Bruton Tyrosine Kinase Is a Therapeutic Target in Stem-like Cells from Multiple Myeloma

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Abstract

Ibrutinib (Imbruvica), a small-drug inhibitor of Bruton tyrosine kinase (BTK), is currently undergoing clinical testing in patients with multiple myeloma, yet important questions on the role of BTK in myeloma biology and treatment are outstanding. Using flow-sorted side population cells from human myeloma cell lines and multiple myeloma primary samples as surrogate for the elusive multiple myeloma stem cell, we found that elevated expression of BTK in myeloma cells leads to AKT/WNT/β-catenin–dependent upregulation of key stemness genes (OCT4, SOX2, NANOG, and MYC) and enhanced self-renewal. Enforced transgenic expression of BTK in myeloma cells increased features of cancer stemness, including clonogenicity and resistance to widely used myeloma drugs, whereas inducible knockdown of BTK abolished them. Furthermore, overexpression of BTK in myeloma cells promoted tumor growth in laboratory mice and rendered side population–derived tumors that contained high levels of BTK more sensitive to the selective, second-generation BTK inhibitor, CGI1746, than side population–derived tumors that harbored low levels of BTK. Taken together, these findings implicate BTK as a positive regulator of myeloma stemness and provide additional support for the clinical testing of BTK-targeted therapies in patients with myeloma.

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Introduction

Following in the footsteps of exciting clinical data on the efficacy of the small-drug inhibitor of Bruton tyrosine kinase (BTK; refs. 1, 2) ibrutinib, in patients with mantle cell lymphoma (MCL; ref. 3) and chronic lymphocytic leukemia (CLL; ref. 4) ibrutinib is now undergoing phase II clinical testing for multiple myeloma registered online at ClinicalTrials.gov as NCT01478581 and NCT01962792. The promise of BTK as a new therapeutic target in multiple myeloma was first suggested by 3 concurrent preclinical studies demonstrating that inhibition of BTK with 3 different small molecules, ibrutinib (5), LFM-A13 (6), and CC-292 (7), effectively targets both myeloma cells, including cancer stem cell (CSC)-like cells commonly referred to as multiple myeloma stem cells (MMSC), and nonmalignant bystander cells in the tumor microenvironment, such as osteoclasts (5–7).

BTK (8) is an X chromosome-encoded member of the Tec protein tyrosine kinase family that has long been known to play a central role in B-cell receptor (BCR) signaling (9, 10). More recent findings, though, place BTK in strategic positions of additional signal transduction pathways important for mature B lymphocytes and plasma cells; for example, CXCR4-CXCL12 (SDF1) chemokine (11) and Toll-like receptor (12) signaling. BTK’s pleiotropic functionality in the B-lineage may complicate efforts to elucidate the mechanisms by which targeted inhibition of BTK suppresses neoplastic growth; that is, the mode of action in multiple myeloma (5–7) may differ in some respects from that in Waldenström macroglobulinemia (13), CLL (14), diffuse large B-cell lymphoma (15), and other blood cancers (16). It is also possible that different subpopulations of tumor cells in the same patient exhibit different degrees of BTK dependency, as suggested by this study, in which MMSCs are shown to be more sensitive to BTK inhibition than bulk myeloma cells. Elevated levels of BTK in myeloma have been reported to prognosticate poor outcome (17), although connections to increased cancer stemness have not been established yet.

The available pipeline of selective BTK inhibitors, such as PCI-32765 (ibrutinib; ref. 18) and CC-292 (19), has recently been enriched by CGI1746, a promising compound that binds reversibly to BTK (20). With regard to the latter, CGI1746 exhibits minimal, if any, cross-reactivity with the Tec family kinases, IL2-inducible T-cell kinase (ITK) and BMX nonreceptor tyrosine kinase (BMX; ref. 20). The high target selectivity of CGI1746 may render it useful for development as second-generation BTK inhibitor able to overcome primary and acquired resistance to ibrutinib (21) as recently reported for MCL (22) and CLL (23), respectively.
In this study, we concentrate on the role of BTK in myeloma stemness, and show that BTK is upregulated in side population cells, a widely used surrogate for the elusive MMSC (24, 25). We demonstrate that enforced expression of BTK in HMCLs leads to elevated clonogenic growth, increased expression of pluripotent/embryonic stem cell (iPS/ES) genes, and heightened tolerance to myeloma drugs. Conversely, knockdown of BTK abrogates these changes. We also show that overexpression of BTK in myeloma accelerates tumor formation in NOD/SCID mice, yet renders the tumors more sensitive to CGI1746. Our findings on BTK’s involvement in myeloma stemness led us to postulate that therapeutic targeting of BTK in patients with myeloma may be most effective in an adjuvant setting, when tumor mass is low and MMSC-targeted drugs may be most effective.

Materials and Methods

Myeloma cell lines

The mouse cell line 5TGM1 was obtained from Dr. Gregory R. Mundy (University of Vanderbilt, Nashville, TN). Human cell lines H929, 8226, JNJ3, and XG1 were kindly provided by Dr. Simona Colla (M.D. Anderson Cancer Center, Houston, TX). ARP1, OCI-MY5, ARK, OPM2, and KMS28PE were kind gifts from Dr. John Shaughnessy (University of Arkansas for Medical Sciences, Little Rock, AR). Cell lines were confirmed to be mycoplasma-free. All myeloma cells were maintained at 37°C and 5% CO2, using RPMI1640 (Gibco) supplemented with 10% heat-inactivated FCS (Gibco) and penicillin/streptomycin (100 mg/mL).

Flow cytometry

Side population cells were enumerated and flow-sorted as described previously (26). Briefly, 1 x 106 cells were suspended in 1 mL RPMI1640 media with 10% FBS and 5 µg/mL Hoechst 33342, and incubated in 37°C water bath for 90 minutes. Cells treated with 100 µmol/L verapamil were used as negative control. After incubation, cells were stained with CD138-FITC, RPE antibodies and resuspended in ice-cold RPMI1640 media with propidium iodide (2 µg/mL) for flow analysis or sorting. Cell death assays were performed using Annexin V Apoptosis Detection Kit APC (eBioscience), eFlux-ID Multidrug resistance assay was performed according to the protocol (26).

Quantitative real time-PCR

Total RNA was extracted using RNeasy Kit (Qiagen) and reverse transcribed using oligo dT primers and SuperScript III RT (Invitrogen; ref. 26). Target gene-specific primers and primers for reference genes were purchased from Integrated DNA Technologies or Invitrogen.

Lentiviral gene transduction

Genes were overexpressed or silenced as described previously (27, 28). Transduction efficiency was determined by flow cytometry, using a green fluorescent reporter protein (GFP).

The BTK promoter region positioned from −596 to 52 of the transcriptional start site was cloned into lentiviral backbone vector driven by GFP. Human NANOG Reporter lentivirus (System Biosciences) was transfected according to the protocol.

Clonogenicity assay

Clonogenic growth was evaluated by seeding 1 x 10^4 myeloma cells in 0.5 mL RPMI1640 containing 0.33% agar and 10% FBS (29). MethoCult H4535 without EPO (Stem Cell Technologies) was used for serial replating.

Cell proliferation and viability assay

Cells were enumerated by Trypan blue staining using hemoctyeter. IC50 of CGI1746 on multiple myeloma cells was determined by PrestoBlue reagent (Invitrogen).

Western blotting and coimmunoprecipitation assays

Protein levels in myeloma cells were determined using immunoblotting (26). β-Actin and histone 2B were used as benchmarks for total and nuclear proteins, respectively. Coimmunoprecipitation (co-IP) assays were performed as described previously (29).

Myeloma xenograft tumors mouse model

Six-week-old NOD.Cg-Rag1tm1Monastikita Ilt2gtm1wjl/Sgl mice were purchased from the Jackson Laboratory. Experiments were approved under the protocol of the Institutional Animal Care and Use Committee of the University of Iowa (Iowa City, IA; IACUC 1203033). Briefly, 1 x 10^6 multiple myeloma cells of ARP1-EV, APR1-BTK OE, H929-SCR, and H929-BTK shRNA cells were injected subcutaneously into each mouse (n = 5). Seven days after injection, mice receiving H929 cells were given water containing doxycycline to induce the expression of BTK-shRNA. Tumor size was measured twice a week, and tumor volume (mm^3) was calculated as 4π/3 x (tumor diameter/2)^2. Studies were terminated once the tumors diameter reached 20 mm.

CGI1746 treatment on the mouse 5TGM1 myeloma model

Around 1 x 10^5 5TGM1 cells were injected into 10 C57BL/ KaLwRij mice from Harlan Laboratories through tail-vein injection. Mice were divided into 2 groups. CGI1746 (100 mg/kg, s.c.) treatment group and control group treated with cremophor/ethanol solution for serial injection. Mice were injected subcutaneously into each mouse (n = 5). Seven days after injection, mice receiving H929 cells were given water containing doxycycline to induce the expression of BTK-shRNA. Tumor size was measured twice a week, and tumor volume (mm^3) was calculated as 4π/3 x (tumor diameter/2)^2. Studies were terminated once the tumors diameter reached 20 mm.

Statistical analysis

The survival data were plotted using Kaplan–Meier curve and analyzed by log-rank test. Multiple groups (≥3) were analyzed by one-way ANOVA, and paired groups were analyzed by two-way ANOVA or Student t test. P ≤ 0.05 was considered as significant.
Results

BTK protein levels in patients with myeloma and inhibition of myeloma cells by CGI1746

Microarray-based global gene expression profiling (6) and quantitative real-time PCR (qPCR; ref. 17) have been recently used by other investigators to convincingly demonstrate that BTK mRNA levels are elevated in myeloma cells compared with normal plasma cells. To complement these findings with BTK protein expression data, we immunostained bone marrow biopsies of 34 patients with newly diagnosed myeloma using an antibody to BTK. Designating immunoreactivity in ≥25% of myeloma cells as cutoff for BTK expression, we found 27 (~80%) cases to be positive and 7 (~20%) cases negative. Semiquantitative evaluation of tissue sections by a hematopathologist identified 3, 9, and 15 cases as BTKHigh, BTKFair, and BTKLow, respectively. An example of moderate BTK expression is shown in Fig. 1A. Examples of BTKHigh and BTKLow myelomas are depicted in Supplementary Fig. S1. Next, we asked whether CGI1746 inhibits HMCLs in vitro. Treatment of ARP1 and OPM2 cells with a dose range of drug (0.2–50 μmol/L; 48 hours) resulted in similar levels of cytotoxicity in both cell lines (IC50 ≈ 10 μmol/L; Fig. 1B). We further tested CGI1746 effect in 5TGM1 mouse model. Figure 1B (left) shows that mice receiving BTK inhibitor survived significantly longer (median survival 57 days) than mice left untreated (median survival 39 days). The survival results were corroborated by measurements of serum levels of IgG2b, a biomarker of 5TGM1 tumor burden. IgG2b rose earlier and more quickly in the control rather than drug-treated mice (Fig. 1C, right).

Elevated BTK expression is a feature of putative MMSCs

The CD138− subpopulation of cells, which exhibits features of cancer stemness, has been used as a stand-in for studies on MMSCs (30). To determine the expression of BTK in this subpopulation, we used CD138 magnetic beads to fractionate CD138− and CD138+ cells from 10 different cell lines. RT-PCR analysis demonstrated in all cases that BTK was more highly expressed in the CD138− fraction. The magnitude of the increase ranged from a modest 1.5-fold in KMS28PE to nearly 50-fold in OCI-MY5 (Fig. 2A). To confirm these findings with a method of MMSC isolation that is widely considered to be more stringent and reliable, we obtained the small but consistently detected side
population from two cell lines with moderate to low elevations of BTK: OPM2 and ARP1 (24, 26). RT-PCR measurements showed that, compared with main population cells, side population cells harbored considerably higher BTK mRNA levels than seen in the CD138 assay: an approximately 150-fold increase in ARP1 and a approximately 35-fold increase in OPM2 (Fig. 2B, top rows). Be this as it may, elevated BTK expression was associated with a marked upregulation of three stem cell genes, \(\text{NANOG}, \text{MYC}, \) and \(\text{SOX2}\), and a moderate upregulation of four additional stem cell genes: \(\text{SMO}, \text{GLI1}, \text{NOTCH1},\) and \(\text{OCT4}\) (Fig. 2B). To translate this investigation to patient-derived myeloma samples, we compared the expression of BTK in flow-sorted IgL-restricted (IgLR) side-population cells with that in CD138\(^+\) main population cells: (26) BTK mRNA levels in the former were on average 2.5 times higher than in the latter (Fig. 2C).

To complement the results described above with a method that yields larger samples of cells than possible using CD138\(^+\) or side population fractionations, we developed a reporter-based genetic method for flow sorting of myeloma cells according to BTK promoter activity. OCI-MY5, ARP1, and OPM2 cells were transduced with a lentivirus-encoded GFP reporter gene under transcriptional control of the BTK promoter. Cells were flow sorted to collect the top and bottom deciles of GFP expressors (Fig. 2D). RT-PCR analysis validated the method by demonstrating that GFPH\(_{\text{High}}\) cells harbored approximately five times more BTK message than GFPLow cells (Fig. 2E). Next, we performed serial colony formation assays using three consecutive passages of ARP1 cells to evaluate the possibility that BTK promotes clonogenicity. Compared with GFPLowBTKLow cells, GFPH\(_{\text{High}}\)BTKHigh cells not only exhibited significantly increased clonogenic potential upon initial plating (110 ± 23 vs. 58 ± 13 colonies, \(P < 0.05; \) Student t test), but also greater capacity for further increase upon second and third replating (\(P = 0.012; \) one-way ANOVA; Fig. 2F).

Enforced expression of BTK enhances myeloma stemness

To prove BTK is a driver rather than a consequential phenomenon in keeping features of cancer stemness in myeloma, ARP1 and OPM2 cells were transfected with lentiviral particles that encoded a BTK cDNA gene. Western blotting showed that compared with cells infected with noncoding “empty” virus (BTK\(^{\text{WT}}\))
used as control), cells overexpressing BTK (BTK<sup>OE</sup>) contained elevated amounts of (i) total and phosphorylated BTK, (ii) total and phosphorylated PLC<sub>γ2</sub>, a downstream substrate of BTK in the BCR signaling pathway, and (iii) NANOG, a master regulator of stemness (Fig. 3A). RT-PCR analysis of the IPS/ES genes OCT4, SOX2, NANOG, and LIN28A revealed 5-fold to 8-fold increases in mRNA levels in BTK<sup>OE</sup> cells compared with BTK<sup>WT</sup> cells (Fig. 3B). Soft agar clonogenicity assays demonstrated BTK-dependent elevations of colony numbers in BTK<sup>OE</sup> versus BTK<sup>WT</sup> cells: 12.9% versus 8.63% in case of ARP1 and 13.7% versus 9.94% for OPM2 (results not shown). Next, using the flow cytometric, we found that overexpression of BTK in both ARP1 and OPM2 cells led to a approximately 3-fold increase in the abundance of side population cells (Fig. 3C). To determine whether enforced expression of BTK also increased tumorigenicity of myeloma cells, we subcutaneously injected ARP1 cells into NOD/SCID mice. BTK<sup>OE</sup> cells generated tumors more effectively than their BTK<sup>WT</sup> counterparts (Fig. 3D).

**BTK promotes drug resistance in myeloma**

Because CSCs have been implicated in acquisition of drug resistance in patients with cancer, we investigated whether enforced expression of BTK blunts the response of myeloma cells to widely used myeloma drugs. Clonogenic growth assays demonstrated that regardless of experimental conditions, BTK<sup>OE</sup> cells produced more colonies than BTK<sup>WT</sup> cells (Fig. 4A). Compared with BTK<sup>WT</sup> cells, the growth advantage of BTK<sup>OE</sup> cells treated for 2 weeks with bortezomib, doxorubicin, or etoposide was more pronounced (2.5- to 5.3-fold) than that of BTK<sup>OE</sup> cells left untreated (1.57-fold; Fig. 4A). Flow cytometric determination of immunoreactivity to Annexin V, a marker of apoptotic cell death, revealed a similar picture, as the survival advantage of untreated BTK<sup>OE</sup> compared with BTK<sup>WT</sup> cells (33%) increased to levels that ranged from 74% (0.5 nmol/L bortezomib) to 81% (5 nmol/L bortezomib) under conditions of treatment (Fig. 4B). To elucidate the mechanism by which BTK promotes drug resistance in myeloma, we determined the activity of the ABC transporter efflux pump with the assistance of the eFluxx-ID Gold MDR assay. The mean fluorescence intensity exhibited a strong verapamil-dependent increase in BTK<sup>OE</sup> cells (50%–65%; Fig. 4C, left). In keeping with that, Western blotting demonstrated increased expression of the transporter protein, ABCB1, in BTK<sup>OE</sup> compared with BTK<sup>WT</sup> cells, and an increase of pBCL2<sub>Ser70</sub>, a phosphorylated and hyperactive form of the antiapoptotic protein (Fig. 4C, right), while the total form of other BCL2 family genes, BCL2, BCL-X<sub>L</sub>, and MCL1, showed no change (data not shown). Next, we performed colony formation assays to assess the possibility that drug efflux pump inhibition restores the sensitivity of BTK<sup>OE</sup> cells to bortezomib. Figure 4D shows that exposure of cells to verapamil alone caused little if any change in the clonogenic growth of BTK<sup>OE</sup> compared with BTK<sup>WT</sup> cells. However, combination treatment was more effective because the corresponding BTK<sup>OE</sup> to BTK<sup>WT</sup> ratios dropped from 5.30 in cells treated with bortezomib alone to 2.20 in cells treated with bortezomib and verapamil.

**Inducible downregulation of BTK reduces growth, survival, stemness, and tumorigenicity of myeloma cells**

We further infected OCI-MY5 and H929 cells with lentivirus that encoded a BTK-targeted shRNA under control of a doxycycline-inducible promoter (BTK<sup>CID</sup>). Cells transduced with a doxycycline-inducible nontargeting shRNA were used as control (BTK<sup>WT</sup>). Immunoblotting of whole-cell lysates demonstrated
decreases in BTK, pBTK, PLCγ2, and pPLCγ2 in BTKKD relative to BTKWT cells (Fig. 5A), indicating that BTK-targeted shRNA knockdowns the same genes found to be overexpressed in BTKOE cells (Fig. 3A). Activation of PARP and caspases 3, 8, and 9 by proteolytic cleavage in BTKKD cells showed that knockdown of BTK may trigger both intrinsic and extrinsic pathways of apoptosis, and that OCI-MY5 and H929 cells may in fact be "addicted" to high endogenous levels of BTK.

Figure 4.

Overexpression of BTK in myeloma cells promotes drug resistance. A, bar diagram depicting percent clonogenic growth of ARP1 myeloma cells that either overexpress BTK (BTKOE) or contain normal levels (BTKWT). Right, representative photomicroscopic images of two soft-agar dishes that contain myeloma cell colonies derived from untreated (top) or bortezomib (Bz)-treated (bottom) BTKOE cells. B, diagrammatic representation of percent apoptotic cell death of BTKOE/BTKWT ARP1 cells treated with the same drugs used in A. Right, a typical set of flow histograms, using again treatment with 5 nmol/L bortezomib as an example. C, gold eFluxx assay data demonstrating that overexpression of BTK results in heightened drug efflux in myeloma cells. Right, Western blot demonstrates BTK-dependent upregulation of ABCB1 and pBCL2 in ARP1 and OPM2 cells. D, bar diagram summarizing results of colony formation assays using ARP1 BTKOE/BTKWT ARP1 cells treated with 10 μmol/L verapamil, 5 nmol/L bortezomib, or combination of both drugs. Cells left untreated were used as control. The BTKOE to BTKWT ratios are indicated, analogous to A and B.
Consistent with that, loss of BTK reduced growth and survival of myeloma cells in vitro (Fig. 5B and C) and in xenotransplanted NOD/SCID mice (Fig. 5D).

The apparent linkage of BTK and NANOG expression in side population myeloma cells (Fig. 2B) and our published findings on the role of NANOG in maintaining myeloma stemness (26)
led us to evaluate whether inducible knockdown of BTK in myeloma cells results in downregulation of NANOG and loss of clonogenicity. OCI-MY5 cells were cotransfected with lentivirus encoding a NANOG promoter–driven GFP reporter and the doxycycline-inducible BTK shRNA or control knockdown constructs used above (Fig. 5E, top left). Cells were fractionated by flow sorting according to reporter gene expression (GFP+/GFP−; Fig. 5E, top right), treated with doxycycline for 48 hours to downregulate BTK, and harvested for gene expression analysis using RT-PCR (Fig. 5E, bottom left). We observed a significant shRNA-dependent reduction of BTK message in both GFP+ and GFP− BTKKD cells compared with BTKWT. Changes in NANOG expression exhibited the same trend: a 5.5-fold drop in GFP+BTKKD versus GFP+BTKWT cells and a approximately 13-fold reduction in GFP− BTKKD relative to GFP+ BTKWT cells. Clonogenic growth was also dependent on BTK and NANOG levels, as GFP+BTKWT and GFP− BTKWT cells exhibited clonogenicity more or less robustly, yet BTKKD cells failed in that regard (Fig. 5E, bottom right).

BTK governs, in part, AKT and WNT/β-catenin signaling in myeloma

To elucidate the mechanism by which BTK regulates signal transduction in myeloma cells, we sought to identify BTK-interacting proteins using co-IP analysis in BTKOE ARP1 and OPM2 cells. Figure 6A shows that antibody to BTK (bait) pulled down the WNT/β-catenin stabilizer, CDC73 (31). Conversely, antibody to CDC73 pulled down BTK. This suggested physical interaction of the two proteins in myeloma cells. Next, we interrogated the WNT/β-catenin pathway in the paired BTKOE/BTKWT and BTKKD/BTKWT cell lines described in Figs. 3–5. Immunoblotting showed that BTKOE cells harbored increased amounts of β-catenin, phosphorylated AKT (pAKT), and phosphorylated GSK3β (pGSK3β), whereas BTKKD cells contained reduced amounts of these proteins relative to WT controls (Fig. 6B). BTK directly phosphorylates AKT in B cells. Following up on the results presented in Fig. 4C and previous work from our laboratory suggesting that AKT-dependent activation of ABCB1 and BCL2 underlies drug resistance in myeloma (29), we performed a dye efflux assay using the AKT inhibitor, LY294002. Figure 6C shows that BTKOE cells responded to that treatment by retaining more dye in the cytoplasm, an indication that ABCB1 and related drug efflux proteins require upstream AKT signaling to operate properly in myeloma cells. Immunoblotting supported this interpretation as BTKOE cells treated with LY294002 contained less ABCB1, pBCL2, and β-catenin than their BTKWT counterparts (Fig. 6D). Because we have recently shown that CSC-like myeloma cells may overexpress NANOG in a WNT/β-catenin–dependent manner (26), we treated BTKOE cells with the β-catenin inhibitor CAY10404. Figure 6E shows that this treatment led to decreased NANOG protein levels.

CGII1746 inhibits myeloma-like tumors in mice

We treated BTKOE cells with 10 μmol/L CGII1746 to test the possibility that selective inhibition of BTK affords a treatment with the indicated AKT inhibitor (bottom) or left untreated (top). D, immunoblots indicating AKT inhibitor–dependent reductions in ABCB1, pBCL2, and β-catenin levels in BTKWT ARPI and OPM2 cells. β-Actin and HIS2B were used as loading controls. E, immunoblots indicating WNT inhibitor–dependent reductions in NANOG in BTKOE ARPI and OPM2 cells.
option for myeloma including BTK^{high} MMSCs (20). Flow cytometric determination of drug efflux capacity using eFluxx dye analysis showed that treatment with CGI1746 significantly reduced this capacity in myeloma cells (Fig. 7A). In agreement with that, clonogenic growth of GFP^{high}BTK^{high} cells was more compromised by CGI1746 than that of GFP^{low}BTK^{low} cells used

**Figure 7.** Preclinical testing of CGI1746. A, flow histograms of eFluxx dye retention assays using BTK^{OE} ARPI and OPM2 cells treated with CGI1746. B, bar diagram of colony formation results indicating CGI1746-dependent reductions in clonogenicity of GFP^{high}BTK^{high} versus GFP^{low}BTK^{low} myeloma cells. C, Kaplan-Meier survival curve (left) of C57BL/KaLwRij mice treated with intravenous injections of either side population (SP) or non-side population 5TGM1 cells, followed by treatment of mice with CGI1746 or solvent control and the right panel is a graph of mean serum IgG2b levels of tumor-bearing mice. D, column diagram presenting results of colony formation assays of HSCs obtained from BTK-deficient Xid or normal mice. n.s., nonsignificant. E, working model on the putative mechanisms by which BTK promotes stemness and drug resistance in myeloma.
as control. In case of ARP1, 1.61% and 7.88% of BTK\textsuperscript{High} cells either treated with inhibitor or left untreated gave rise to colonies, a ratio of 0.204. The same ratio for BTK\textsuperscript{Low} cells was 0.533, indicating that treatment with CGI1746 affected BTK\textsuperscript{High} cells 2.6 times more severely than BTK\textsuperscript{Low} cells. OPM2 cells showed the similar trend as ARP1 cells (Fig. 7B). To extend these observations in vivo, we transferred 5TG1M1 cells, previously flow-sorted as side population and main population. Figure 7C (left) shows that untreated mice harboring side population-derived tumors exhibited the shortest median survival (61 days), followed by untreated mice containing main population-derived tumors (89 days). Treatment of mice with CGI1746 prolonged the median survival in both cases: 119 days in the side population group and “undefined” (after 140 days of observation, at which time the study was terminated) in the main population group. The survival results were corroborated by changes in serum IgG2b M-spike levels, an imperfect but widely used biomarker of tumor burden (Fig. 7C, right). Figure 7D shows that CGI1746 was also effective in preliminary studies comparing hematopoietic stem cells (HSC) from BTK-deficient Xid and normal mice.

Discussion

The main finding of this study is experimental evidence for an important role of BTK in myeloma stemness. A working model of BTK’s function in MMSC is depicted in Fig. 7E. Using flow-sorted myeloma side population cells as surrogate for MMSCs, we showed that elevated expression of BTK is associated with both upregulation of key iPS/ES genes (e.g., OCT4, SOX2, NANOG, MYC) and enhanced self-renewal (serial passage of clonogenicity). Enforced expression of BTK in myeloma cells increased features of stemness (e.g., side population, clonogenicity, iPS/ES genes, drug resistance), whereas knockdown of BTK abolished them. In vitro results of this sort were underlined by in vivo studies using laboratory mice, demonstrating that overexpression of BTK in myeloma cells promotes tumor growth and renders SP/BTK\textsuperscript{High} tumors derived from 5TG1M1 myeloma more sensitive to BTK inhibitor, CGI1746, than their MP/BTK\textsuperscript{Low} tumor counterparts. Although the growth inhibition in vivo was not as potent as in vitro study, there is statistically significant inhibition effect in vivo ($P < 0.05$). The discrepancy of BTK-shRNA between in vitro and in vivo may be caused tumor environment, because some factors from the environment may rescue partial effects induced by BTK-shRNA.

BTK can now be added to the growing list of candidate myeloma stemness and drug resistance genes that have been uncovered by us (26, 28, 29, 32, 33) and others (34). The master stem cell factor NANOG, which is positively regulated by BTK according to this study and by RAR\alpha2 according to our previous report (26), illustrates this point. Upregulation of NANOG, which is often attributed to constitutive WNT/\beta-catenin signaling under conditions of heightened cancer stemness (35), is tightly associated with adverse outcome in solid and hematologic cancers (36). \beta-Catenin promotes NANOG expression directly, by virtue of binding to the NANOG promoter, and also indirectly by suppressing TCF-3, a negative regulator of NANOG (37). Our finding that myeloma cells contained increased levels of \beta-catenin in a BTK-dependent manner, and that inhibition of WNT/\beta-catenin signaling using CAY10404 suppressed NANOG in myeloma cells, suggests that inhibition of the WNT/\beta-catenin/NANOG axis may be a promising MMSC-targeted therapy.

In addition to testing promising myeloma drug candidates, such as CGI1746, in tumor transplantation-based mouse models of myeloma, the compounds should also be evaluated in genetically engineered mouse models (GEMM) in which myeloma-like tumors arise spontaneously. A number of GEMMs driven by constitutive oncogenes, including MYC, BCL2L1 (Bcl-\textsubscript{XL}), IL6, CCND1, and XBP1, are available for myeloma drug testing, with some of them, particularly Vk-MYC (38), iMyc-Bcl-\textsubscript{XL}, (39), and IL6Myc (40), already having demonstrated capability to assess objective treatment responses. The genetically and environmentally controlled setting of a preclinical mouse drug study may provide significant benefits compared to a clinical trial.

In conclusion, this study provides preclinical evidence for a role of BTK in myeloma stemness, and directs attention to including BTK inhibitors in MMSC-targeted drug cocktails aimed at eradicating MMSCs and curing myeloma in clinics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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