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

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Key Points

- Inhibition of Bruton’s tyrosine kinase is as effective in-vitro against acute myeloid leukemia (AML) as chronic lymphocytic leukemia (CLL)
- Ibrutinib shows activity in AML because Bruton’s tyrosine kinase is constitutively active

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 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 ibrutinib binds at nanomolar range to BTK. Furthermore, we also showed ibrutinib’s anti-proliferative effects in AML are mediated via an inhibitory effect on 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 efficacious in chronic lymphocytic leukemia (CLL). These results provide a biologic rationale for clinical evaluation of BTK inhibition in AML patients.
Introduction

Acute myeloid leukemia (AML) is primarily a disease of the elderly\(^1\). In younger patients (<65 years, median diagnosis-age 72), there is improved survival over the decades, however older patients have seen no similar improvement, with intensive cytotoxic treatment a dilemma\(^2\). AML comprises a heterogenous group of tumors. Despite this diversity, AML relies on common programs of self-renewal downstream of the driver oncogenes suggesting disease is caused by only a few mutations\(^3\), and mechanistically common therapeutic approaches are broadly useful despite oncogenic involvement\(^4\).

Tyrosine kinases (TK) are attractive drugable targets in cancer. In AML TK activating mutations occur in 50% of patients\(^5,6\). Furthermore TK-dependent cell survival pathways are dysregulated in most cases\(^7-10\). Bruton’s tyrosine kinase (BTK) has been identified as functionally important in malignant haematopoietic cells. BTK, originally identified functionally in B-cell receptor (BCR) signaling, with mutations blocking B-cell development\(^11-13\). Other receptors (including toll-like receptors (TLR)) are BTK-dependent. Recent phase 1&2 studies of the irreversible BTK inhibitor, ibrutinib have demonstrated promising activity and tolerability against B-cell malignancies including, chronic lymphocytic leukemia (CLL), mantle cell lymphoma, hairy cell leukaemia, multiple myeloma and diffuse large B-cell lymphoma\(^14-20\). In addition to lymphoid cells, BTK expression has also been found in hematopoietic stem cells (HSC), multipotent progenitors, and several other haematopoietic cells including erythroid and megakaryocytic cells\(^21\). Furthermore, it known that BTK-deficiency/inhibition affects myeloid cells including macrophage LPS/TLR-induced TNF production\(^22\), dendritic cell function via IL-10 and Stat3\(^23\), neutrophil development\(^24,25\), and collagen-induced platelet aggregation\(^26,27\). Moreover high BTK phosphorylation/expression are observed in AML\(^28,29\). Here we explain BTK
function in human AML and describe the pharmacological effects of BTK inhibition by ibrutinib on AML proliferation and bone marrow adhesion.
Methods

Materials

AML-derived cell lines were obtained from European Collection of Cell Cultures, authenticated by DNA-fingerprinting. They are used at low passage for 6 months maximum post-resuscitation, with regular Mycoplasma testing. Anti-NF-κB/AKT antibodies were from Cell Signaling Technologies. Total-BTK [D3H5], p-Y223-BTK [EP420Y] and p-Y551-BTK [EP267Y] antibodies were from Abcam. Other antibodies were from Santa Cruz Biotechnology. Ibrutinib was obtained from Selleck Chemicals. Stem cell factor (SCF), interleukin-1 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF were from Invitrogen. All other reagents obtained from Sigma-Aldrich, unless indicated.

Cell culture

AML cells were obtained from patient’s bone marrow or blood following informed consent in accordance with the Declaration of Helsinki 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 density-gradient centrifugation. AML samples >80% blasts were purified using CD34+-selection kit (Miltenyi Biotec)(denoted by * in Supplementary Table 1). We obtained hematopoietic CD34+ cells from two sources, Stem Cell Technologies and volunteers. For all CD34+ experiments at least three different donors were used to obtain the results presented. Cell type was confirmed by microscopy and flow cytometry.

Human bone marrow stromal cells (BMSCs) were isolated from AML patient bone marrow aspirates. Mononuclear cells were collected by gradient
centrifugation and plated in growth-media. 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 expression of CD105, CD73, CD90 and lack of CD45 and CD34 expression$^{30,31}$. 

Real-time PCR
Total RNA was extracted from $5 \times 10^5$ cells using the Nucleic acid Prep Station. Reverse transcription was performed using RNA PCR core-kit (Applied Biosystems). Relative quantitative real-time PCR used SYB-green technology (Roche) on generated cDNA. After preamplification (95°C/60s), PCRs were amplified for 45 cycles (95°C/15s, 60°C/10s, 72°C/10s) on a Roche 384-well LightCycler480. mRNA expression was normalized against GAPDH.

Western and NF-κB-binding assays.
Western analyses were performed as described$^{32,33}$. NF-κB-binding in AML cells used TransAM NF-κB family Transcription Factor assay kit (ActiveMotif).

Immunocytochemistry
Primary CD34+ cells, AML lines and primary samples were fixed, permeabilised and blocked with goat serum. Samples were stained with primary antibodies against BTK or phosphorylated-Y223-BTK (Cell Signaling Technology), and visualised with secondary Alexa Fluor® 568- or 488-conjugated IgG (H+L)(Invitrogen), respectively. Cell nuclei were visualised with DAPI before samples were mounted with FluoromountTM aqueous mounting medium. Cells were imaged by AxioCam ICM1 monochrome CCD camera attached to the Apotome.2 Imaging System using Axiovision 4.8.2 software (Carl Zeiss). Image staining intensities were analysed with ImageJ software (n=20 per sample).
Proliferation/death assays
Cells were treated with different doses of ibrutinib then viable numbers measured with Cell-Titre GLO (Promega). For pro-survival assays, cells were cultured in serum-free media as described\textsuperscript{34}, with indicated ibrutinib concentrations and cytokines, then measured for proliferative activity using Cell-Titre GLO or BrdU proliferation assay (Cell Signalling). Apoptosis measurements were performed by Accuri-C6 flow cytometry (BD biosciences), with annexin-V/propidium iodide (PI) (Abcam). For AML-BMSC co-cultures, AML cell viability was measured by flow cytometry with BMSC forward-scatter exclusion gating.

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 negative control. MicroRNA-encoding sequences were cloned into Block-iT Pol II miR-RNAi vector (Invitrogen) then EmGFP-pre-miRNA fragments subcloned into \textit{BamHl/Xhol} site of LNT/SffvMCS (gift from Penny Powell, UEA). MicroRNA-encoding viruses were produced as described\textsuperscript{35}, using packaging plasmids pCMVΔR8.91 (expressing gag-pol) and pMD.G (expressing VSV-G) (provided by Ariberto Fassati, UCL). Lentiviral stocks were concentrated using Lenti-X\textsuperscript{TM} Concentrator (Clontech) and titers obtained with Lenti-X\textsuperscript{TM} qRT-PCR Titration kit (Clontech). For transduction, cells plated onto 12-well plates (5x10\textsuperscript{4} cells/well/0.5ml) were infected with lentiviral stocks (MOI of 15) with 8 μg/ml Polybrene\textsuperscript{®}.
Clonogenic methylcellulose assays
Control CD34+ HSC, AML cell lines and primary AML cells (1-50x10^3 cells) were plated in methylcellulose (R&D). Colonies were visualized and counted after 10-15 days.

BMSC-AML cell adhesion assay
BMSCs were grown in 96-well plates. AML cell lines and primary cells were incubated for 1 h with 2.5 µM calcein-AM. Fluorescently-labeled AML cells were added onto BMSCs and incubated for the indicated times. Gentle washing removed non-adherent calcein-labeled cells. Adherent cells were quantitated with a fluorescence plate reader.

PCR gene array
CD34+ control cells, AML cell lines and primary were pretreated +/- 5 µM ibrutinib (16 h). Resultant cDNA was incubated on an NF-κB Signaling Pathway RT2 qPCR array (SABiosciences, UK), containing 84 key NF-κB genes, normalized to GAPDH expression.

BTK occupancy assay
Primary AML cells and U937 were treated with increasing concentrations of ibrutinib (0.3-1000 nM) for 1 h. Cells were then washed in PBS, stored at -80°C until BTK occupancy assay was performed as described36.

Statistics
Student’s T-test was performed with P<0.05 considered statistically significant (*). Results represent means ± SD of 3 independent experiments. Western blotting data is representative 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, play an important role in normal B-cell differentiation and hematopoietic signalling, are potential targets for AML therapy. As both LYN and SYK are known to activate BTK in hematologic cells and BTK mRNA had previously been reported to be expressed in AML we examined basal activity levels of BTK in human AML. We determined the level of BTK expression in human primary AML blasts comparing to normal CD34+ HSC. Real-time PCR assessment of BTK mRNA in 25 primary human AML patients and 3 AML cell lines, showed BTK expressed at comparable levels to control CD34+ HSC (not shown). Furthermore using phosphorylation at Y223 as a marker of BTK activation (Figure 1A), we identified a significant correlation between p-BTK and total-BTK expression in AML samples. Immunocytochemical analysis showed a median 3.1 fold greater p-BTK activity in primary AML cells than in comparator non-malignant CD34+ cells (Figure 1B and 1C). We also tested phosphorylation at Y551 as a marker of BTK activity. Although we could not detect a signal by Western blotting, immunocytochemical analysis of AML cell lines showed similar results to Y223 probe (not shown). These observations show BTK is ubiquitously expressed in AML, with increased activity (as measured by phosphorylation relative to non-malignant CD34+ cells) in over 90% of the primary AML patient samples tested. Together this implies BTK is functionally significant in human AML.

Pharmacological inhibition of BTK in primary AML blasts.

Since the irreversible BTK inhibitor, ibrutinib has been shown to inhibit proliferation in-vitro in CLL, MCL and MM, we tested whether BTK inhibition would reduce cell viability in primary AML blasts. First we established the level of occupancy of the BTK active site by ibrutinib in AML.
cells. To do this, we treated primary AML sample #22 and AML cell line U937 with increasing concentrations of ibrutinib (3-1000 nM) for 1 h. Using the fluorescently-tagged ibrutinib derivative PCI-33380\textsuperscript{36}, we found that 10 nM ibrutinib was sufficient to fully occupy BTK in both AML#22 and U937 cells (Figure 2A). EC50s between 0.3 and 3 nM were observed in AML cells, which is comparable to BTK occupancy data in CLL\textsuperscript{36}. Next, we tested in-vitro activity of ibrutinib in primary AML cells from a broad age-range of adult patients (26-92 years) and across a spectrum of WHO AML subclasses (Supplementary Table 1). AML cells from 25 patients were treated with increasing concentrations of ibrutinib for 72 h and compared with non-malignant CD34+ cells. We found ibrutinib exhibited concentration-dependent cytotoxicity in AML patient cells (Figure 2B). IC50s were calculated for all AML patient samples with AML cells exhibiting greater sensitivity to ibrutinib than comparator non-malignant CD34 positive cells (Supplementary Tables 1&2). Separate annexin-V/PI staining found 1-5 µM ibrutinib-induced AML apoptosis (Supplementary Figure 1). Examining viability of 4 AML cell lines showed only U937 had significant cytotoxic-response to ibrutinib (IC50 ~2.6 µM)(Figure 2C, Supplementary Table 2). Correlation analysis of BTK phosphorylation (Figure 1C) and ibrutinib IC50s (Supplementary Tables 1&2) showed high correlation between high-p-BTK and sensitivity towards ibrutinib (Figure 2D). As a control/comparator we tested ibrutinib cytotoxicity in six primary human CLL samples that showed comparable sensitivity (IC50s ~5 µM) to that achieved in AML (Supplementary Figure 2) and compatible with in-vitro CLL data previously published (cytotoxic IC50s 5-50 µM)\textsuperscript{43}.

**Ibrutinib inhibits AML proliferation**

We explored ibrutinib’s effect on AML blast colony formation in methylcellulose compared to normal CD34+ myeloid progenitor cells. We assayed colony formation across a range of AML samples that are both
sensitive and resistant to ibrutinib in assays (Supplementary Table 1). We found in AML samples and U937 cells that ibrutinib inhibited colony forming cellular proliferation (Figure 3A). Since ibrutinib was shown to inhibit pro-survival signals derived from CLL microenvironment, we examined ibrutinib’s effects on AML pro-survival signals\textsuperscript{34,43,44}. AML cells were pre-treated with ibrutinib (10-1000 nM) for 1 h then cultured with IL-3, GM-CSF, SCF or TNF for 72 h. Ibrutinib inhibited proliferative responses to exogenous IL-3, GM-CSF, SCF but not TNF (Figure 3B). Similarly, a BrdU assay measured AML cell proliferation with 0.5 µM ibrutinib pretreatment before addition of IL-3, GM-CSF, SCF or TNF (Supplementary Figure 3), also showing that ibrutinib-pretreatment inhibited proliferative responses to IL-3, GM-CSF, SCF but not TNF.

**Genetic inhibition of BTK reduces AML cell colony formation**

Genetic inhibition of BTK in AML cell lines and AML blasts was achieved by lentiviral-mediated long-term BTK knockdown using targeted artificial microRNA (BTK-targeted miRNA) and visualisation of infected cells via a concurrently-expressed GFP signal. These constructs induced GFP expression and BTK knockdown for up to 16 days (Figure 4A&B). The role of BTK in cell viability clonogenicity was assessed. Introduction of BTK-targeted miRNA dramatically inhibited U937 proliferation but had no effect on TF-1 cells (Figure 4C) which show control levels of p-BTK activity. With BTK-miRNA-targeted knockdown we observed reduced methylcellulose colony-formation in high-p-BTK-expressing primary AML blasts and U937 [3/3 samples tested] but not in low-p-BTK-expressing AML blasts and TF-1 [3/3 samples tested] or non-malignant CD34+HSC, compared to control-miRNA targeted cells (Figure 4D). This suggests p-BTK plays a central role in AML proliferation and maintenance.
**Ibrutinib inhibits AML NF-κB survival genes**

We and others previously reported BTK is involved in p65-mediated transactivation during NF-κB activation in macrophages and malignant plasma cells\(^{14,45}\). As p65 phosphorylation is necessary for induction of NF-κB/p65-dependent gene expression in other haematologic cells\(^9\), we determined if a similar role for BTK exists in AML. We employed PCR-based NF-κB gene expression array to examine expression of 84 NF-κB genes from control CD34+ HSC, primary AML cells and AML cell lines, treated with 5 µM ibrutinib for 16 h. We found ibrutinib dramatically reduced expression of NF-κB target genes from AML patient cells with high-BTK phosphorylation but not CD34+ HSC and low-BTK phosphorylation level AML cells (Supplementary Figure 4A). 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 5). To validate PCR NF-κB array results, we examined expression of TNF, FLIP, AKT and NF-κB1 (which the array highlighted as BTK-dependent) by qRT-PCR. This analysis confirmed initial observations that decreased NF-κB survival genes and NF-κB transcription factors are seen in response to ibrutinib (Supplementary Figure 4B). Thus, BTK expression or activity regulates selective known NF-κB target survival genes.

To further verify the involvement of NF-κB, and specifically p65 we treated AML cells with ibrutinib and examined p65 phosphorylation. In primary AML blasts and cell lines, ibrutinib was found to inhibit p-p65 expression in high-p-BTK cells (2/2 samples tested) but not in low-p-BTK 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 activities in AML blasts and cell lines. Ibrutinib significantly inhibited p50 and p65 κB binding activity but not c-Rel (Figure 5C). Taken
together, these results confirm BTK is involved in p65-mediated transactivation during NF-κB activation in human AML.

**Silencing BTK inhibits AKT pathway in AML cells**

The PI3K/AKT pathway is frequently activated in AML\(^46,47\). AKT phosphorylation on Ser473 can be detected in 50-80% of AML patients\(^48,49\). Mechanisms leading to PI3K/AKT activation in AML are unclear. The p110\(\delta\) isoform of class-IA PI3K, is always expressed in AML cells, whereas p110\(\alpha\) and p110\(\beta\) isoforms are heterogeneously expressed, with frequency of p110\(\gamma\) isoforms unknown\(^50,51\). Moreover, inhibition of BTK by ibrutinib has been shown to inhibit AKT phosphorylation in CLL and MCL\(^43,52\).

We hypothesised BTK was upstream of PI3K/AKT. To verify the role of BTK in constitutively-active PI3K/AKT, we treated U937 and TF-1 cells with 1 µM ibrutinib for various times, then analysed extracts for ser473 p-AKT. Since ERK signaling was also inhibited by ibrutinib, we examined p-ERK too. Figure 5D shows that expression of p-AKT and p-ERK in U937, but not TF-1, is reduced by ibrutinib when compared to total-AKT, total-ERK and \(\beta\)-actin. To validate the results obtained with ibrutinib we also analysed p-AKT in U937 with BTK437 knockdown. This showed that U937 treated with BTK437 knockdown had significantly reduced p-AKT (Figure 5E). To further validate these observations we examined p-AKT and p-ERK levels in primary AML in response to increasing concentrations of ibrutinib (3-1000 nM). Figure 5F shows low concentrations of ibrutinib inhibit p-AKT and p-ERK in primary AML (Supplementary Figure 6). Moreover, to determine if ibrutinib had off-target effects at concentrations of between 30-10000 nM we examined p38MAPK activity in AML cells, as ibrutinib doesn’t directly inhibit p38 activity\(^53\). Ibrutinib had no effect on p-p38 in AML cells (Supplementary Figure 7A&B). These
data place BTK upstream of constitutively-active PI3K/AKT and p-ERK signalling in human AML.

**BTK inhibition enhances conventional chemotherapy’s effects in AML**

Chemotherapeutics frequently act synergistically and are often used clinically in combination. Here we determined if ibrutinib could act synergistically with either cytarabine or daunorubicin, two widely used AML front-line treatments. In AML patient’s cells and cell lines, ibrutinib significantly increased cytotoxic responses, in combination with cytarabine (0.1-0.5 μM) or daunorubicin (0.05-0.1 μM) in high-p-BTK AML cells (Figure 6A). Furthermore, in high-p-BTK 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), or when added to daunorubicin, reduced IC50s by a median of 3.1 fold (range 0.72-15.14, n=5). Importantly, ibrutinib had no effect on cytarabine and daunorubicin IC50s in non-malignant CD34+ cells (Supplementary Table 3). Similarly, ibrutinib augmented cytotoxic effects of both cytarabine and daunorubicin to high-p-BTK AML cells in colony forming assays, but had no additional effect on non-malignant CD34+ cells (Figure 6B).

**Ibrutinib inhibits AML cell adhesion to BMSCs**

Interaction between AML blasts and its BM micro-environment is critical in regulating tumor survival and chemotherapy-resistance. Inhibiting AML blast adhesion to BMSCs is associated with improved tumor cytotoxicity. Because we found BTK functions directly upstream of AKT in AML blasts, and as BTK inhibition with ibrutinib is known to perturb interactions between CLL and myeloma tumor cell and micro-environment, we established whether BTK also functions between AML tumor cells and their BM-stromal niche that protects them. A calcein-AM fluorescence-based adhesion assay determined if BTK inhibition by ibrutinib affects AML cell lines and blasts binding to
BMSCs. Figure 7A shows a representative example of calcein-AM-treated THP-1 cells on BMSCs after 8 h co-culture +/- ibrutinib. Overall, treatment with ibrutinib concentrations ≥ 0.1 µM significantly reduced adhesion of AML cell lines and primary blasts to BMSCs (Figure 7B&C) regardless of BTK phosphorylation status. Moreover, concentrations needed to 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&C), demonstrating that ibrutinib effectively disrupts interactions between AML cells and BMSC independent of AML p-BTK expression, and at concentrations much lower than those required to cause cytotoxicity.

**BMSCs provide no protection for AML blasts from ibrutinib-induced apoptosis**

Because others showed BMSC can protect AML cells from chemotherapy-induced apoptosis\(^5^4-^5^6\), we assessed the anti-leukemic efficacy of ibrutinib in AML blasts under BMSC co-culture conditions. We cultured AML blasts from AML#17 (low-p-BTK) and AML#19 (high-p-BTK) alone or co-cultured with the AML patient’s very own BMSCs, with or without ibrutinib. Ibrutinib-induced apoptosis in a concentration-dependent manner in AML#19 cells cultured alone or co-cultured with their isotypic BMSCs (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 ibrutinib inhibition of BTK activity induces apoptosis in AML cells 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 its protective microenvironment and (ii) anti-proliferative/cytotoxic responses, independent of any stromal detachment.
Discussion

Outcomes for the 75% of patients diagnosed with AML aged over 60 remain generally poor, largely because of intensity and side effects of existing curative therapeutic strategies (which are commonly used to treat younger fitter patients) coupled to patient co-morbidities, which 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 observations that SYK and LYN have been identified as possible AML targets.\textsuperscript{37,38} Downstream of SYK and LYN is BTK, widely expressed in hematopoietic cells and long known in B-cell differentiation and survival. A Btk/Tec member\textsuperscript{57}, it contains a pleckstrin-homology (PH) domain and SH2\&3 Src-homology domains\textsuperscript{21}. 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\textsuperscript{20}. Our studies demonstrate BTK is expressed and constitutively active (p-BTK) in circa 90% of AML samples tested (relative to normal CD34+ cells) and furthermore, that AML proliferation and survival appears partly dependent on BTK.

Ibrutinib (formally PCI-32765) is a selective covalent inhibitor of BTK. It is rapidly absorbed, potently irreversibly binds to BTK, then is eliminated primarily through metabolism, and shows selectivity for BTK against a panel of kinase enzymes\textsuperscript{36}. We have determined BTK target occupancy of ibrutinib in primary AML and an AML cell line (Figure 2A), showing that 99% of BTK is occupied by ibrutinib at 10 nM in AML. We have correlated this with partial inhibition of SCF-, GM-CSF- and IL-3-induced AML proliferation at 10 nM.
Moreover, downstream signalling pathways including AKT and ERK are also inhibited at 10 nM ibrutinib (Figure 5F). Together, these results show a correlation between BTK-ibrutinib binding with a cellular responses in AML.

Ibrutinib reduced IC50s of daunorubicin and cytarabine by 3.5 and 1.5 fold respectively in primary AML samples, but did not change sensitivity of control non-malignant CD34+ cells to these drugs. This leads us to hypothesise whether future regimens may include BTK inhibition to permit dose reductions of cytotoxic drugs but maintain cytotoxic efficacy. If such approaches were associated with more favourable side effect profiles, there would be scope for their use across a greater spectrum (including age and co-morbidities) of AML patients than the present standard dose cytotoxics alone.

In this study we describe a significant correlation between BTK Y223 phosphorylation and viability of AML cells to ibrutinib, (Figures 2B-D and 3A-B). 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 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 to hijack normal homeostatic processes, leading to enhanced self-renewal, proliferation and chemotherapeutic-resistance\textsuperscript{56}. Moreover, drug strategies disrupting interactions between AML tumor cell and its microenvironment appear to increase cytotoxicity of conventional chemotherapies\textsuperscript{54,55}. In the lymphoid malignancies, ibrutinib appears to function in-part by disrupting interaction between tumor cell and bone marrow/lymph node niche. In AML we report that ibrutinib as well as inhibiting cytokine/chemokine-induced proliferation in AML cells in vitro, also inhibits cell adhesion to BMSCs, and that the same BMSCs do not confer any protection from ibrutinib-induced apoptosis. This data leads us to hypothesize that ibrutinib will improve efficacy of standard chemotherapeutic drugs in AML patients, not only by directly inhibiting proliferation, but additionally by perturbing tumor cell adhesion to microenvironment stromal cells that protect and maintain them.

In conclusion, we show BTK is activated and functional in primary AML blasts. We demonstrate the majority of primary AML blasts display therapeutic responses to BTK inhibition, efficacious on cell growth, adhesion and colony-formation. With well-tolerated BTK inhibitors currently in clinical trials in other haematological malignancies, these results should have immediate relevance for clinical testing in AML patients of ibrutinib and/or other BTK inhibitors either alone or combined.
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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 reprobed for wild type BTK and β-actin to show BTK expression and sample loading respectively. Correlation analysis of p-BTK/BTK expression using densitometry is shown. (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, p-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. Pharmacological inhibition of BTK in 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 occupancy of the BTK active site. (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) 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. (D) Correlation analysis of IC50 values of AML blasts treated with ibrutinib and % BTK phosphorylation.
Figure 3. Ibrutinib inhibits AML 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 or colony forming cells (CFC). 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 (10 ng/ml), IL-3 (10 ng/ml), SCF (50 ng/ml), and TNF (10 ng/ml) for 72 hours and then assessed by Cell TitreGlo. 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 p-BTK 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, ERK and NF-κB activity in AML cells is augmented by BTK inhibition. (A) AML cell lines and AML blasts were treated with 0.5 and 1 µM of ibrutinib for 8 h and then whole cell extracts were prepared and Western blot analysis was conducted for p-p65, 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 0.1-5 µM of ibrutinib for 8 h and then nuclear extracts were prepared and NF-κB binding assay was performed for p50, p65 and c-Rel. (D) AML cell lines and were treated with 1 µM of ibrutinib for various times and then whole cell extracts. Western blot analysis was conducted for p-AKT-S473, total AKT, p-ERK and total ERK and β-actin protein levels. (E) U937 were transduced with miRNA GFP-tagged lentiviral constructs (Neg-miRNA and BTK437-miRNA) for up to 8 days. Whole cell extracts were prepared and Western blot analysis was conducted for p-AKT-S473, total AKT and β-actin protein levels. (F) Primary AML (AML#22 and AML#24) were treated with 3-1000 nM of ibrutinib for 8 h. Whole cell extracts were prepared and Western blot analysis was conducted for p-AKT-S473, total AKT, p-ERK and total ERK 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 48 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-p-BTK) and AML#19 (high-p-BTK) 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.
References


2. Burnett A. Ham-Wasserman Lecture.


42. Burger JA, Keating MJ, Wierda WG, et al. The Btk inhibitor Ibrutinib (PCI-32765) in combination with Rituximab is well tolerated and


49. Gallay N, Dos Santos C, Cuzin L, et al. The level of AKT phosphorylation on threonine 308 but not on serine 473 is associated


57. de Weers M, Verschuren MC, Kraakman ME, et al. The Bruton's tyrosine kinase gene is expressed throughout B cell differentiation, from early precursor B cell stages preceding immunoglobulin gene
Rushworth et al, Figure 1

A

<table>
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<tr>
<th>CD34+ #1</th>
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<th>TF-1</th>
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<th>U837</th>
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R^2 = 0.766

B

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C

ratio (pBTK/BTK)

CD34+ AML cell lines AML patients

32
Rushworth et al., Figure 2

A

AML#22

U937

% BTK occupancy to no treatment

ibrutinib (nM)

B

AML blasts

CD34+ HSC

% cell viability

normalized to DMSO

ibrutinib (µM)

C

% cell viability

normalized to DMSO

ibrutinib (µM)

D

% phospho-BTK

IC50 values (µM)

R² = 0.649
Rushworth et al, Figure 4

A

events

uninfected control
BTK437
BTK1092

FL1

BTK437-targeted miRNA
non-silencing control miRNA

B

0 1 2 4 8 (days)

0 1 2 4 8 (days)

BTK

β-actin

TF-1

C

Normalized as a % to uninfected cells at day 0

time (days)

D

CFU (% of DMSO

treated control)

neg
BTK437
BTK1092
Rushworth et al, Figure 6

A

\[
\begin{align*}
\text{CD34+} & \quad \text{low pBTK} & \quad \text{high pBTK} \\
\text{cell viability of DMSO treated control cells} & \\
\end{align*}
\]

ib 1 μM — + — + + — + — + — + daun 0.05 μM

cyt 0.1 μM — + — + — + — + — — — —
cyt 0.5 μM — + — + — + — + — — — —

B

\[
\begin{align*}
\text{untreated} & \quad \text{cytarabine} & \quad \text{daunorubicin} \\
\text{CFC (cells per million DMSO treated control)} & \\
\end{align*}
\]

CD34#1 | CD34#2 | AML#8 | AML#12 | AML#17 | AML#18 | AML#2 | AML#8 | AML#14 | AML#19

low pBTK | high pBTK

* * *
Identification of Bruton’s tyrosine kinase as a therapeutic target in acute myeloid leukemia

Stuart A. Rushworth, Megan Y. Murray, Lyubov Zaitseva, Kristian M. Bowles and David J. MacEwan