BTK inhibitor ibrutinib is cytotoxic to myeloma and potently enhances bortezomib and lenalidomide activities through NF-κB

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ABSTRACT

Ibrutinib (previously known as PCI-32765) has recently shown encouraging clinical activity in chronic lymphocytic leukaemia (CLL) effecting cell death through inhibition of Bruton’s tyrosine kinase (BTK). In this study we report for the first time that ibrutinib is cytotoxic to malignant plasma cells from patients with multiple myeloma (MM) and furthermore that treatment with ibrutinib significantly augments the cytotoxic activity of bortezomib and lenalidomide chemotherapies. We describe that the cytotoxicity of ibrutinib in MM is mediated via an inhibitory effect on the nuclear factor-κB (NF-κB) pathway. Specifically, ibrutinib blocks the phosphorylation of serine-536 of the p65 subunit of NF-κB, preventing its nuclear translocation, resulting in down-regulation of anti-apoptotic proteins Bcl-2, FLIP, and survivin and culminating in caspase-mediated apoptosis within the malignant plasma cells. Taken together these data provide a platform for clinical trials of ibrutinib in myeloma and a rationale for its use in combination therapy, particularly with bortezomib.

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1. Introduction

Multiple myeloma (MM) is a progressive malignant disorder, characterized by accumulation of plasma cells in the bone marrow. MM represents about 10% of haematological malignancies and 1% of all cancers. Across Europe there are about 21000 new patients per year diagnosed with MM and approximately 16000 deaths per year from the disease [1]. Despite a variety of therapeutic options, MM remains an incurable disease with a 5 year survival of about 40%. As such considerable effort has been invested in evaluating a number of novel therapies and in the last 10 years, the proteasome inhibitor, such considerable effort has been invested in evaluating a number of novel therapies and in the last 10 years, the proteasome inhibitor, bortezomib as well as thalidomide and its derivative lenalidomide activities through NF-κB have been shown to be effective in the treatment of MM. Unfortunately however despite these new drugs relapse remains inevitable [2,3].

Multiple myeloma (MM) cells are derived from a post germinal centre B-cell [4], and are supported by growth factors that signal through cell surface receptors which activate the NF-κB pathway [5,6]. NF-κB is a member of the Rel family proteins, including RelA (p65), RelB, c-Rel, p50 (NF-κB1), and p52 (NF-κB2), which regulate protein expression mediating proliferation, anti-apoptosis, and cytokine secretion [7,8]. It is typically a heterodimer composed of p50 and p65 subunits, which in the cytosol is inactivated by its association with the inhibitor of NF-κB (IκB), which has a crucial role in regulating NF-κB activation. After stimulation IκB is phosphorylated by IκB kinases (IKKs) followed by its proteosomal degradation, thereby allowing nuclear translocation of NF-κB via either canonical or non-canonical cascades. Although the precise role of NF-κB activation in the pathogenesis of MM has not been fully characterized, recent analysis of MM genomes has confirmed previous observations of the pivotal role and dependence of MM on the NF-κB pathway [9,10].

In normal B-cell development the receptor for B cell-activating factor (BAFF) of the TNF family (BAFF-R) is coupled to the NF-κB pathway by Bruton’s tyrosine kinase (BTK) [11]. Loss of BTK results in defective BAFF-mediated activation of both canonical and non-canonical NF-κB pathways. Moreover, BTK directly regulates the canonical pathway in response to BAFF such that BTK-deficient B cells exhibit reduced IKK kinase activity and defective IκB degradation [12]. Thus, BAFF-induced signalling to NF-κB via BTK serves to promote B-cell survival. These findings are in keeping with the clinical observations that genetic defects in BTK are associated with a profound deficiency of B-cells [13], and that the BTK inhibitor ibrutinib is cytotoxic to chronic lymphocytic leukaemia (CLL) cells [14,15].

As the NF-κB pathway is central to myeloma cell survival, and BTK couples cell surface survival signals to the NF-κB pathway in B-cell development, as well as the BTK inhibitor ibrutinib being highly effective
in killing CLL cells, we therefore investigated the effects of BTK inhibition in MM.

2. Materials and methods

2.1. Materials

The MM-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 postresuscitation, testing regularly for mycoplasma infection. Anti-NF-κB antibodies were purchased from Cell Signaling Technologies (Cambridge, MA). All other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Ibrutinib was obtained from Selleck Chemicals and LFM-A13 was obtained from Tocris Biosciences. All other reagents were obtained from Sigma-Aldrich (St Louis, MO), unless indicated.

2.2. Cell culture

MM cells were obtained from the bone marrow of previously untreated patients under ethical approval (LREC ref07/H0310/146). After initial purification, MM samples with >80% cells expressing CD138, were purified using a CD138+ selection kit (Miltenyi Biotec). Cell type was confirmed by flow cytometry. For primary cell isolation, heparinized blood was collected from volunteers and human peripheral blood mononuclear cells (PBMCs) isolated by Histopaque (Sigma-Aldrich) density gradient centrifugation.

2.3. RNA extraction and real-time PCR

Total RNA was extracted from 5 x 10^5 cells using the Nucleic acid PrepStation from Applied Biosystems, according to the manufacturer’s instructions. Reverse transcription was performed using the RNA polymerase chain reaction (PCR) core kit (Applied Biosystems). Real-time PCR primers for GAPDH, BTK, FLIPβ, BCL-XL, and survivin were purchased from Invitrogen. 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 min), the PCRs were amplified for 45 cycles (95 °C for 15 s and 60 °C for 10 s and 72 °C for 10 s) on a LightCycler 480 (Roche). Each mRNA expression was normalized against GAPDH mRNA expression using the standard curve method.

2.4. Western immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western analyses were performed as described previously. Briefly, whole cell lysates were extracted using radioimmunoprecipitation assay (RIPA) buffer method and sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation performed [16]. Protein was transferred to nitrocellulose and Western blot analysis performed with the indicated antisera according to their manufacturer’s guidelines.

2.5. Proliferation/death assays

MM cells and monocytes were treated with different doses of ibrutinib, bortezomib or lenalidomide then viable numbers measured with Cell-Titer GLO (Promega). Flow cytometry for measuring apoptosis was performed on the Accuri C6 flow cytometer (Accurri). Samples were collected and stained with Annexin-V and Propidium Iodide (PI), followed by detection.

2.6. Statistical analyses

Student’s t-test was performed to assess statistical significance from controls. Results with p less than 0.05 were considered statistically significant. Results represent the mean ± standard deviation of 3 independent experiments. For Western blotting experiments, data are representative of 3 independent experiments.

![Fig. 1. BTK is expressed by malignant plasma cells and ibrutinib induces cytotoxicity in MM patient cells. (A) BTK mRNA and protein expression in control B-cells and MM patient cells and MM cell lines as measured by real-time PCR and Western blotting. (B) MM cells (n = 11) were treated with two doses of ibrutinib (1 and 10 μM), bortezomib (10 and 20 nM) and lenalidomide (1 and 10 μM) for 48 h and then assessed for cell death/proliferation by Cell Titer GLO assay. (C and D) MM cell lines were analysed for cell death in response to ibrutinib (1 and 10 μM), bortezomib (10 and 20 nM) and lenalidomide.](image-url)
Fig. 2. Ibrutinib induces increased cytotoxicity in combination with lenalidomide and bortezomib in MM patient cells. (A) MM patient cells were treated with various combinations of ibrutinib (1 and 10 µM), bortezomib (10 and 20 nM) and lenalidomide (1 and 10 µM) for 48 h and then assessed for cell death/proliferation by Cell Titer GLO assay. (B) MM cell lines were analysed for cell death in response to various combinations of ibrutinib (1 and 10 µM), bortezomib (10 and 20 nM) and lenalidomide. (C) RPMI8226 cells were analysed for cell death in response to ibrutinib (1 and 10 µM), bortezomib (20 nM) and lenalidomide (10 µM) and combinations thereof, and then analysed for apoptosis using annexin-V/propidium iodide staining and flow cytometry. (D) Primary human monocytes were treated with two doses of ibrutinib (1 and 10 µM) and then in combination with bortezomib (20 nM) and lenalidomide (10 µM) for 48 h and then assessed for cell death/proliferation by Cell Titer GLO assay.
3. Results

3.1. Expression of BTK in malignant plasma cells

It has previously been reported that non-malignant plasma cells have low or no BTK protein or RNA expression [17,18]. However, since BTK expression is known to be controlled by NF-κB and that the NF-κB pathway is central to MM cell survival and malignant B-cells in CLL express higher levels of BTK protein than comparator non-malignant B-cells, we sought to determine the expression profile of BTK in MM cells [14,19]. We found that malignant plasma cells, from MM patients and cell lines, express BTK mRNA at levels that approximate to about 20% of that seen in normal peripheral blood B-cells (Fig. 1A). Furthermore, we found variable levels of BTK protein expression in malignant plasma cells from MM patients and cell lines that poorly correlated with BTK mRNA expression (Fig. 1B), therefore implying BTK has a functional role in MM cell survival.

3.2. Ibrutinib induces cytotoxicity in MM patients

To determine if the BTK inhibitor, ibrutinib, could induce cytotoxicity in human myeloma we treated MM patient cells with ibrutinib at 1 µM and 10 µM concentrations for 48 h then measured plasma cell survival. Ibrutinib induces significant cell death (7–46%) in MM cells at 10 µM (Fig. 1C) as measured by the Cell Titer GLO assay. We also observed a similar effect with ibrutinib in MM cell lines (Fig. 1D). Therefore, despite relatively low levels of BTK protein expression in MM (when compared to primary human B-cells), ibrutinib is able to kill malignant plasma cells when used at a concentration similar to that which is effective in CLL [14,15].

3.3. Ibrutinib augments the cytotoxic effects of lenalidomide and bortezomib in MM patients and cell lines

As anti-cancer therapeutics frequently act synergistically and are often used clinically in combination we looked to determine if ibrutinib could act synergistically with either lenalidomide or bortezomib, two widely used drugs in the treatment of MM [20,21]. At in-vitro concentrations of bortezomib and lenalidomide that approximate to levels that can be achieved in-vivo we observed, that in 48 h cultures of MM patients cells and cell lines, ibrutinib significantly increased cytotoxicity of malignant plasma cells, with the effect in-vitro appearing greater in combination with bortezomib (Fig. 2A). We also observed a similar effect with ibrutinib in combination with either lenalidomide or bortezomib in MM cell lines (Fig. 2B). These observations were further confirmed using an annexin-V/propium iodide apoptosis assay (Fig. 2C). Furthermore, we observed that BTK inhibition had no cytotoxic effects on primary monocytes suggesting the effect on malignant plasma cells is not through non-specific cytotoxicity (Fig. 2D).

3.4. BTK inhibitor, LFM-A13, shows similar activity to ibrutinib in MM

In order to determine whether the cytotoxicity observed with ibrutinib on MM cells was specific to this BTK inhibitor alone or is more likely to be a class effect, we repeated the survival assays with another BTK inhibitor LFM-A13. As with ibrutinib we found that LFM-A13 on its own was toxic to MM cells and in combination with lenalidomide and bortezomib significantly increased the potency of bortezomib (Fig. 3A and B).

3.5. Ibrutinib inhibits phosphorylation of p65 in MM cells

It has previously been reported that BTK is involved in p65-mediated transactivation during NF-κB activation by lipopolysaccharide (LPS) in macrophages [22]. As p65 phosphorylation is therefore necessary for induction of NF-κB/p65 dependent gene expression in monocytes, we sought to determine if a similar role for BTK exists in malignant plasma cells. Accordingly, we treated MM cells with ibrutinib for various times and by Western blotting examined p65 phosphorylation, as well as IκBα phosphorylation and p100 and p52 expression in the MM cell lines RPMI8226 and U266. We found that ibrutinib can inhibit basal p65 and IκBα phosphorylation, but phosphorylation was inhibited by the addition of ibrutinib (Fig. 4D). Bortezomib is known to induce IκBα-dependent down-regulation of IκBα, thereby activating NF-κB via the canonical pathway in MM [23].

3.6. Ibrutinib downregulates anti-apoptotic proteins and induces caspase mediated apoptosis in MM

NF-κB regulated anti-apoptotic proteins have been shown to protect a number of different types of cancer cells from drug-induced cell death [24,25]. Therefore we examined the expression profile of known NF-κB regulated anti-apoptotic proteins in response to ibrutinib alone and in combination with bortezomib. We found that FLIPL, Bcl-xL and survivin are inhibited by ibrutinib alone and in combination with bortezomib (Fig. 5A and B). Since both FLIPL and Bcl-xL negatively regulate caspase-induced cell death we looked to establish whether cell death observed in the MM cells was the result of caspase activation. The pan-caspase inhibitor zVAD-fmk was able to protect MM cells from cell death induced by ibrutinib alone or in combination with bortezomib establishing that ibrutinib induced MM cell death is caspase-dependent (Fig. 5C).

4. Discussion

The BTK inhibitor, ibrutinib, has been shown to have excellent clinical activity in CLL. Here we describe for the first time that BTK is expressed in malignant plasma cells and that ibrutinib is cytotoxic to these cells. Furthermore, treatment with ibrutinib significantly augments the cytotoxic activity of bortezomib and lenalidomide. We report that the cytotoxicity of ibrutinib in MM is mediated via an inhibitory effect on the canonical

Fig. 3. LFM-A13 induces apoptosis in MM cells both alone and in combination with lenalidomide and bortezomib. (A) MM patient cells and RPMI8226 cells were analysed for cell death in response to LFM-A13 (50 and 100 µM). (B) MM patient cells and RPMI8226 cells were analysed for cell death in response to LFM-A13 (50 and 100 µM), bortezomib (20 nM) and lenalidomide (10 µM) and combinations thereof, for 48 h and then assessed for cell death/proliferation by Cell Titer GLO assay.
NF-κB pathway. Specifically, ibrutinib blocks the phosphorylation of serine-536 of the p65 subunit of NF-κB, preventing its nuclear translocation, resulting in down-regulation of anti-apoptotic proteins and ending in caspase-mediated apoptosis. Taken together these data provide a platform for clinical trials of ibrutinib in MM and a rationale for its use in combination therapy, particularly with bortezomib.

In normal non-malignant plasma cells BTK expression has been shown to be low or undetectable [17,18]. However here we showed that BTK is expressed in malignant plasma cells as detected by real-time PCR and Western blotting. We therefore hypothesize that BTK expression is detectable in MM and not in non-malignant plasma cells because of the elevated NF-κB levels observed in MM together with the knowledge that BTK expression is known to be controlled by NF-κB [19]. Although normal B-cells show little variation between BTK mRNA and protein expression, malignant B-cells from patients with CLL show poor correlation between mRNA and protein expression [14]. Similarly, we found variable levels of BTK protein expression in malignant plasma cells from MM patients and cell lines that poorly correlated with BTK mRNA expression, therefore implying that deregulation of BTK protein expression must be occurring at a
apoptosis (Fig. 6). The clinical observations that ibrutinib rarely causes myelotoxicity in B-cell lymphoma patients, and infrequently causes (grade ≥ 3) toxicities in CLL patients [26,27], taken together with our data here, provide a platform for clinical trials of ibrutinib in myeloma and a rationale for its use in combination therapy, particularly with bortezomib.

**Authorship contributions**

SAR, KMB and DJM designed the research. SAR, LNB and MVM performed the research. LZ 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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**References**


**Fig. 6.** Schematic diagram of how ibrutinib may enhance chemotherapeutic-induced cell death in MM.