Activity of ibrutinib, a BTK inhibitor, in patients with CD117 positive acute myeloid leukaemia – a pre-clinical study

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Background
As approximately 80% of patients with acute myeloid leukaemia (AML) have high activity of Bruton's Tyrosine Kinase (BTK) in their blasts, which renders them sensitive to the oral BTK inhibitor ibrutinib in-vitro, we sought to develop the biologic understanding of the BTK pathway in AML to identify clinically relevant diagnostic information that may define a subset of patients that should respond to ibrutinib treatment.

Methods
We determined the activity of BTK in response CD117 activation in primary AML blasts. Furthermore we investigated the effects of ibrutinib on CD117 induced BTK activation, downstream signalling, adhesion to primary bone marrow mesenchymal stromal cells and proliferation of AML.

Findings
Ibrutinib inhibits CD117 driven proliferation of primary AML blasts. CD117 activation increases BTK activity in human AML. Furthermore ibrutinib inhibits CD117 induced activity of BTK and downstream kinases. Functionally we report that in-vitro activation of CD117 in CD117 expressing AML blasts which leads to adherence of the blasts to the cytoprotective bone marrow microenvironment stromal cells is inhibited by ibrutinib.

Interpretation
As we begin first in man clinical trials of ibrutinib in AML the pre-clinical data suggest not all patients will respond. Here we provide evidence that in AML, BTK has specific pro-tumoral biologic actions downstream of surface CD117 activation which are inhibited by ibrutinib. Accordingly we propose that patients with AML whose blasts express CD117 should be considered for forthcoming clinical trials of ibrutinib.

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Introduction

Acute myeloid leukemia (AML) comprises a morphologically and biologically heterogeneous group of tumours. However despite this apparent diversity comprehensive analysis of AML genomes has suggested that AML is not a disease caused by hundreds of mutations, but in fact only a few (1). Furthermore AML appears to rely on common programs of self-renewal downstream of the driver oncogene (2). Taken together these observations would suggest that mechanistically common therapeutic approaches are likely to be applicable to broad populations of patients with AML, regardless of the identity of the driver oncogene/s involved.

Bruton's Tyrosine Kinase (BTK) is a non-receptor tyrosine kinase that belongs to the Tec family and has an important function in a number of benign and malignant cells of the haematopoietic system (3-5). Moreover, recent phase 1 and 2 studies of the irreversible oral BTK inhibitor, ibrutinib have demonstrated promising clinical activity and tolerability against a variety of B-cell malignancies including, chronic lymphocytic leukemia (CLL), mantle cell lymphoma, hairy cell leukaemia, multiple myeloma and diffuse large B-cell lymphoma in younger and older patients alike (6-14). Furthermore, it is now clear that the mechanism of action [MOA] of ibrutinib is multifactorial in nature with a significant component of its function in lymphoid malignancy involving disruption of the interaction between the tumour cell and the microenvironment that protects it. Recently our group and others have shown that there is high BTK phosphorylation and RNA expression in AML (15-17). Moreover, our recent study described for the first time that ibrutinib and BTK-targeted RNA interference functionally reduced factor-induced proliferation of both AML cell lines and primary AML blasts, as well as reducing AML blast adhesion to BMSC (17). First in man studies of ibrutinib treatment in AML are due to begin in the near future.

CD117 is a precursor stage marker expressed on circa 80% of all AML blasts (18, 19) and in routine clinical practice commonly forms part of the immunophenotyping panel for AML diagnosis (20). CD117 functions to enhance the adhesion of AML blasts to fibronectin and augments fibronectin-mediated anti-apoptotic and proliferative signals (21, 22). In non-malignant myeloid cells CD117 coordinates mast cell activation (23) and stimulates the chemotaxis, integrin up-regulation, and survival of basophils (24). Moreover, CD117 mediates BTK phosphorylation and downstream survival signals in erythroid progenitors which protects against apoptosis signals (25).

In this study we dissect the intracellular signalling cascade downstream of CD117 activation, specifically the effects on fibronectin (FN)-mediated AML blast proliferation and adhesion. Furthermore in light of upcoming studies of ibrutinib in AML and the ready availability to the physician on the day of diagnosis of the presence or absence of CD117 expression on the AML blast surface we investigate the function of CD117 activation on AML survival in response to BTK inhibition.

Methods

Primary human AML

AML blasts were obtained from 29 unselected patients attending our hospital (between January 2010 and February 2014) who consented to give bone marrow or blood for research following informed consent and under approval from the UK National Research Ethics Service (LRECref07/H0310/146). Patients characteristics including WHO diagnosis, previous treatment (or not) and age were collected (Table1). For primary cell isolation, heparinized blood was collected from patients and human peripheral blood mononuclear cells (PBMCs) isolated by Histopaque (Sigma-Aldrich, Gillingham, UK) density gradient centrifugation. AML samples that were less than 80% blasts were purified using the CD34 positive selection kit (denoted by * in Table 1). Cell type was confirmed by microscopy and flow cytometry. We obtained hematopoietic CD34+ cells from two sources, Stem Cell Technologies (Cambridge, UK) and patients. Positive selection of CD34+ cells was isolated from PBMCs using a CD34 positive selection kit (Miltenyi Biotec, Bisley, UK) as previously described (26). Cell type was confirmed by microscopy and flow cytometry.

Human bone marrow stromal cells (BMSC) were isolated by bone marrow aspirates from AML patients. Mononuclear cells were collected by gradient centrifugation and plated in growth medium containing RPMI and 20% FBS and 1% l-glut (Life Technologies, Paisley, UK) . The non-adherent cells were removed after 2 days. When 60%-80% confluent, adherent cells were trypsinised and expanded for 3-5 weeks. BMSCs were checked for positive expression of CD105, CD73, and CD90 and the lack of expression of CD45 and CD34 by flow cytometry as previously described (17).

Materials

Anti-phosphorylated and pan CD117, AKT, BTK and MAPK antibodies were purchased from Cell Signaling Technology (Cambridge, USA). We also purchased anti-BTK (phospho Y223) antibody from R&D Systems (Abingdon, UK). Anti-CD34-PE, anti-CD90-FITC, anti-CD73-PE, anti-CD105-APC antibodies were purchased from Miltenyi Biotec . All other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Ibrutinib was obtained from Selleck Chemicals. Stem cell factor (SCF) and fibronectin were purchased from Life Technologies. All other reagents were obtained from Sigma-Aldrich , unless indicated.

Western immunoblotting.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analyses were performed as described previously. Briefly, whole cell lysates as well as nuclear and cytosolic were extracted and sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation performed (27). Protein was transferred to nitrocellulose and Western blot analysis performed with the indicated antisera according to their manufacturer's guidelines

Proliferation/death assays

Cells were treated with different doses of ibrutinib then viable numbers measured with Cell TiterGlo (Promega, Southampton, UK). BrdU Cell Proliferation Assay Kit was purchased from Cell Signaling Technology. Flow cytometry for measuring apoptosis was performed on the Accuri C6 flow cytometer (BD Biosciences, Oxford, UK). Samples were collected and stained with annexin- V and propidium Iodide (PI) (Abcam, Cambridge, UK), followed by detection. For the AML-BMSC co-cultures AML cell viability was measured using flow cytometry. After exclusion of BMSC by electronic gating using forward scatter the extent of AML cells apoptosis was measured using annexin-V.

BMSC/fibronectin-AML cell adhesion assay

BMSCs were grown in 96-well plates. Primary AML cells were incubated with 2.5 µM calcein-AM for 1 h at 37°C and 5% CO_2. The fluorescence-labeled AML cells were added into stromal cell coated 96-well plates and incubated for the indicated time points. Non-adherent calcein-labeled cells were removed by gently washing and adherent cells were quantitated in a fluorescence multi-well plate reader. For AML blast adhesion onto fibronectin (FN), 96 well plates were coated with 10 mg/ml FN for 1 h before the fluorescence-labeled AML cells were added. Again non-adherent calcein-labeled cells were removed by gently washing and adherent cells were quantitated in a fluorescence multi-well plate reader.

Quantification of BTK activity by immunocytochemistry

Relative activity of phosphorylated versus total BTK, was measured by immunocytochemistry as previously described (20). Briefly, 2 x 10^4 AML cells were fixed onto microscope slides using 4% (w/v) paraformaldehyde. Fixed cells were incubated with rabbit anti-human primary monoclonal antibodies (mAbs) against pBTK

(Tyr223) and goat anti-human primary mAbs against total BTK, or goat anti-human primary followed by incubation with Alexa Fluor goat anti-rabbit and rabbit anti-goat IgG secondary antibodies (Molecular Probes, Life Technologies). Image analyses and quantification of pBTK and BTK staining intensity was performed using Image J 1.46 software (National Institute of Health, Bethesda, USA).

Statistical analyses

The Mann-Whitney test was used to compare test groups where stated. Results where $P < 0.05$ were considered statistically significant. Pearson's correlation analysis was used to investigate the relationship between phosphorylated BTK and % CD117 expression. For Western blotting the data are representative images of 3 independent experiments.

Role of the funding source

The funding sources for this study had no involvement in study design or in the collection, analysis, and interpretation of the data or in the writing of the report or in the decision to submit the paper for publication.

Results

Ibrutinib inhibits CD117 driven proliferation of primary AML blasts

We recently reported that nanomolar concentrations of ibrutinib inhibit primary human AML proliferation and adhesion in-vitro in approximately 80% of non-selected primary AML patient samples tested (17). Here we first examined the role of ibrutinib in regulating CD117 induced proliferation. CD117 mediated proliferation (as measured by BrdU incorporation) in primary CD117 expressing AML blasts is inhibited by ibrutinib (Figure 1A). Conversely CD117 negative AML blasts did not proliferate in response to CD117 activation and ibrutinib had no significant effect on the proliferation of these AML cells (Figure 1B). In addition, CD117 activation increased normal CD34+ haematopoietic progenitor cell (HPC) proliferation, which was also significantly inhibited by ibrutinib (Figure 1C). Data were normalised to DMSO treated cells (The line through the data indicates the median and the Mann Whitney test was used to determine statistical significance between SCF and ibrutinib+SCF treatment groups). We next showed that as little as 100nM of ibrutinib inhibits CD117 induced proliferation in CD117 positive AML. An anti-proliferative effect was only seen in CD117 negative AML at a concentration of 5000nM and is likely non-specific (Figure 1D and 1E). Data were normalised to DMSO treated cells. The line through the data indicates the median. Proliferation at varying concentrations of ibrutinib were compared with DMSO treated control by Mann Whitney test (* = $P<0.05$). A scatterplot (Figure 1F) shows the relationship between pBTK and percentage CD117 (%CD117) expression in human AML blasts (patients n=24; correlation coefficient r=0.63). Table 1 shows the half maximal inhibitory concentration (IC50) of ibrutinib induced AML cell death (measured by by CellTitre-GLo as described in our previous manuscript (17)). For concentrations of ibrutinib <10μM %CD117 expression is variable (range 18 to 92), whereas for concentrations of ibrutinib >10μM %CD117 expression was zero (Figure 1G). Taken together these results show that ibrutinib inhibits CD117 mediated AML proliferation. (8, 14)

CD117 activation increases BTK activity in human AML

As CD117 activation has been shown to regulate BTK activity in haematopoietic progenitor cells (HPC) we investigated BTK activation in AML and downstream pro-survival and proliferation signalling. CD117 is expressed in circa 80% of our AML patient samples in keeping with previously published observations (18), where CD117 positivity is defined as > 10% (table 1) (28). Recently we reported that approximately 80% of AML have constitutive BTK activity as measured by levels of phosphorylated BTK (pBTK) expression in primary samples (17). Next to determine whether CD117 activation increases activity of BTK in AML we serum starved CD117 positive AML blasts for 4 hours before treatment with stem cell factor (CD117 ligand or SCF) for various times up to 30 minutes. Western blot analysis demonstrates that CD117 activation induces pBTK from 5-30 minutes above control untreated cells in CD117 positive AML but not in CD117 negative AML (Figure 2A and 2B).

Ibrutinib inhibits CD117 induced activation of BTK and downstream kinases.

Next we asked whether ibrutinib can inhibit CD117 mediated proliferation signals. To do this we examined the effect of increasing doses of ibrutinib on CD117 activated AML and analysed phosphorylation of CD117, BTK, AKT and MAPK in CD117 positive AML and CD117 negative AML samples. The results show that CD117 activation induced phosphorylation of CD117 which was not inhibited by ibrutinib however downstream pBTK, pAKT and pMAPK were all inhibited at 100 nM or above of ibrutinib in CD117 positive blasts (Figure 3). In contrast in the CD117 negative AML we found that pBTK and CD117 levels were undetectable and that the levels of pAKT and pMAPK were not affected by any dose of ibrutinib up to 1000 nM (Figure 3). Taken together these observations confirm that in CD117 positive AML CD117 mediated activation of pBTK, pAKT and pMAPK is inhibited by treatment with ibrutinib.

Ibrutinib blocks adhesion of AML blasts to cytoprotective bone marrow stromal cells (BMSC)

CD117 activation functions in part by supporting AML adhesion to BMSC (21). Furthermore BMSC provide protection from standard cytotoxic chemotherapy (17, 22). Therefore we examined the role of ibrutinib in regulating CD117 mediated adhesion to cytoprotective BMSC. AML/BMSC interaction was assessed by calcein AM cell adhesion assay. As previously reported, in our primary samples CD117 activation increases adhesion of CD117 expressing AML blasts to BMSC (Figure 4A). Next we examined the effect of ibrutinib on CD117 mediated AML adhesion to BMSC. CD117 positive and CD117 negative AML blasts were pre-treated with ibrutinib (500 nM) and then co-cultured with BMSC in the presence of or absence of CD117 activation. CD117 mediated AML blast adhesion is significantly inhibited by ibrutinib at 500 nM (Figure 4B). Ibrutinib also inhibited CD117 mediated adhesion of non-malignant CD34+ HPC to BMSC (Figure 4C). Next we examined the apoptotic response of CD117 expressing AML blasts co-cultured with BMSC in the presence of CD117 activation and ibrutinib for 72 hours. The co-culture was removed from the 12 well plate and antibody to CD34 was used to distinguish the AML blasts from the BMSC and annexin V was used to examine apoptosis. Figure 4D shows that ibrutinib alone increased AML blast apoptosis over control untreated co-cultures. Taken together

we find that ibrutinib inhibits AML blast adhesion to the underlying cytoprotective BMSC through its action on CD117 mediated signalling.

The interaction between AML blasts and the bone marrow microenvironment is critical in regulating tumour survival and chemotherapy resistance. Inhibiting AML blast adhesion to BMSC via the VLA4-VCAM/fibronectin interactions is associated with improvements in tumour cytotoxicity and increasing sensitivity to chemotherapy (22). To determine if ibrutinib functions to inhibit VLA4-VCAM/fibronectin interactions downstream of CD117 activation in AML we examined primary AML adhesion to fibronectin (FN) coated plates. Figure 4E shows that CD117 positive AML blasts (but not primary non-malignant CD34+ HPC or CD117 negative AML; supplementary Figure 1 A and C) increase adhesion to FN in the presence of CD117 activation. When we repeated these experiments in the presence of ibrutinib, CD117 mediated FN adhesion was inhibited in CD117 expressing AML (Figure 4F) (but not in primary CD34+HPC or CD117 negative AML; supplementary Figure 1 B and D). To demonstrate the specificity of the observed ibrutinib effect, ibrutinib-treated cells were then treated with PMA (which results in adhesion via protein kinase C-activated integrin activation). Figure 4G shows that ibrutinib cannot inhibit PMA induced AML adhesion to FN. Finally others have reported that CD117 activation in combination with culture on FN-coated plates increases AML proliferation (21). Therefore we examined the effects of ibrutinib on CD117 mediated and FN-mediated proliferation. AML proliferation in response to CD117 activation in combination with FN was inhibited by ibrutinib (supplementary Figure 2).

Discussion

Here we report that in human AML ibrutinib inhibits the CD117 activation pathway and functions to disrupt pro-survival signals from the microenvironment to promote de-adhesion of malignant cells from the BMSC and subsequent cell death.

Currently available curative therapeutic strategies for AML are intensive and toxic and generally only widely applicable to younger fitter patients. However AML is primarily a disease of the elderly with a median age at diagnosis in the Swedish Acute Leukaemia Registry of 72 years (quartile values, 60-79 years; range, 16-97 years) (29). Intensive chemotherapy given with curative intent is not easily deliverable to older patients and as such outcomes presently remain dismal for the majority group of older patients with the disease (30). Accordingly to improve the prognosis for all patients, (including older patients with AML), there is a requirement for tumour targeted treatments with improved efficacy and reduced toxicity. Such therapies will result from a better understanding of the biology of AML.

BTK is a cytoplasmic tyrosine kinase widely expressed in hematopoietic cells and long known to be critical in B cell differentiation and survival pathways. BTK is a member of the BTK/Tec family of tyrosine kinases (31). BTK activation has been implicated in a variety of haematopoietic cellular responses and there is a growing literature defining the role of BTK in HPC and cells of the myeloid compartment (25, 32, 33). In our previous work we showed that ibrutinib could inhibit AML colony formation and survival (17). In this study we develop on these findings to investigate upstream mediators (including cell surface receptors) and downstream consequences of BTK activation and inhibition.

CD117 is a type III receptor tyrosine kinase operating in cell signal transduction in several cell types(23, 34, 35). Upon activation (phosphorylation) CD117 initiates a downstream phosphorylation cascade ultimately activating various transcription factors depending on the different cell type. In a number of cell types phosphorylated CD117 has been shown to use multiple non-receptor tyrosine kinases including BTK, SHIP1/2, PLCY2, PI3K, SYK and SRC kinases (25, 36, 37), to regulate apoptosis, cell differentiation, proliferation, chemotaxis, and cell adhesion (38). On activation BTK auto-phosphorylates at tyrosine Y223. We were able to detect phosphorylation at BTK Y223 in response to activation of CD117 and furthermore this was inhibited by ibrutinib. The BTK tyrosine Y551 residue is phosphorylated directly by upstream tyrosine kinases and phosphorylation at Y551 should not be affected by ibrutinib, however, immunoblots for BTK Y551 phosphorylation were not informative. CD117 is expressed on the cell surface of approximately 80% of cases of AML (18), and here we report that CD117 activation in AML links via BTK to downstream pro-tumoral signals.

The oral BTK inhibitor ibrutinib appears active and well tolerated in patients with chronic lymphocytic leukemia (CLL) and mantle cell lymphoma with limited grade 3 and 4 toxicity (13, 14). Ibrutinib functions primarily in these B cell malignancies by perturbing the interactions between the tumour and its microenvironment (39). Here we found that ibrutinib was able to inhibit CD117 mediated BMSC adhesion to

AML cells at concentrations similar to those that have been reported to inhibit adhesion of CLL cells to BMSC (8). Others have also shown that CD117 activation can increase AML adhesion to fibronectin (21), and we have previously shown that inhibition of AML/BMSC interactions using ibrutinib can increase AML susceptibility to daunorubicin and cytosine arabinoside (17). Taken together this argues that ibrutinib may show clinical efficacy in patients at least in part by disrupting the CD117 mediated AML/BMSC interaction.

In patients with CLL receiving ibrutinib the disruption of tumour/BMSC interaction is associated with a (usually) transient rise in the circulating leukaemia cell count in the peripheral blood (14). Given the relatively small size of CLL cells this is unlikely to have a negative clinical consequence. AML blasts are however larger and a high circulating white count in AML can be associated with leucostasis and consequent risk of micro-infarction, local hypoxemia, bleeding and death. Therefore because of these in-vitro observations regarding AML and the BMSC it seems likely that if ibrutinib is effective in AML patients we will observe a rising blast count in the peripheral blood in some patients and care should be taken at least initially to monitor for and prevent/treat drug-induced hyperleucostasis. Ultimately though, release of AML leukemic cells from their bone marrow microenvironment niche should destabilise their survival mechanisms and render them more sensitive to chemotherapy. This function may be particularly efficacious in dealing with minimal residual disease in the consolidation cycles of intensive chemotherapy regimens.

We found that ibrutinib inhibits CD117 mediated non-malignant CD34+ cell proliferation, however the degree of inhibition was not as pronounced as that observed in the AML blasts. In addition approximately half of the CD117 negative AML samples also showed active pBTK. This data suggest the presence of additional receptor tyrosine kinases and signalling proteins functioning upstream of BTK in AML. The identification of additional receptor tyrosine kinases that signal through BTK in AML may point to other subsets of AML patients who may benefit from BTK inhibition. Furthermore it is becoming apparent that ibrutinib appears to inhibit kinases other than BTK including mutant EGFR and ITK (40, 41). We and others have previously found that in AML ibrutinib treatment mimics data from BTK RNA interference experiments, which argues that at least in AML the mechanism of action of ibrutinib is via its inhibitory effect on BTK (17, 42). It is also likely that there are other upstream drivers of BTK in AML including FLT3, LYN and SYK (42-44). Ultimately the clinical significance of the BTK signalling pathway and its inhibition by ibrutinib in patients with AML can only fully be defined in the context of clinical trials.

To our knowledge we show for the first time that in human AML ibrutinib inhibits the CD117 activation pathway and functions to disrupt pro-survival signals from the microenvironment to promote de-adhesion of malignant cells from the BMSC and subsequent cell death. This study further validates an emerging focus of targeting kinases critical for the survival of malignant cells and gives us a clearer understanding of how inhibition of BTK can be harnessed therapeutically in AML. In summary, we provide further biologic rationale for clinical trials of ibrutinib to treat patients with AML.

Research in Context

- BTK is a non-receptor tyrosine kinase overexpressed in 80% of AML (17, 42).
- Ibrutinib shows efficacy and tolerability (and is licenced for use) in patients with chronic lymphocytic leukaemia (CLL) and mantle cell lymphoma and functions through inhibition of BTK downstream of the B cell receptor in those diseases.
- Here we show that in AML, BTK signals downstream of surface CD117 activation and that inhibition of BTK by ibrutinib is anti-proliferative and disrupts the interaction between the blasts and the bone marrow stromal cells in a mechanism similar to that observed in CLL.
- In summary we provide biologic data to further support the clinical trial of ibrutinib in patients with AML, including those patients whose blasts express CD117.

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Authorship Contributions
SAR, KMB and DJM designed the research. SAR, GP, AAA, MSS, RP, LZ and MYM performed the research. MJL and KMB provided essential reagents. SAR, KMB and DJM wrote the paper.

Disclosure of Conflicts of Interest
The authors declare no conflicts of interest.

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Legends

Figure 1. SCF/CD117-induced proliferation is inhibited by ibrutinib in primary human AML blasts. Primary AML blasts which were (A) $CD117^{pos}$ (n=2) and (B) $CD117^{neg}$ (n=2) and (C) CD34+ HPC (n=4) were pre-treated with ibrutinib (500 nM) for 1 hour and then treated with SCF (10 ng/ml) for 72 hours and then assessed by BrdU assay. Primary AML patient blasts which were (D) $CD117^{pos}$ (n=11 patient samples) and (E) $CD117^{neg}$ (n=8 patient samples) were pre-treated with increasing doses of ibrutinib for 1 hour and then treated with SCF (10 ng/ml) for 72 hours and then assessed by CellTiterGlo. (F) Scatterplot of phosphorylated BTK and % CD117 expression on AML blasts. (G) Scatterplot of phosphorylated % CD117 expression and ibrutinib IC50 [μM] in AML blasts.

Figure 2. SCF/CD117-induced BTK phosphorylation in AML. (A) AML#21 ($CD117^{pos}$) and AML#17 ($CD117^{neg}$) were treated with SCF (10 ng/ml) for up to 30mins. Protein extracts were obtained and Western blot analysis was conducted for pCD117, CD117, pBTK, BTK. (B) Primary AML blasts which were $CD117^{pos}$ and $CD117^{neg}$ were treated with SCF (10 ng/ml) for 5 min and then whole cell extracts were prepared and Western blot analysis was conducted for pBTK and total BTK.

Figure 3. SCF/CD117-mediated AML proliferation signals are inhibited by ibrutinib. (A) Primary AML blasts which were $CD117^{pos}$ and $CD117^{neg}$ were pre-treated with increasing doses of ibrutinib for 1 hour and then treated with SCF (10 ng/ml) for 5 min and then whole cell extracts were prepared and Western blot analysis was conducted for pCD117, CD117, pBTK, BTK , pAKT, AKT, pMAPK and MAPK protein levels.

Figure 4. Ibrutinib inhibits SCF/CD117-induced AML adhesion to BMSC. (A) Primary AML blasts which were $CD117^{pos}$ (n=4) were either untreated or treated with SCF (10 ng/ml) and then placed onto BMSC for 4 hours. Cell adhesion was then assessed and expressed as percentage of total cells in the assay. (B) Cell adhesion of AML#17 ($CD117^{pos}$) and AML#23 ($CD117^{neg}$) AML blasts were pre-treated with ibrutinib (500 nM) for 1 hour and then treated with SCF (10 ng/ml) and then placed onto BMSC for 4 hours and is expressed as percentage of total cells in the assay. (C) CD34+ HPC were pre-treated with ibrutinib (500nM) for 1 hour and then treated with SCF (10 ng/ml) and then placed onto BMSC for 4 hours. Cell adhesion was then assessed and expressed as percentage of total cells in the assay. (D) Flow cytometry (72 h) showing co-cultured AML blasts ($CD117^{pos}$) and BMSC. (E and F) Primary AML blasts which were $CD117^{pos}$ were treated with various concentrations of (E) SCF (0-50 ng/ml) or (F) ibrutinib (0-1000nM) plus SCF (10ng/ml) and then placed onto FN coated plates for 4 hours. Cell adhesion was then assessed and expressed as percentage of total cells in the assay. The experiments were repeated 4 times using the blasts from AML#26.(G) $CD117^{pos}$ AML blasts were pre-treated with ibrutinib (500nM) for 1 hour and then treated with PMA (100nM) and then placed on FN coated plates for 1 hour. Cell adhesion was then assessed and expressed as percentage of total cells in the assay. Individual results are represented as circles, squares or triangles and median as a horizontal line. The Mann-Whitney test was used to for comparison between treatment groups (* = $p<0.05$).

Number	Age	Gender	WHO diagnosis	Cytogenetics	%Blasts	% pBTK/ tBTK	% c-CD117 expression	Ibrutinib IC50 (μM)
AML#1	78	M	AML with myelodysplasia related changes	not available	85	54	56	3
AML#2	57	M	Relapsed AML without maturation	not available	95	97	90	5.6
AML#3	25	M	AML with maturation	Normal	50*	52	73	-
AML#4	61	M	Relapsed AML without maturation	not available	Na	38	59	-
AML#5	26	M	AML with t(8;21)(q22;q22); RUNX1-RUNX1T1	t(8;21)	60*	80	69	0.7
AML#6	28	F	Acute monoblastic and monocytic leukaemia	Normal	95	54	16	1.8
AML#7	40	M	AML without maturation	Constitutional XXY	95	95	57	2.6
AML#8	84	M	AML, NOS	not available	75*	29	0	26
AML#9	53	M	AML with t(6;9)(p23;q34);DEK-NUP214	t(6;9)	70*	32	69	9.8
AML#10	51	F	AML with maturation	Normal	40*	51	57	5.4
AML#11	85	F	AML NOS	not available	60*	65	0	35.6
AML#12	82	M	AMLNOS	not available	65*	63	29	2
AML#13	70	M	Therapy-related myeloid neoplasm'	Not available	n/a	15	8	-
AML#14	47	M	AML without maturation	not available	90	77	29	6.6
AML#15	59	F	Acute myelomonocytic leukaemia	Normal	20*	13	0	27.8
AML#16	64	F	AML with myelodysplasia related changes	Complex	20*	56	70	3.7
AML#17	55	F	Acute monoblastic and monocytic leukaemia	Normal	80	60	0	58.2
AML#18	82	M	AML with maturation	Normal	40*	42	66	7.8
AML#19	41	F	AML with t(6;9)(p23;q34);DEK-NUP214	t(6;9)	>95	76	77	2
AML#20	76	M	AML with myelodysplasia-related changes	Normal	20*	85	67	8
AML#21	77	F	AML with maturation	Normal	65*	81	89	1.6
AML#22	62	M	AML with maturation	Complex	55*	-	96	-
AML#23	70	M	AML with minimal differentiation	Normal	>95	81	90	-
AML#24	65	F	AML with maturation	Normal	36*	-	77	-
AML#25	40	M	AML with minimal differentiation	Normal	90	-	65	-
AML#26	70	M	AML without maturation	Complex	95	-	93	-
AML#27	91	F	AML NOS	Not available	60*	33	16	-
AML#28	59	F	AML with t(8;21)(q22;q22); RUNX1-RUNX1T1	t(8;21)	80	39	50	-
AML#29	51	M	Acute monoblastic and monocytic leukaemia	Normal	95	26	0	-

Table 1. AML patient sample information including relative pBTK levels and CD117 expression. This table describes the AML patient samples used. Data show WHO diagnosis, cytogenetic results, percent BTK

phosphorylation, percent CD117 expression (surface expression on the AML blasts by immunophenotyping) and ibrutinib IC50 (μM) where avaiable. "-" represents data not available.