Ibrutinib significantly inhibited Bruton’s tyrosine kinase (BTK) phosphorylation, in-vitro proliferation and enhanced overall survival in a preclinical Burkitt lymphoma (BL) model

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ABSTRACT
Pediatric and adult patients with recurrent/refractory Burkitt lymphoma (BL) continue to have poor outcomes, emphasizing the need for newer therapeutic agents. Bruton’s tyrosine kinase (BTK) is activated following B-cell receptor stimulation and in part regulates normal B-cell development. Ibrutinib, a selective and irreversible BTK inhibitor, has been efficacious in chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), Waldenström’s macroglobulinemia, and marginal zone lymphoma. In this study, we investigated the efficacy of ibrutinib alone and in selective adjuvant combinations against BL in-vitro and in a human BL xenografted immune-deficient NOD.Cg-Prkdcscid Il2rgtm1Wjl/Sjcl (NSG) mouse model. Our data demonstrated that phospho-BTK level was significantly reduced in BL cells treated with ibrutinib (p < 0.001). Moreover, we observed a significant decrease in cell proliferation as well as significant decrease in IC50 of ibrutinib in combination with dexamethasone, rituximab, obinutuzumab, carfilzomib, and doxorubicin (p < 0.001). In-vivo studies demonstrated ibrutinib treated mice had a significantly prolonged survival with median survival of mice following ibrutinib treatment (32 days) (24 days) (p < 0.02). In conclusion, our findings demonstrate the significant in-vitro and preclinical in-vivo effects of ibrutinib in BL. Based on our preclinical results in this investigation, there is an on-going clinical trial comparing overall survival in children and adolescents with relapsed/refractory BL treated with chemoimmunotherapy with or without ibrutinib (NCT02703272).

Introduction
Burkitt lymphoma (BL) represents the most common subtype of childhood and adolescent non-Hodgkin lymphoma (NHL) (40%) but an incidence of less than 5% of adults with NHL.1–3 We and others have demonstrated that the prognosis of pediatric BL (PBL) has significantly improved over the last 40 years through the use of short and intense multi-agent chemotherapy and immunotherapy with a current 90% 5-year event-free survival (EFS).2,4–9 Specifically, we previously demonstrated 90% 5-year overall survival (OS) following treatment with a short, intensive course of chemotherapy in a cooperative international childhood B-cell NHL (B-NHL) trial (French-American-British/mature lymphoma B [FAB/LMB] 96) involving the Children’s Cancer Group (CCG), French Pediatric Oncology Group (SPOP) and United Kingdom Children’s Cancer Study Group (UKCCSG).5 However, children and adolescents with recurrent/refractory PBL have a significantly decreased and dismal prognosis and, moreover, current upfront chemotherapy protocols are associated with serious acute and long-term morbidities.5,8 It is therefore critical to investigate and to develop targeted translational therapeutic strategies in PBL with the goal of reducing acute morbidities, decreasing late effects in newly diagnosed patients, and/or providing new therapeutic options for those with chemo-immunotherapy resistant relapsed/refractory disease.

Bruton’s tyrosine kinase (BTK) is a regulator of normal B-cell development and is activated upon B-cell receptor (BCR) stimulation. Activation of the BCR signaling pathway has now emerged as a central oncogenic pathway that promotes growth and survival in both normal and malignant
B-cells. Antigenic activation of the dimeric membrane immunoglobulin B-cell receptor, which induces phosphorylation of BTK and phospholipase Cγ2 (PLCγ2), results in the activation of a number of signaling pathways including mitogen-activated protein kinase (MAPK), nuclear factor (NF)-κB (xB) and AKT (protein kinase B). Chronic active BCR signaling through BTK activation and downstream pathways including the NF-κB pathways can be inhibited by the selective and covalent BTK inhibitor, ibrutinib. Ibrutinib is a selective and irreversible small molecule inhibitor of BTK and covalently binds to cysteine residue 481 on BTK, thereby inhibiting the autophosphorylation of tyrosine 223 on exon 8 resulting in irreversible inhibition of BTK enzymatic activity. Ibrutinib has been demonstrated to be an active agent in activated B cell-like diffuse large B-cell lymphoma (ABC-DLBCL), an NHL subtype that is characterized by constitutively activated NF-κB signaling.

Preclinical studies of ibrutinib in chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL) suggested that ibrutinib inhibits cell proliferation in vitro in the range of 1.0 μM to 25.0 μM. Despite high ibrutinib half maximal inhibitory concentration (IC₅₀) values in preclinical studies of CLL and MCL, ibrutinib has been highly effective in the treatment of refractory and relapsed adult patients with CLL and MCL. A recent study demonstrated that the combination of ibrutinib and B-cell lymphoma-2 (BCL2) inhibition using venetoclax was highly effective in patients with MCL. Ibrutinib was originally approved by the FDA in adult patients with relapsed/refractory CLL or MCL who have received at least one prior therapy (USPI) and now is approved in all lines of therapy in CLL. BL, however, is associated with tonic or possibly chronic active BCR signaling. Most recently, we demonstrated that expression of total BTK and phospholipase Cγ2 (PLCγ2) are significantly overexpressed in BTK (9 fold) in patients with sporadic form BL treated on the Children’s Oncology Group (COG) protocol 5961. Bouska et al demonstrated that adult BL cells shares commonly mutated genes in the chronic BCR/BTK/NF-κB signaling pathway, which could be targeted by ibrutinib.

Dexamethasone is often administered in conjunction with rituximab to enhance rituximab-mediated cytotoxicity. Carfilzomib is a second-generation proteasome inhibitor. It was identified as a significantly cytotoxic agent against CLL cells isolated from ibrutinib-treated patients, suggesting that carfilzomib can potentially complement ibrutinib’s anti-tumor activity.

Idelalisib is a potent, selective small-molecule inhibitor of phosphoinositide 3-kinase delta (PI3Kδ). Since BTK and PI3K differentially regulate BCR signaling, the combination of ibrutinib and idelalisib may synergistically target BCR positive tumor cells such as CLL and MCL and other B-cell lymphomas. Doxorubicin has been widely used as a chemotherapeutic agent in BL to induce tumor cell death by intercalation into DNA and disruption of topoisomerase II-mediated deoxyribonucleic acid (DNA) repair or generation of free radicals and their damage to cellular membranes, DNA and proteins. The results from an early phase 1 trial indicate that the combination of ibrutinib with the first-line therapy rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) potentially improve response rates in adults with B-NHL. The antitumor activity of ibrutinib alone and more importantly in combination with these regimens against BL is currently unknown. We hypothesized that ibrutinib would be an efficacious small molecule inhibitor alone and/or in selective combination with other active therapies in BL and could potentially be utilized in the future treatment of BL. Here, we investigated the in-vitro and in-vivo efficacy of ibrutinib in human BL cell xenografted immune-deficient mouse NOD.Cg-Prkdcs-def/Il2rgtm1Wji/SzJ (NSG) mouse model.

Results

Ibrutinib inhibits the expression of p-BTK protein in BL cells

We first demonstrated that the expression of total BTK protein in BL was similar in Raji and Ramos BL cell lines following ibrutinib treatment with varying doses (0, 0.1, 0.2, 0.5, 1.0, 5.0, and 10 μM) for five days (Figure 1A and B). The medium was refreshed daily with ibrutinib. In both Raji and Ramos BL cell lines, p-BTK at Tyr 223 was significantly decreased following exposure to ibrutinib at all doses (Figure 1A and B) (p < 0.005, p < 0.0005, p < 0.00001), respectively.

Ibrutinib inhibits cell proliferation in BL cells

Next, we demonstrated that in comparison to control dimethyl sulfoxide (DMSO) (1.00 ± 0.00), 5 days of ibrutinib treatment significantly inhibits proliferation of Raji cells at 5.0 μM (0.561 ± 0.170, p = 0.023) and 10 μM (0.409 ± 0.115, p = 0.044). The IC₅₀ for inhibition of Raji cell proliferation was 5.20 μM (Figure 2A). There was a significant inhibition of cell proliferation in ibrutinib-treated Ramos vs DMSO-treated Ramos (1.00 ± 0.000) with five days of ibrutinib treatment course at 0.25 μM (0.626 ± 0.015, p = 0.0002), 0.5 μM (0.511 ± 0.131, p = 0.011), 1.0 μM (0.442 ± 0.091, p = 0.004), 5.0 μM (0.248 ± 0.056, p = 0.0009) and 10.0 μM (0.109 ± 0.018, p = 0.004). The ibrutinib IC₅₀ for inhibition of Ramos cell proliferation was 0.868 μM (Figure 2B) (p < 0.05).

The efficacy of ibrutinib in combination with dexamethasone, rituximab and obinutuzumab against BL

We investigated the efficacy of ibrutinib in combination with dexamethasone in Raji and Ramos BL cells. Dexamethasone (1μM) was broadly used in vitro alone or in combination with other drugs to inhibit proliferation or induce apoptosis in lymphoid malignancies. Different doses of ibrutinib and 1μM dexamethasone were co-cultured with Raji or Ramos for five days. The medium was refreshed daily with ibrutinib+ dexamethasone. Interestingly, we observed a significant decrease of cell proliferation with the combination of ibrutinib and 1μM dexamethasone in both Raji and Ramos cells and a significant reduction of IC₅₀ compared to ibrutinib alone (Raji IC₅₀: ibrutinib alone 5.2μM vs ibrutinib
+ dexamethasone 0.34 μM, p < 0.01 and Ramos IC_{50}: ibrutinib alone 0.87μM vs ibrutinib + dexamethasone 0.13 μM, p < 0.01, respectively (Figure 2C and 2D). To determine if anti-CD20 antibodies are additive to ibrutinib-mediated growth inhibition, next, we extended our investigation to determine the efficacy of ibrutinib in BL cells with anti-CD20 antibodies rituximab or obinutuzumab. We observed a significant decrease in cell proliferation following ibrutinib and rituximab (20ug/ml) or obinutuzumab (20 ug/ml) combination-treated Raji vs ibrutinib alone-treated Raji following 5 days of treatment (Figure 3A). The ibrutinib IC_{50} values for inhibition of cell proliferation in Raji were significantly decreased from 4.55 ± 4.038 μM (ibrutinib alone) to 0.67 ± 0.364 μM with 20 ug/ml rituximab (p < 0.0005) and 0.16 ± 0.095 μM with 20 μg/ml obinutuzumab (p < 0.00005) combination following 5 days of ibrutinib treatment. In addition, we observed a significant decrease in cell proliferation following ibrutinib and rituximab (0.2μM) and rituximab (20 μg/ml) or obinutuzumab (20 μg/ml) combination-treated Ramos vs ibrutinib alone-treated Ramos following ibrutinib at 5 days of treatment (Figure 3B). The ibrutinib IC_{50} values for inhibition of cell proliferation in Ramos were significantly decreased from 1.41 ± 1.372 μM (ibrutinib alone) to 0.16 ± 0.174 μM with 20 μg/ml rituximab (p < 0.0001) and 0.01 ± 0.005 μM with 20 μg/ml obinutuzumab (p < 0.001) (Figure 3B). Our results suggest in part that by combining with rituximab or obinutuzumab, we may reduce the dose of ibrutinib on BL to limit the potential toxicity of ibrutinib.

Figure 1. Significant inhibition of BTK phosphorylation in ibrutinib treated BL cell lines. Raji (A) and Ramos (B) BL cell lines were treated with ibrutinib at varying doses (0, 0.1, 0.2, 0.5, 1.0, 5.0, and 10 μM) for five days. Ibrutinib was dissolved in DMSO. DMSO (ibrutinib dose at 0) was used as control. The total levels of BTK protein and phosphorylated BTK protein (p-BTK) was examined by western blot analysis with specific antibody against BTK and phospho-BTK (Tyr223). GAPDH was used as loading control. Representative Western blot results are shown in the left panels of (A) and (B). Intensities of immunoreactive phospho-BTK (Tyr223) bands on Western blots shown in (A) were quantified by densitometric analysis as shown in the middle panels of (A) and (B). Intensities of immunoreactive BTK bands on Western blots shown in (A) were quantified by densitometric analysis as shown in the right panels of (A) and (B). n = 3, ***p < 0.00001, **p < 0.0005, *p < 0.005.

The efficacy of ibrutinib alone and in combination with carfilzomib, idelalisib, and doxorubicin on cell proliferation in Raji BL cells

Carfilzomib, idelalisib, and doxorubicin have been previously demonstrated to have significant activity against a variety of histological subtypes of B-NHL. With fixed doses of each single agent, we also investigated the efficacy of different doses of ibrutinib and in combination with fixed doses of carfilzomib, idelalisib, or doxorubicin with prolonged exposure for five days in Raji BL cells. The medium was refreshed daily with ibrutinib and in combination with carfilzomib, idelalisib, or doxorubicin. We observed a significant decrease in cell proliferation following ibrutinib and carfilzomib (2 nM and 5 nM) combination-treated Raji vs ibrutinib alone-treated Raji following 5 days of treatment (Figure 4A). Previously, it was reported that in-vitro exposure of BL cell lines to idelalisib in concentrations from 0.1-100µM resulted in a dose and time-dependent decrease in viable cells.31 We therefore investigated the efficacy of ibrutinib with a fixed dose of idelalisib at 10 μM. Raji cells were treated with different doses of ibrutinib with 10
μM idelalisib for 5 days. There was a significant decrease in cell proliferation following ibrutinib-treated Raji vs ibrutinib and idelalisib (10 μM) combination-treated Raji compared to DMSO-treated Raji (1.000 ± 0.000) with 0.2 μM (0.94 ± 0.036 vs 0.743 ± 0.025, p > 0.05), 0.5 μM (0.89 ± 0.019 vs 0.736 ± 0.032, p < 0.01), 1.0 μM (0.850 ± 0.013 vs 0.707 ± 0.016, p < 0.005), 5.0 μM (0.742 ± 0.015 vs 0.626 ± 0.023, p < 0.005) and 10 μM (0.468 ± 0.007 vs 0.424 ± 0.027, p < 0.05). The irbutinib IC_{50} values for inhibition of cell proliferation in Raji were decreased from 13.809 ± 2.843 μM (ibrutinib alone) to 10.04 ± 1.696 μM (10 μM idelalisib) with this combination following 5 days of irbutinib treatment, however, the differences were not statistically significant (p = 0.076) (Figure 4B).

Next, we also examined the efficacy (IC_{50}) of irbutinib alone and in combination with doxorubicin. We observed a significant decrease in cell proliferation following irbutinib and doxorubicin (0.01 μM and 0.1 μM) combination-treated Raji vs irbutinib alone-treated Raji following 5 days of treatment. The irbutinib IC_{50} values for inhibition of cell proliferation in Raji were decreased from 7.20 ± 1.531 μM (ibrutinib alone) to 6.78 ± 1.730 μM (0.01 μM doxorubicin, p = 0.1) and significantly decreased to 0.01 ± 0.009 μM (0.1 μM doxorubicin, p < 0.005) in combination following 5 days of irbutinib treatment (Figure 4C).

### The dose-dependent efficacy of irbutinib alone and in combination on tumor progression and survival in Raji BL xenografted NSG mice

We first compared the efficacy of irbutinib in a dose dependent manner on tumor progression and survival. Ibrutinib (12.5 mg/kg) treated mice had a significantly prolonged survival compared to phosphate-buffered saline (PBS) control mice (p < 0.02) with median survival of mice following irbutinib treatment (32 days) compared to PBS control (24 days) (Figure 5A). The treatment experimental schema is shown in Figure 5B. Furthermore, we observed a significant decrease of tumor progression by measuring tumor luminescence signal intensity following irbutinib treated Raji BL xenografted NSG mice at day 20 (p < 0.001) and at day 25 (p < 0.05) compared to control (Figure 5C and 5D).

Cyclophosphamide is an active agent in the treatment of BL and has been widely used in combination with doxorubicin, vincristine, prednisone, and rituximab to treat patients with BL. We investigated and compared the efficacy of irbutinib (12.5 mg/kg) vs cyclophosphamide (CTX, 25mg/kg) vs obinutuzumab (ObiN, 30mg/kg) in Raji BL xenografted NSG mice. We observed that there was a similar median survival between the irbutinib vs cyclophosphamide vs obinutuzumab treated Raji xenografted NSG mice (Figure 6).
Discussion

We evaluated the in-vivo and in-vivo efficacy of ibrutinib, a FDA approved BTK inhibitor, against BL. Our data demonstrated that ibrutinib significantly inhibited BL cell proliferation with concomitant decrease in phosphorylation of BTK in-vitro and secondly that ibrutinib was synergistic with either dexamethasone, rituximab, obinutuzumab, carfilzomib, or doxorubicin leading to BL growth inhibition and cell death in-vitro. Furthermore, our data demonstrated that ibrutinib alone significantly decreased tumor progression and significantly increased the survival of BL xenografted mice and resulted in similar efficacy compared to therapeutic doses of cyclophosphamide or obinutuzumab.

Ibrutinib’s unique biochemistry and in-vivo activities in mice and dogs paved the way for human clinical trials in adults with mature B-cell lymphomas. In a phase I study, ibrutinib was well tolerated in fifty evaluable adults with relapsed/refractory B-cell lymphomas including MCL, follicular lymphoma, DLBCL, marginal zone lymphoma, and CLL and was associated with an objective response rate (ORR) of 60%, including complete response (CR) 16%. BL is the most common subtype of pediatric NHL. Schmitz et al reported that 6 of 9 BL cells were clearly BCR-dependent, based on a time dependent decrease in their viability following knockdown of either CD79A or the BCR associated tyrosine kinase SYK. However, there is a paucity of studies investigating the efficacy of ibrutinib in BL in-vitro and in-vivo. In our preclinical studies, we demonstrated that in-vitro, ibrutinib significantly inhibited the levels of p-BTK protein in BL cells and significantly inhibited the proliferation of BL (Figure 1 and Figure 2A-B). Consistently, in Raji xenografted NSG mice, ibrutinib significantly reduced tumor burden and extended BL xenografted NSG mice survival following ibrutinib at 12.5mg/kg daily (Figure 5). Furthermore, ibrutinib resulted in significant survival compared to cyclophosphamide treated group or obinutuzumab treated group in BL xenografted NSG mice (Figure 6).

Accumulating evidence demonstrates that BTK expression and activity is crucial for the survival or proliferation of malignant B cells. Activation of BTK usually correlates with an increase in the phosphorylation of two regulatory BTK tyrosine residues: transphosphorylation at Y551 within the Src homology type 1 (SH1) domain by the Src family tyrosine kinases and autophosphorylation at Y223. We demonstrated that BTK was highly expressed and...
phosphorylated in BL cells, similarly as reported previously in CLL and MCL cells.\(^\text{15,35}\) Ibrutinib treatment resulted in diminished cell survival and proliferation and abolished BCR-stimulated AKT and extracellular signal-regulated kinase phosphorylation and BTK autophosphorylation in CLL and MCL.\(^\text{16,35}\) Consistently, our studies demonstrated that ibrutinib significantly reduced autophosphorylation in BL cells and significantly inhibited BL proliferation. Most importantly, dexamethasone significantly reduced the IC\(_{50}\) of ibrutinib \emph{in-vitro} to less than 1 \(\mu\)M in BL (Figure 2C-D). Since steroids are active agents of BL chemoimmunotherapy regimens, the possibility of clinical synergy exists.\(^\text{4-9}\)

In previous studies, MCL cell lines showed differential sensitivity to BTK inhibition.\(^\text{36}\) Sensitive MCL cells showed a 50% inhibitory concentration (IC\(_{50}\)) of ibrutinib at 0.115 \(\mu\)M. Intermediate sensitive cells had IC\(_{50}\) at 0.491 \(\mu\)M while the

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**Figure 4. Inhibition of BL cell proliferation by ibrutinib in combination with carfilzomib, idelalisib or doxorubicin.** Raji cells were treated with ibrutinib (Ibr) with varying doses (0, 0.25, 0.5, 1.0, 5.0, and 10 \(\mu\)M) in combination with carfilzomib (CFZ) (A), idelalisib (B), or doxorubicin (dox) (C) for five days. The doses of CFZ, idelalisib, and dox were indicated in the figures. Ibrutinib was dissolved in DMSO. DMSO (ibrutinib dose at 0 without any other drug) was used as control. The second and the third grey bars in A with ibrutinib dose at 0 represent the cells treated with carfilzomib alone. The second and the third grey bars in B with ibrutinib dose at 0 represent the cells treated with idelalisib alone. The second and the third grey bars in C with ibrutinib dose at 0 represent the cells treated with doxorubicin alone. Cell proliferation was examined by CellTiter 96® Aqueous One solution cell proliferation assays. Cell proliferation fold change was calculated by the equation: treated A490 reading/DMSO A490 reading. DMSO A490 reading was set as 1. IC\(_{50}\) was determined with CompuSyn software.

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**Figure 2C-D.**
least sensitive cell line with no detectable inhibition until the concentration of ibrutinib reached 16 μM. In our study, we demonstrated that ibrutinib significantly inhibits Raji and Ramos BL cell growth following five days of treatment. The IC\textsubscript{50} of ibrutinib is 5.20 μM against Raji and 0.868 μM against Ramos cells (Figure 2A-B). The pharmacokinetic and pharmacodynamic studies derived from a human trial for patients with relapsed/refractory B-cell malignancies suggested that the \textit{in-vivo} maximal achievable concentration of ibrutinib is 0.408 μM with a once daily dose of 560 mg. In the studies in BL cell lines, ibrutinib doses as low as 0.2 μM resulted in complete inhibition of BTK phosphorylation and with the addition of dexamethasone, the IC\textsubscript{50} of ibrutinib was further reduced \textit{in-vitro} to between 0.13 and 0.34 μM, a concentration achievable \textit{in-vivo}.

Although ibrutinib demonstrates an impressive clinical activity in relapsed or refractory CLL, some patients achieve partial remission and the development of resistance is now well known. A combination of ibrutinib with other drugs such as dexamethasone targeting alternative pathways that are independent of BTK signaling could be additive or synergistic. In a recent \textit{in-vitro} study, Manzoni et al showed that dexamethasone potentiated anti-proliferative effects of ibrutinib. Consistent with the previous studies, we demonstrated a significant decrease of cell proliferation with the combination of ibrutinib and dexamethasone in both Raji and Ramos with a significant reduction of IC\textsubscript{50} compared to ibrutinib alone (Figure 2C-D). Our data indicates that the use of a dexamethasone and ibrutinib combination may be an active combination in patients with BL. Ex-vivo drug screens using targeted agents on primary CLL cells have also been performed in order to identify additional pharmacologic agents that can complement ibrutinib therapy. Carfilzomib (PR-171), a second-generation proteasome inhibitor, was identified as a synergistic cytotoxic agent combined with ibrutinib against primary CLL. Consistent with this report, we demonstrated that carfilzomib significantly reduced ibrutinib IC\textsubscript{50} for inhibition of cell proliferation in Raji (Figure 4A), providing some foundation to further investigate carfilzomib-ibrutinib combination therapies in patients with relapsed/refractory BL.

Another approach to improve the activity of ibrutinib and circumvent ibrutinib resistance is to investigate the efficacy of combining ibrutinib with anti-CD20 monoclonal antibodies (mAb). While there are some reports that ibrutinib may interfere with anti-CD20 mAb activity under some experimental conditions, growing clinical evidence has demonstrated that the two drugs in combination can be more effective than either agent alone. It is notable that ibrutinib induced lymphocytosis in patients and potentially released MCL and CLL cells into the blood from tissue sites and thus anti-CD20 mAb may potentially facilitate the clearing of...
these tumor cells in the blood. In our in-vitro studies, we demonstrated that ibrutinib IC$_{50}$ values for inhibition of cell proliferation in Raji and Ramos cells were significantly decreased with 20 ug/ml obinutuzumab to 0.16 ± 0.095 μM and 0.01 ± 0.005 μM, respectively (Figure 3), suggesting that both rituximab and obinutuzumab in fact may enhance the sensitivity of BL cells to ibrutinib.

Even though the primary target of ibrutinib is BTK and it leading to development as a treatment for B-cell malignancies, interestingly, ibrutinib is also shown as an irreversible molecular inhibitor of interleukin-2-inducible kinase (ITK). ITK is an enzyme required by Th2 T cells, allowing a shift of T-cell immune responses to a Th1 T cells and potentiating Th1-based immune responses. Second-generation BTK inhibitors, such as ACP-196, ONO/GS-4059, and BGB-3111 have been developed and are being investigated in clinical trials. These inhibitors have been suggested to have fewer off-target effects in early clinical trials. Immune checkpoint inhibitors, or CAR-T/CAR-NK will be critical to improve the success of treatment in patients with B cell malignancies such as BL.

In summary, we have demonstrated the significant in-vitro and in-vivo efficacy of ibrutinib against BL. Complete inhibition of phosphorylation of BTK occurs with doses as low as 0.2 μM and the efficacy of ibrutinib is significantly enhanced with reduced the IC$_{50}$ in combination with dexamethasone. ibrutinib showed synergistic effects in combination with other active drugs in B-NHL such as obinutuzumab, carfilzomib, or doxorubicin, and the combination significantly reduced the IC$_{50}$ of ibrutinib. Most importantly, in-vivo studies show significant improvement in OS in BL xenografted NSG mice (Figure 5) and similar efficacy to cyclophosphamide or obinutuzumab (Figure 6). Based on these preliminary results, there is an on-going clinical trial comparing OS in children and adolescents with relapsed/refractory BL with chemoimmunotherapy with or without ibrutinib (NCT02703272). The result of this study will assist in the development of subsequent clinical trials to test the efficacy of ibrutinib in BL.
Materials and methods

Cell lines and cell culture

Raji and Ramos human BL cell lines were purchased from ATCC and cultured under RPMI1640 supplemented with 10% Fetal Bovine Serum and 2mM Glutamine in a 37°C incubator at 5% CO₂ as we have previously described. The survival rate was performed using the Kaplan-Meier method.

Cell proliferation assay

Cell growth was determined by using the non-radioactive CellTiter 96® Aqueous One solution cell proliferation assay (Promega, Madison, WI) and measured by a Multilabel Counter (Perkin Elmer, Massachusetts, USA) at OD490.

Western blotting

Western blotting was performed as we have previously described. In brief, cell lysates were prepared in ice-cold lysis buffer (50 mmol/L Tris–HCl, pH 7.4, 350 mmol/L NaCl, 1% Triton-X-100, 0.5% Nonidet P-40, 10% glycerol, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L Na₃PO₄, 1 mmol/L phenylmethysulphonyl fluoride (PMSF) with a phosphatase inhibitor cocktail. The total lysates were then separated into SDS-PAGE and then transferred to nitrocellulose membrane (Bio-Rad) and incubated with primary antibodies according to the manufacturer’s instructions. Membrane was developed using Enhanced Chemiluminescence Reagent (GE Healthcare Biosciences). Antibodies specific for phospho-BTK at Tyr 223 (cat# 5082) and total BTK (cat# 3533) were purchased from Cell Signaling Technology. GAPDH was purchased from Santa Cruz. Band intensities on SDS-PAGE gels were measured using ImageJ software program.

Reagents and antibodies

Ibrutinib was generously provided by Janssen R & D, LLC. Carfilzomib (PR-171) was obtained from Chemi Tek (Indianapolis, IN, USA). Idelalisib (GS-1101/CAL-101) was purchased from ApexBio Technology, LLC (Boston, MA, USA) and cyclophosphamide was purchased from Sigma-Aldrich (St. Louis, MO, USA). Doxorubicin was purchased from Selleck Chemicals (Houston, TX, USA). Obinutuzumab (GA101) was generously provided by Dr. Christian Klein, PhD from Roche (Basel, Switzerland) and rituximab was purchased from Genentech Inc. (South San Francisco, CA, USA).

In-vivo xenograft models

All experimental animal procedures, animal protocols and care were approved by the Institutional Animal Care and Use Committee at New York Medical College.

Ffluc/Zeo-labeled Raji cells were generated as we previously described. 1 × 10⁶ genetically modified fluc/Zeo-labeled Raji cells were administered by sterile subcutaneous (s.c) injections in the lower flank of 6–8 week-old female mice as we have previously described. Stable engraftment of the genetically engineered cell line was also verified by bioluminescence imaging as we previously described. The total light emissions of the tumor cells in the animal were quantified using the Living Image software (Xenogen IVIS-200, Caliper Life Science). Tumor progression was monitored at day 7 and then every five days by BLI. Therapy was administered daily for 10 days and in combination with other drugs. Tumor size and whole-body tumor burden were assessed and survival was measured for 60 days as we have previously described. The survival rate was performed using humane endpoints and all mice were sacrificed when tumor size was greater than 2.0 cm³ [(length × width²)/2] or with signs of ulceration, or if mice were moribund.

Statistical analysis

Average values are reported as the mean ± SD. Significant differences between two groups were determined by using one-tailed paired Student’s t-test and p-values less than 0.05 were considered significant. CompuSynTM software was utilized for the determination of IC₅₀ value and quantitation of synergism and antagonism in drug combination. Kaplan-Meier method was performed for survival rate and differences were evaluated by log-rank test using the Prism Version 6.0 software.

Authorship contributions

YC analyzed and organized the data and wrote the manuscript. SL performed the experiments, analyzed the data and wrote the manuscript. CY performed experiments, analyzed the data and wrote the manuscript. JA, EM, CV, MB, TS, RRM, PG, STG, MSL, MH, LML, LGR, SLP, MB, TS, RRM, PG, STG, MSL, MH, LML, LGR, SLP reviewed and approved the manuscript. MSC designed the study, analyzed the data and wrote the manuscript.

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Conflict of Interest Statement

All authors have no potential conflicts of interest to disclose.
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