Identification of Bruton's tyrosine kinase as a therapeutic target in acute myeloid leukemia.

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Abstract

Bruton's tyrosine kinase (BTK) is a cytoplasmic protein found in all hematopoietic cell lineages except for T cells. BTK mediates signalling downstream of a number of receptors. Pharmacological targeting of BTK using ibrutinib (previously PCI-32765) has recently shown encouraging clinical activity in a range of lymphoid malignancies. This study reports for the first time that ibrutinib is inhibits blast proliferation from human acute myeloid leukaemia (AML) and that treatment with ibrutinib significantly augmented cytotoxic activities of standard AML chemotherapy cytarabine or daunorubicin. Here we describe that BTK is constitutively phosphorylated in the majority of AML samples tested, with BTK phosphorylation correlating highly with the cell's cytotoxic sensitivity towards ibrutinib. BTK targeted RNAi knock-down reduced colony forming capacity of primary AML blasts and proliferation of AML cell lines. We showed this anti-proliferative affects of ibrutinib in AML is mediated via an inhibitory effect on AKT and downstream nuclear factor-κB (NF-κB) survival pathways. Moreover, ibrutinib inhibited AML cell adhesion to bone marrow stroma. Furthermore, these effects of ibrutinib in AML were seen at comparable concentrations that are efficacious in chronic lymphocytic leukemia (CLL). These results reported here provide a biologic rationale for the clinical evaluation of BTK inhibition in patients with AML.

Key Points

- Inhibition of Bruton's tyrosine kinase is as effective against acute myeloid leukemia (AML) as chronic lymphocytic leukemia (CLL) in vitro.
- Ibrutinib shows similar activity in AML due to its constitutively active Bruton's tyrosine kinase activity.

Introduction

Acute myeloid leukemia (AML) is primarily a disease of the elderly with a median age at diagnosis in the Swedish Acute Leukemia Registry of 72 years (quartile values, 60-79 years; range, 16-97 years) [1]. In younger patients (<65 years old), there is clear evidence of improved survival in the last two or three decades, however in the majority group of older patients, there is little evidence of similar improvement, and the appropriateness of intensive cytotoxic treatment represents a current dilemma [2]. Accordingly to improve outcomes for all patients (including older patients with AML) there is a requirement for more effective treatments with reduced toxicity. Such therapies will result from a better understanding of the biology of AML.

Clinically, morphologically and biologically, AML seems to comprise of a heterogenous group of tumors. However despite this apparent diversity, AML relies on common programs of self-renewal downstream of the driver oncogene and further more comprehensive analysis of AML genomes has suggested that AML is not a disease caused by hundreds of mutations, but in fact only a few [3]. 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 [4].

Tyrosine kinases (TK) are proving to be attractive drugable targets in cancer. In AML activating mutations of receptor tyrosine kinases have been identified in circa 50% of all patients [5,6]. Furthermore cell survival and proliferation pathways dependent on TK activation, including MAPK, phosphoinositide 3-kinase (PI3K)/AKT, mTOR, NF-κB and STATs are deregulated in most, if not all, cases of AML [7-10]. These observations suggest that a therapeutic strategy

targeting key tumor specific, deregulated receptor TKs and/or non-receptor TKs may be both widely applicable and effective in AML patients.

Bruton tyrosine kinase (BTK) belongs to the Tec family of tyrosine kinases and clearly has an important function in a number of benign and malignant cells of the haematopoietic system. BTK was originally identified in BCR signalling as mutations in BTK block B cell development causing a lack of circulating immunoglobulins {Vetrie, 1993 #8500}{Tsukada, 1993 #8249}. Since then It has been shown an essential mediator of normal B-cell development linking the receptor for B cell-activating factor (BAFF) receptor (BAFF-R) of the TNF receptor superfamily to the NF-κB pathway [11]. The properties of other receptors (including some toll-like receptor responses) are dependent on BTK. Recent phase 1 and 2 studies of the irreversible BTK inhibitor, ibrutinib have demonstrated promising 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 [12-18]. In addition to its function in lymphoid cells BTK expression has also been found in hematopoietic stem cells (HSC), multipotent progenitors, and in several other cells of the haematopoietic system including those of erythroid and megakaryocytic lineage [19]. Furthermore it has become evident that BTK-deficiency significantly affects cells in the myeloid compartment including LPS-induced toll-like receptor-induced TNF production by monocytes [20], regulation of dendritic cell maturation and function via IL-10 and Stat3 [21], as well as neutrophil development and function [22,23]. Moreover high BTK phosphorylation and RNA expression has been observed in AML [24,25]. Here we explain the function of BTK in human AML and describe the pharmacological effects of BTK inhibition by ibrutinib on AML proliferation and bone marrow adhesion.

Methods

Materials

The AML-derived cell lines were obtained from the European Collection of Cell Cultures where they are authenticated by DNA-fingerprinting. In the laboratory they are used at low passage number for a maximum of 6 months post-resuscitation, testing regularly for Mycoplasma infection. Anti-NF-κB and AKT antibodies were purchased from Cell Signaling Technologies (Cambridge, MA). Anti-BTK (phospho Y223) antibody [EP420Y] and (phospho Y551) antibody [EP267Y] was purchased from Abcam (Cambridge, UK). All other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Ibrutinib was obtained from Selleck Chemicals. Stem cell factor (SCF), interleukin-1 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF were purchased from Invitrogen. All other reagents were obtained from Sigma-Aldrich (St Louis, MO), unless indicated.

Cell culture

AML cells were obtained from patients bone marrow or blood following informed consent and under approval from the UK National Research Ethics Service (LRECref07/H0310/146). For primary cell isolation, heparinized blood was collected from volunteers and human peripheral blood mononuclear cells (PBMCs) isolated by Histopaque (Sigma-Aldrich, UK) density gradient centrifugation. AML samples that were less than 80% blasts were purified using the CD34 positive selection kit (denoted by * in Supplementary Table 1). Cell type was confirmed by microscopy and flow cytometry We obtained hematopoietic CD34+ cells from two sources, Stem Cell Technologies and volunteers. Positive selection of CD34+ cells were isolated from PBMCs using a CD34 positive selection kit (Miltenyi Biotec, Auburn, CA). For all CD34+ experiments at least three different donors were used to obtain the

results presented in this paper. Cell type was confirmed by microscopy and flow cytometry.

Human bone marrow stromal cells (BMSCs) 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. 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 [26,27].

RNA extraction and real-time PCR

Total RNA was extracted from 5 x 10^5 cells using the Nucleic acid Prep Station from Applied Biosystems (Paisley, UK), according to the manufacturer's instructions. Reverse transcription was performed using the RNA polymerase chain reaction (PCR) core kit (Applied Biosystems). Relative quantitative real-time PCR used SYBR green technology (Roche) on cDNA generated from the reverse transcription of purified RNA. After preamplification (95°C for 2 minutes), the PCRs were amplified for 45 cycles (95°C for 15 seconds and 60°C for 10 seconds and 72°C for 10 seconds) on a 384-well LightCycler 480 (Roche, Burgess Hill, UK). Each mRNA expression was normalized against GAPDH mRNA expression using the standard curve method.

Western immunoblotting and NF-κB binding assay.

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 [28,29]. Protein was transferred to nitrocellulose and Western blot analysis performed with the

indicated antisera according to their manufacturer's guidelines. To examine NF-κB binding in AML cells we used The TransAM NF- κB family Transcription Factor assay kit from ActiveMotif.

Immunolocalisation and quantitation of total versus phopho-BTK

Primary CD34+ cells, AML cell lines and AML primary samples were fixed in 4 % (w/v) paraformaldehyde, permeablised with 0.1 % (v/v) Triton-X100 and blocked with 10 % (v/v) normal goat serum. Samples were stained with primary antibodies against total BTK (D3H5) and phosphorylated BTK (Y223) (Cell Signalling Technology). Primary antibody binding was visualised with secondary Alexa Fluor® 568 and 488 conjugated IgG (H+L) antibodies (Invitrogen Molecular Probes), respectively. Cell nuclei were visualised with 4',6-diamidino-2-phenylindole (DAPI) before samples were mounted with FluoromountTM aqueous mounting medium. Cells were stained with secondary antibody only to determine non-specific binding which was subtracted from the staining intensity analysed for pBTK and total BTK. Cells were imaged with the AxioCam ICm 1 monochrome CCD camera attached to the Apotome.2 Imaging System using Axiovision 4.8.2 software (all Carl Zeiss Ltd). Images were analysed for staining intensity of total versus phosphoBTK with ImageJ open source software (NIH) and Area Calculator plugins before percentage phopshoBTK was calculated (n=20 per sample).

Proliferation/death assays

Cells were treated with different doses of ibrutinib then viable numbers measured with Cell-Titre GLO (Promega, Southampton, UK). 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), 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.

Virus construction and infection

MicroRNA sequence miRNA-BTK437 (5'-TTCACTGGACTCTTCACCTCT-3') and miRNA-BTK1092 (5'-TGACAATGAAACCTCCTTCTT-3') targeting human BTK was selected with Invitrogen Block-iT RNAi Designer software (www.invitrogen.com/rnai) and plasmid pcDNATM6.2-GW/EmGFP-miR-neg (Invitrogen) was used as source for negative control. MicroRNA-encoding sequences were cloned into Block-iT Pol II miR-RNAi vector (Invitrogen) and then EmGFP-pre-miRNA fragments were subcloned into the *BamHI*/*Xho I* site in the LNT/SffvMCS plasmid (kind gift from Penny Powell, University of East Anglia, UK). MicroRNA-encoding viruses were produced in 293T cells as described previously [30], using packaging plasmids pCMVΔR8.91 (expressing gag-pol) and pMD.G (expressing VSV-G) (kindly provided by Dr Ariberto Fassati, University College London, UK). Lentiviral stocks were concentrated using Lenti-X^{TM} Concentrator (Clontech, St-Germain-en-Laye, France) and titers were obtained with Lenti-X^{TM} qRT-PCR Titration kit (Clontech). For transduction, AML and control cells were plated onto 12 well plates at 5 x 10^4 cells/well/0.5ml. Cells were infected with lentiviral stocks at an MOI of 15 in presence of 8 µg/ml Polybrene®. Transduced cells were analysed by flow cytometry (Accuri, BD biosciences), real-time-PCR (Roche) and Western blotting.

Clonogenic methylcellulose assays

Control CD34+ HSC, AML cell lines and primary AML cells (1×103 to 5×104 cells) were plated in methylcellulose medium (R&D systems, Abingdon, UK) and colonies were visualised, measured and counted after 10-15 days.

BMSC-AML cell adhesion assay

BMSCs were grown in 96-well plates. AML cell lines and 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. Nonadherent calcein-labeled cells were removed by gently washing and adherent cells were quantitated in a fluorescence multiwell plate reader.

PCR gene array expression analysis

CD34+ control cells, AML cell lines and primary AML cells were left untreated or pretreated with 5 µM ibrutinib for 16 h. mRNA was isolated and reverse-transcribed to obtain cDNA. cDNA was incubated on an NF-κB Signaling Pathway RT2 qPCR array (SABiosciences, Crawley, UK) according to the manufacturer's instructions. This array contains 84 key genes involved in the NF-κB signal transduction pathway and several controls. Target genes were normalized to control GAPDH expression and expressed as fold change relative to unstimulated control.

BTK occupancy

Primary AML cells and U937 were treated with increasing doses of ibrutinib (1nM-1000nM) for 1 hour. Cells were then washed in PBS and frozen at -80C. BTK occupancy was performed as described previously {Honigberg, 2010 #8165}.

Statistical analyses

Student's T test was performed to assess statistical significance from controls. Results with $P < 0.05$ were considered statistically significant (*). Results represent the mean ± SD of 3 independent experiments. For Western blotting, data are representative images of 3 independent experiments.

Results

BTK is expressed and constitutively phosphorylated in AML.

It has been recently reported that the non-receptor tyrosine kinases LYN and SYK, which have been shown to play an important role in normal B cell differentiation and hematopoietic signalling, are potential targets for AML therapy [31,32]. As both LYN and SYK are known to activate BTK in haematologic cells [33,34] and BTK mRNA had previously been reported to be expressed in AML [24] we examined the basal activity levels of BTK in human AML. Initially we determined the frequency of BTK expression in human primary AML blasts and how its level of expression compares to normal CD34+ HSC. Real-time assessment of BTK mRNA in 25 primary human AML patients and 3 AML cell lines, showed it to expressed at comparable levels to control CD34+ HSC (not shown). Indeed, total BTK protein expression in AML samples (Figure 1A) shows its expression in these cells. However, using phosphorylation at Y223 as a marker of BTK activation (Figure 1A), we identified a significant correlation between phospho BTK and total BTK expression in the AML samples. Immunocytochemical analysis showed (in all but one case) there was an average 3 times greater BTK activity in AML cells than was present in comparator non-malignant CD34+ cells (Figure 1B and 1C). We also tested phosphorylation at Y551. Although we could not detect a signal by Western blotting, immunocytochemical analysis of AML cell lines showed similar results to that observed with Y223 (data not shown). These observations showed that BTK was not only ubiquitously expressed in our patients AML but had increased activity (as measured by phosphorylation relative to non-malignant CD34+ cells) in over 90% of the primary AML patient samples tested, Taken together this implies that BTK may be functionally significant in human AML

Pharmacological inhibition of BTK induces cell death in primary AML blasts.

Since the irreversible BTK inhibitor, ibrutinib has been shown to be cytotoxic in-vitro in CLL, MCL and MM [18,35,36], we next asked whether BTK inhibition would reduce cell viability in primary AML blasts. However, we first wanted to determine the level of occupancy of BTK by ibrutinib in AML cells. To do this we treated AML#22 and U937 with increasing doses of ibrutinib (3-1000nM) for 1 hour, cells were washed in PBS and frozen at 80C. By using the fluorescently tagged ibrutinib derivative PCI-33380 {Honigberg, 2010 #8165}, we found that 10 nM of ibrutinib was sufficient to fully occupy the active site of Btk in AML#22 and U937. These results mimic the results observed in CLL. Next, we tested the in-vitro activity of ibrutinib in a spectrum of primary AML cells from a wide age spectrum of adult patients and across a range of WHO AML subclasses (Supplementary Table 1). AML cells from 25 patients were treated with increasing concentrations of ibrutinib for 72 h. We found that ibrutinib exhibited a significant induction of cytotoxicity in AML patient cells (Figure 2A). IC50s were calculated for all AML patient samples (Supplementary Table 2). Cell death was examined separately by annexin-V and propidium iodide staining. We found that ibrutinib as a single agent at concentrations of 1 μM and 5 μM induced early apoptosis (annexin-V positive only) in AML (Supplementary Figure 1). We also examined the viability of 4 AML cell lines in response to ibrutinib. The results showed that only U937 had significant induction of cytotoxicity in response to ibrutinib (IC50 ~ 2.6 μM) (Supplementary Table 2). Primary AML blasts were significantly more sensitive to ibrutinib than comparator CD34+ HSC (IC50s > 20 μM) for the control CD34+ HSC shown in Supplementary Table 2). Correlation analysis of the data from BTK phosphorylation (Figure 1C) and ibrutinib IC50 levels (Supplementary Table 2) showed that there was a high correlation between high BTK phosphorylation and cytotoxicity to ibrutinib (Figure 2B). Figure 2C

shows the effect of ibrutinib on AML cell lines. As a control/comparator we tested the cytotoxic effects of ibrutinib in primary human CLL cells and showed levels of cytotoxicity (IC50s ~ 5 μM) in primary CLL cells similar to that achieved in AML at equivalent concentrations of the drug (Supplementary Figure 2) and compatible with in-vitro CLL data previously published (cytotoxic IC50s 5 – 50 μM)[37].

Ibrutinib inhibits AML proliferation

We next explored the effect of ibrutinib on AML blast colony formation in methylcellulose compared to normal CD34+ myeloid progenitor cells. AML samples were more sensitive to the effects of ibrutinib at concentrations of 1 μM, 5 μM and 10 μM than normal CD34 myeloid progenitor cells (Figure 3A). These observations show that in AML cytotoxicity can be achieved at ibrutinib concentrations not toxic to non-malignant normal residual CD34 positive cells.

Next we examined the effects of ibrutinib on AML proliferation in response to cytokines known to induce proliferation in AML blasts {Vellenga, 1987 #8501}{Westermann, 1996 #8502}. We used IL-3, GM-CSF, SCF and TNF to induce proliferation of AML blast. Pre-treatment of these AML blasts with ibrutinib (10-1000 nM) inhibited proliferation in IL-3, GM-CSF, SCF but not TNF treated cells (figure 3B).

Genetic inhibition of BTK reduces colony formation from AML cells

We next evaluated the genetic inhibition of BTK in AML cell lines and AML blasts. To do this we generated lentivirus-mediated long-term BTK knockdown using targeted artificial microRNA (BTK-targeted miRNA) and visualisation of infected cells by a concurrently expressed GFP signal tag. These constructs induced GFP expression and BTK knockdown confirmed for up to 16 days by real-time PCR and protein expression (Figure 4A and B) and the role of BTK

in cell viability and long-term clonogenic assays was assessed. The introduction of BTK-specific miRNA dramatically inhibited the proliferation of U937 but had no effect on TF-1 cells (Figure 4C) which show control levels of pBTK activity. With BTK-miRNA targeted knockdown we saw a reduction in methylcellulose colony formation in high pBTK expressing primary AML blasts and U937 [3/3 samples tested] but not in low pBTK expressing AML blasts and TF-1 [3/3 samples tested] or non-malignant CD34+HSC, compared to control-miRNA targeted cells (Figure 4D) suggesting when present pBTK appears to play a central role in AML proliferation and maintenance in these cells.

Ibrutinib inhibits NF-κB survival genes in AML.

We and others have previously reported that BTK is involved in p65-mediated transactivation during NF-κB activation in macrophages and malignant plasma cells [12,38]. As p65 phosphorylation is therefore necessary for induction of NF-κB /p65-dependent gene expression in other haematologic cells [9], we sought to determine if a similar role for BTK exists in AML. To do this we employed a PCR-based NF-κB gene expression array by examining the RNA expression of 84 NF-κB.genes from control CD34+ HSC, primary AML cells and AML cell lines treated with ibrutinib 5 μM for 16 h. Results showed that ibrutinib dramatically reduced the expression of NF-κB target genes from AML patient cells with high BTK phosphorylation but not CD34+ HSC and AML cells with low BTK phosphorylation levels (Supplementary Figure 3A). A similar pattern of genes regulation was observed in U937 and TF-1 in which BTK was knocked-down by BTK-targeted miRNA (Supplementary Figure 4).

To validate the results obtained with the PCR NF-κB arrays, we examined the expression of the genes highlighted above with quantitative RT-PCR. The quantitative RT-PCR analysis confirmed our initial observation that decreases

in the expression of NF-κB survival genes and NF-κB transcription factors was seen in response to the BTK inhibitor (Supplementary Figure 3B). Thus, modulation of BTK activity or expression results in selective effects on known NF-κB target survival genes.

To further verify the involvement of NF-κB, and specifically p65 we treated AML cells with ibrutinib and by Western blotting examined p65 phosphorylation. In primary AML blasts and AML cell lines we found that ibrutinib can inhibit p-p65 expression in high pBTK cells (2/2 samples tested) but not in low pBTK expression AML cells (2/2 samples tested) (Figure 5A). This observation was confirmed on AML cell lines U937 and TF-1 with BTK knockdown (Figure 5B). To determine if BTK inhibition had an effect on NF-κB nuclear activity we examined p50, p65 and c-Rel binding activity in AML blasts and AML cell lines. We found that ibrutinib significantly effected p50 and p65 binding activity but not c-Rel (Figure 5C).

Silencing BTK inhibits the AKT pathway in AML cells

The PI3K/AKT pathway is frequently found to be activated in AML [39,40]. AKT phosphorylation on Ser473, assayed by Western blotting analysis of purified blast cells, can be detected in 50-80% of AML patients [41,42] . The mechanisms leading to PI3K/AKT activation in AML are not completely clear. The p110δ isoform of class IA PI3K, is always expressed in AML cells, whereas the p110α and p110β isoforms are heterogeneously expressed and the frequency of p110γ isoforms is unknown [43,44]. Moreover, inhibition of BTK by ibrutinib has been shown to inhibit AKT phosphorylation in CLL and MCL {Herman, 2011 #5689}{Chang, 2013 #8503}.

Here we hypothesised that BTK was upstream of PI3K/AKT. To verify the role of BTK in constitutively active PI3K/AKT we treated U937 and TF-1 cell lines

with 1 μM ibrutinib for various times and extracted protein lysates. We then analysed these extracts for phosphorylated AKT at ser473 by Western blotting. Since ERK has also been shown to be inhibited by ibrutinib we also examined pERK levels in the same samples. Figure 5D shows that expression of phospho AKT and phospho ERK in U937, but not TF-1, is reduced in response to ibrutinib when compared to total AKT and ERK and β-actin. To validate the results obtained with ibrutinib we also analysed pAKT in U937 with BTK437 KD. This showed that U937 treated with BTK437 KD had significantly reduced pAKT (Figure 5E). To further validate these observations we examined pAKT and pERK levels in primary AML in response to various concentrations of ibrutinib (3-1000nM). Figure 5F demonstrates that even at low levels of ibrutinib pAKT and pERK are inhibited in primary AML. These results confirm that BTK is upstream of constitutively active PI3K/AKT and pERK signalling in human AML.

Pharmacological inhibition of BTK enhances the effect of conventional chemotherapy in reducing cell viability and colony formation of AML blasts

Chemotherapeutics frequently act synergistically and are often used clinically in combination. Here we looked to determine if ibrutinib could act synergistically with either cytarabine or daunorubicin, two widely used drugs in the treatment of AML. In 48 h cultures of AML patients cells and cell lines, ibrutinib significantly increased cytotoxicity, when in combination with either cytarabine (0.1 and 0.5 μM) or daunorubicin (0.1 and 0.05 μM) (Figure 6A). Furthermore, in assays of primary human AML blasts ibrutinib when added to cytarabine reduced its IC50 by a median of 1.5 fold (range 0.18 – 3.55, n = 5) and when added to daunorubicin reduced the IC50 by a median of 3.1 fold (range 0.72 – 15.14, n = 5). Ibrutinib had no effect on the cytarabine and daunorubicin IC50s of non-malignant CD34+ cells (Supplementary Table 2).

Similarly ibrutinib augmented the cytotoxic effect of both cytarabine and daunorubicin to AML in colony forming cell assays (Figure 6B)

Ibrutinib inhibits AML cell adhesion to BMSCs

The interaction between AML blasts and the BM micro-environment is critical in regulating tumor survival and chemotherapy resistance. Inhibiting AML blast adhesion to BMSCs via the CXCR4-SDF-1 axis or by VLA4-VCAM/fibronectin interactions via the PI-3K/AKT/Bcl-2 signalling pathway is associated with improvements in tumor cytotoxicity [45,46]. Because we found BTK functions directly upstream of AKT in AML blasts and as BTK inhibition with ibrutinib is known to perturb the interaction between the tumor cell and micro-environment in CLL and myeloma [15,37], we sought to establish whether BTK functions in the important relationship between the AML tumor cells and the BM stromal niche that protect them. To this end we used a calcein-AM fluorescence-based adhesion assay to determine if BTK inhibition by ibrutinib affects binding of AML cell lines and AML blasts to BMSCs. Figure 7A shows a representative example of the calcein-AM treated THP-1 cells on BMSCs after an 8 h co-culture with and without ibrutinib treatment. Overall treatment with various ibrutinib concentrations ≥ 0.1 μM significantly reduced the adhesion of AML cell lines and primary AML blasts to BMSCs (Figure 7B and 7C) regardless of the BTK phosphorylation status of the AML cells. Moreover, the concentration needed to significantly inhibit AML-BMSC adhesion of 0.1 μM has little or no effect on cytotoxicity of primary AML cell and AML cell lines (Figure 2A and C). These results demonstrated that ibrutinib effectively disrupts the interactions between AML cells and BMSC.

BMSCs provide no protection for AML blasts from ibrutinib induced apoptosis

Because others have shown that BMSC can protect AML cells from chemotherapy-induced apoptosis [45-47], we wanted to assess the anti-leukemic efficacy of ibrutinib in AML blasts under BMSC co-culture conditions. To do this we cultured AML blasts from AML#17 (low pBTK) and AML#19 (high pBTK) alone or co-cultured with BMSCs, in the presence or absence of ibrutinib. Ibrutinib induced apoptosis in a concentration-dependent manner in AML#19 cells cultured alone or co-cultured with BMSCs, as determined by annexin-V staining (Figure 7D). No apoptosis was observed in AML#17 cells treated with ibrutinib either cultured alone or co-cultured with BMSCs. The effect of ibrutinib on AML#17 was anticipated as ibrutinib had not previously been shown to be cytotoxic to this sample. These findings show that inhibition of BTK activity with ibrutinib induces apoptosis in AML cells in response to ibrutinib alone even when co-cultured with BMSCs. However, as the stromal cells are known to protect AML from conventional cytotoxic drugs such as cytarabine, ibrutinib delivers a dual anti-leukemic effect by (i) releasing leukemic cells from the cytotoxic protecting microenvironment and (ii) direct cytotoxicity independent of stromal detachment.

Discussion

Outcomes for the 75% of patients who get AML over the age of 60 remain generally poor, largely because the intensity and side effects of existing curative therapeutic strategies (which are commonly used to treat younger fitter patients) coupled to patient co-morbidities, frequently limit their use in this older less fit population. Consequently, there is an urgent need to identify pharmacological strategies in AML, which are not only effective but can be tolerated by the older less well patient. It is envisaged that treatments which target tumor-specific biology will help realise this goal.

In this work, we build upon the observations that SYK and LYN, two non-receptor tyrosine kinases, have been identified as possible AML targets [31,32]. Downstream of SYK and LYN is BTK, 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 [48]. Like other Btk family members, it contains a pleckstrin homology (PH) domain and Src homology SH3 and SH2 domains [19]. Btk activation has been implicated in a variety of hematopoietic cellular responses and there is a growing literature supporting the role of BTK in a spectrum of B cell-derived hematological malignancies [18]. In our studies we demonstrate that BTK is expressed and constitutively active in circa 90% of AML samples tested (relative to normal CD34+ cells) and furthermore AML growth and survival appears to be dependent on the presence of BTK.

Ibrutinib (formally PCI-32765) is a selective covalent inhibitor of BTK. It is rapidly absorbed, potently irreversibly binds to BTK then is rapidly renally excreted [49], and it shows selectivity for BTK against a panel of kinase enzymes. To recapitulate this on/off phenomena in vitro, a 1 h pulse exposure of cells to 5 μM ibrutinib followed by washout has been shown to fully inhibit

BTK gene expression and furthermore minimises in vitro non-specific toxic effects in these experiments. Early phase studies in CLL and lymphoma have produced not only particularly encouraging results but have also shown the drug to be generally well tolerated with limited toxicity [14,50]. In our studies, the majority (12/21) of primary patient AML samples responded in a concentration-dependent manner, with an IC50 on or below the concentrations used in similar CLL studies [36,50]. Thus, these observations suggest that inhibition of BTK in AML could be achieved with the doses currently used in clinical trials for CLL and lymphoma.

Ibrutinib enabled us to reduce the IC50 of daunorubicin and cytarabine by approximately 3.5 and 1.5 fold respectively in primary AML samples but did not change the sensitivity of control non-malignant CD34+ cells to these cytotoxic drugs. This leads us to hypothesise whether in the future regimens including BTK inhibition may permit dose reductions of the cytotoxic drugs but maintain cytotoxic efficacy. If such approaches were associated with a more favourable side effect profile, there would be scope for their use across a greater spectrum (including age and co-morbidities) of AML patients than standard dose cytotoxics alone.

In this study we describe a significant correlation between BTK Y223 phosphorylation and the cytotoxicity of ibrutinib as measured by viability, apoptosis, and colony formation in methylcellulose (Figures 2A-D). In contrast, ibrutinib had minimal effects on apoptosis in control CD34+ HSC, AML samples and cell lines with low BTK Y223 phosphorylation. However, some AML samples that exhibited Y223 phosphorylation, such as AML#17 were relatively insensitive to ibrutinib whereas AML#7 with lower levels of Y223 phosphorylation were very sensitive, suggesting that at least in vitro there are

factors other than the degree of BTK phosphorylation that may modify responsiveness to ibrutinib.

Interestingly, we showed that AML blasts express BTK mRNA or protein at levels comparable to that observed in human CD34+ HSC. This demonstrates that the high BTK activity seen in this study is not primarily due to differential BTK expression, but probably more the result of upstream BTK regulators. Likely candidates include SYK and LYN, both of which have been shown to have constitutive activity in AML. Determining the mechanism of activation of BTK in AML is under investigation, however, we have demonstrated evidence of both AKT signalling alteration and NF-κB gene expression and activity after ibrutinib treatment or BTK knockdown. This suggests that BTK is involved in pro-survival signals within AML.

Leukaemia stem cells can infiltrate the bone marrow niche and may hijack the normal homeostatic processes, leading to enhanced self-renewal, proliferation and resistance to chemotherapeutic agents [47]. Moreover drug strategies that disrupt the interaction between the AML tumor cell and its microenvironment appear to increase the cytotoxicity of conventional chemotherapies [45,46]. Ibrutinib in the lymphoid malignancies appears to function in-part by disrupting the interaction between the tumor cell and the bone marrow/lymph node niche. In AML we report that ibrutinib as well as being directly cytotoxic to AML in vitro also inhibits cell adhesion to bone marrow stromal cells and that the same BMSC do not confer any protection from ibrutinib-induced apoptosis. This data leads us to hypothesize that ibrutinib will improve the efficacy of standard chemotherapeutic drugs in patients with AML, not only by directly potentiating cytotoxicity of the cells, but additionally by perturbation of tumor cell adhesion to the microenvironment stromal cells that protect and maintain them.

In conclusion, we show that BTK is activated and functional in primary AML blasts. We demonstrate that the majority of primary AML blasts display a therapeutic response to BTK inhibition, with efficacy on cell growth, adhesion and colony formation capacity. With an orally available, well-tolerated BTK inhibitor currently in clinical trials in other haematological malignancies, the results reported here should have immediate relevance for clinical testing of ibrutinib in patients with AML. Furthermore, these results provide a rationale for combining ibrutinib with conventional AML therapies and exploring the opportunities for lowering the doses of daunorubicin and cytarabine in combination with BTK inhibition.

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Authorship Contributions

SAR, KMB and DJM designed the research. SAR, LZ and MYM performed the research. SAR, KMB and DJM wrote the paper.

Disclosure of Conflicts of Interest

The authors declare no conflicts of interest.

Figure Legends

Figure 1. BTK is highly expressed and constitutively phosphorylated in AML. (A) Control CD34+ cells, AML patient cells and AML cell lines measured for constitutive levels of BTK phosphorylation by Western blotting, blots re-probed for wild type BTK and β-actin to show BTK expression and sample loading respectively. (B) Primary CD34+ cells, AML cells and AML cell lines were analysed for phosphorylated BTK (Y223) (green) and total BTK (red) by immunocytochemistry. DAPI nuclear stain is shown in blue. (C) Using the immunocytochemical images captured, phospho-BTK was calculated as a percentage of total BTK. Values indicate the mean ± SEM from at least 5 individual experiments, sampling at least 10 representative cells from each view. * indicates statistical significance of $P < 0.05$ between the different treatment groups using Student's T test.

Figure 2. BTK occupancy by the BTK inhibitor ibrutinib and pharmacological inhibition of primary AML blasts. (A) AML blasts and the AML cell line U937 were treated with increasing doses of ibrutinib for 1 h and then assayed for BTK occupancy using ..... (B) AML blasts and CD34+ control cells were treated with increasing doses of ibrutinib (0.1 – 10 μM) for 72 h and then assessed by Cell TitreGlo. Data were normalised to DMSO treated cells and represents the means ± SD, n = 3. (C) Correlation analysis of IC50 values of AML blasts treated with ibrutinib and % BTK phosphorylation. (D) AML cell lines were increasing doses of ibrutinib (0.1 – 10 μM) for 72 h and then assessed by Cell TitreGlo. Data were normalised to DMSO treated cells and represents the means ± SD, n = 3.

Figure 3. Ibrutinib antagonises AML colony forming and cell proliferation. (A). AML blasts AML cell lines and CD34+ control cells were treated with 0.5, 1, 5 and 10 μM ibrutinib and colony forming assays were performed to show the number of colonies. Data were normalised to DMSO treated cells (B) Primary AML blasts (n=6) were pretreated with increasing doses of ibrutinib (0.01-1 μM) for 1 hour and then treated with either GM-CSF (ng/ml), SCF (10ng/ml) IL-3 (10ng/ml) and TNF (10ng/ml) for 72 hours. Data were normalised to DMSO treated cells.

Figure 4. Genetic inhibition of BTK inhibits cell viability in AML cell lines. AML cell lines (TF-1 and U937) were transduced with BTK-targeted miRNA GFP-tagged lentiviral constructs. (A) Transfected cells were measured for GFP expression using flow cytometry for two BTK targeted miRNA (BTK437 and BTK1092) in TF-1 cells. RNA was extracted from TF-1 and U937 cells transduced with BTK-targeted and non-silencing miRNA control constructs and examined for BTK expression by real-time PCR at the indicated times. mRNA expression was normalized to GAPDH mRNA levels. (B) Protein extracts were also obtained and Western blot analysis was conducted for pBTK and BTK protein levels. (C) TF-1 and U937 were transduced with either BTK-targeted miRNA or non-silencing control miRNA construct for 72 h, Cell number was assessed by Cell TitreGlo assay. (D) AML blasts, AML cell lines and CD34+ HSC were transduced with BTK-targeted miRNA and control miRNA constructs and colony forming assays were performed to show the number of colonies detected. In all panels values indicate the mean ± SD from 3 independent experiments. * indicates statistical significance of $P < 0.05$ between the different treatment groups.

Figure 5. AKT and NF-κB activity in AML cells is augmented by BTK inhibition. (A) AML cell lines and AML blasts were treated with 1 and 5 μM of

ibrutinib for 4 h and then whole cell extracts were prepared and Western blot analysis was conducted for p-p65 and β-actin protein levels. (B) AML cell lines (TF-1 and U937) were transduced with BTK-targeted miRNA GFP-tagged lentiviral constructs (BTK437 and BTK1092) as well as negative control. Protein extracts were also obtained and Western blot analysis was conducted for p-p65 and β-actin protein levels. (C) AML cell lines and AML blasts were treated with 1 and 5 μM of ibrutinib for 4 h and then nuclear extracts were prepared and Western blot analysis was conducted for nuclear p50 and TBP protein levels. (D) AML cell lines (TF-1 and U937) were transduced with BTK-targeted miRNA GFP-tagged lentiviral constructs (BTK437 and BTK1092) as well as negative control. Nuclear extracts were prepared and Western blot analysis was conducted for nuclear p50 and TBP protein levels. (E) AML cell lines and were treated with 5 μM of ibrutinib for various times and then whole cell extracts were prepared and Western blot analysis was conducted for pAKT-S473, pAKT-T308, total AKT and β-actin protein levels. (F) AML cell line (U937) were transduced with BTK-targeted miRNA GFP-tagged lentiviral constructs (BTK437) as well as negative control. Whole cell extracts were prepared and Western blot analysis was conducted for pAKT-S473, total AKT and β-actin protein levels.

Figure 6. Reduced viability and colony formation of AML cells following inhibition of BTK in combination with conventional chemotherapy. (A) AML blasts and CD34+ control cells were either untreated or treated with ibrutinib (1 μM) for 8 h and then treated with either cytarabine (0.1 μM or 0.5 μM) or daunorubicin (0.05 μM or 0.1 μM) for 72 hours and then assessed by Cell TitreGlo. Values indicate means ± SD, n = 3. (B) AML cells and control cells were either untreated or treated with ibrutinib (1 μM) for 8 h and then treated with either cytarabine (0.1 μM) or daunorubicin (0.05 μM) and then colony forming assays were performed to show the number of colonies. In all

panels values indicate the mean ± S.D. from 3 independent experiments. * indicates statistical significance of $P < 0.05$ between the different treatment groups using Student's T test.

Figure 7. AML-BMSC adhesion and protection is disrupted by Ibrutinib. (A) Light and fluorescence microscopic images show co-cultured calcein-AM treated THP-1 cells and BMSC with and without 0.5 μM ibrutinib treatment for 8 h. (B) Percentage of AML cell lines and (C) primary AML blasts attached to the primary AML BMSCs in the co-culture setting in the presence and absence of various concentrations of ibrutinib for 8 h. (D) AML blasts from AML#17 (low pBTK) and AML#19 (high pBTK) were left alone or co-cultured with BMSCs, in the presence or absence of various concentrations of ibrutinib for 48 h and then stained for annexin-V and analysed by flow cytometry. In all panels values indicate the mean ± SD from 3 independent experiments. * indicates statistical significance of $P < 0.05$ between the different treatment groups.

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