatinib (dose range 0-600nM) in CD34+ normal and CML cells. The graph illustrates the cytotoxic effect of BMS-214662 62.5nM as a single agent in CD34+ CML cells compared to normal CD34+ cells as assessed by total viable cell counts and also the added cytotoxicity of using BMS-214662 in combination with either IM or dasatinib. (D) CI plots calculated using CalcuSyn software for BMS-214662 and IM in an algebraic estimate. Combination of BMS-214662 plus IM indicated synergistic activity (CI values <1) in CD34+ CML cells. CI values are represented by points below the dotted line. (E) A conservative isobologram for CD34+ CML cells indicated synergism between the two drugs.

Figure 2. Survival of total and quiescent CML cells in culture in the presence of drug combinations. Cells were cultured in SFM supplemented with growth factors. (A) Total viable cells (n=4) after 6 days culture in the different treatment arms. Results are expressed as a percentage of the no drug control.
and data are represented as mean ± SEM. (B) Total undivided CD34+\textsuperscript{CFSE}\textsuperscript{max} cells (n=4) present after 6 days culture. In addition to P values shown, P=0.023 and P=0.005 for no drug control versus BMS-214662+IM and BMS-214662+dasatinib, respectively. Results are expressed as a percentage of the no drug control and data are represented as mean ± SEM. (C) Representative FACS dot plots showing the presence of a large quiescent CML stem/progenitor cell population (boxed region) in the IM and dasatinib arms, but not the BMS-214662-containing arms. The percentage value in the lower right corner of each plot represents the number of quiescent CML cells remaining at the end of the culture period expressed as a percentage of the starting CD34\textsuperscript{+} cell number. (D) Representative FACS histogram plot demonstrating the anti-proliferative effect of dasatinib and IM compared to no drug control and BMS-214662. (E) Histogram highlighting the cell frequency for each cell division in each treatment arm. In the no drug control and BMS-214662 arms, there is a relative lack of cells in the early divisions (▼ ≤ 4 divisions) with increased numbers of cells in the later divisions (▼ ≥ 6 divisions), compared to the TKI-containing arms which tended to group together (→→→), with increased cells in the early divisions and fewer cells in later divisions. Results represent mean of 4 experiments.

**Figure 3.** (A) Results of LTC-IC assay in CML (n=3). The addition of BMS-214662 to either IM or dasatinib significantly reduced the number of colonies compared to either drug alone (P=0.032 and P=0.027 respectively). BMS-214662 also significantly reduced the number of colonies compared to the no drug control (P=0.028 for BMS-214662 and BMS-214662+IM arm and
P=0.031 for BMS-214662+dasatinib arm). (B) Results of LTC-IC assay in normal donors (n=3). There was a non-significant increase in LTC-IC in the IM and dasatinib only arms. In the BMS-214662-containing arms, there was a non-significant reduction in LTC-IC compared to control (P=0.079-0.29).

**Figure 4.** Assessment of apoptosis in total CML cells and the quiescent fraction by measurement of caspase-3 activity (n=4). Percentage of (A) total and (B) quiescent (CD34⁺ CFSE\(^{\text{max}}\)) caspase-3⁺ CML cells in the different treatment arms after 72 hours culture. In the quiescent fraction, P=0.045 for BMS-214662-containing versus non-containing arms. Data are represented as mean ± SEM. Illustrative FACS histograms demonstrating (C) Increased TUNEL activity (FL-1 channel) in CD34⁺ CML cells, cultured in SFM only, treated with BMS-214662 compared to no drug control or treatment with BMS-225975 and (D) BMS-214662 induced decrease in the membrane potential, as indicated by a decrease in TMRE signal (FL-2 channel). (E) Percentage of caspase-3⁺ cells in both CML CD34⁺38⁻ and CD34⁺38⁺ cells after 24 and 48 hours of treatment with BMS-214662 in SFM alone. (F) Percentage of caspase-3⁺ cells in normal CD34⁺38⁻ cells after 24 and 48 hours of treatment with BMS-214662. C-F are representative results for three independent experiments. FSC; forward scatter.

**Figure 5.** Comparison of the effects of BMS-214662 with the cytostatic FTI BMS-225975 on quiescent CML stem/progenitor cells (n=4). (A) The effect of BMS-225975 (250nM) was not significantly different to the no drug control. Results are expressed as a percentage of the no drug control and data are
represented as mean ± SEM. (B) Percentage of caspase-3$^+$ cells in CD34$^+$ CML cells (n=6) cultured in the absence of growth factors after 24 hours of treatment with BMS-214662 or BMS-225975.

Figure 6. Effect of BMS-214662 in blast crisis CML (n=4) and IM-resistant Ba/F3 cell lines. (A) Total viable cells in blast crisis CML after 6 days culture in the different treatment arms. The addition of BMS-214662 to either IM or dasatinib significantly reduced the number of total viable cells compared to either agent alone (P=0.04 for both). (B) Total quiescent CD34$^+$ CFSE$^{max}$ cells present after 6 days culture. Results are expressed as a percentage of the no drug control and data are represented as mean ± SEM. (C) Forty-eight hour proliferation assays for Ba/F3 cell lines with different BCR-ABL kinase mutations after treatment with BMS-214662. Results are the mean of 3 experiments with 5 replicates in each experiment.
Table I. D-FISH results for LTC-IC experiments with CD34+ CML samples.

Colonies were harvested after LTC-IC experiments, pooled for each treatment condition and then FISH was performed on the cells from these colonies.

<table>
<thead>
<tr>
<th>UPN&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BCR-ABL positive / total cells (%)</th>
<th>Baseline</th>
<th>No drug</th>
<th>IM</th>
<th>Dasatinib</th>
<th>BMS-214662</th>
<th>BMS-214662 + IM</th>
<th>BMS-214662 + Dasatinib</th>
</tr>
</thead>
<tbody>
<tr>
<td>166</td>
<td></td>
<td>439/463</td>
<td>111/111</td>
<td>ND</td>
<td>74/74</td>
<td>97/99</td>
<td>ND</td>
<td>62/62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(95)</td>
<td>(100)</td>
<td></td>
<td>(100)</td>
<td>(98)</td>
<td></td>
<td>(100)</td>
</tr>
<tr>
<td>189</td>
<td></td>
<td>932/1003</td>
<td>51/78</td>
<td>85</td>
<td>181/291</td>
<td>12/145</td>
<td>8/88</td>
<td>0/335</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(93)</td>
<td>(65)</td>
<td></td>
<td>(71)</td>
<td>(8)</td>
<td>(9)</td>
<td>(0)</td>
</tr>
<tr>
<td>215</td>
<td></td>
<td>104/109</td>
<td>67/71</td>
<td>40</td>
<td>63/66</td>
<td>6/134</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(95)</td>
<td>(94)</td>
<td></td>
<td>(100)</td>
<td>(4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>UPN; unique patient number, <sup>b</sup>ND; not determined.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
BMS-214662 potently induces apoptosis of chronic myeloid leukemia stem and progenitor cells and synergises with tyrosine kinase inhibitors

Mhairi Copland, Francesca Pellicano, Linda Richmond, Elaine K Allan, Ashley Hamilton, Francis Y Lee, Roberto Weinmann and Tessa L Holyoake