Cell-Cycle Reprogramming for PI3K Inhibition Overrides a Relapse-Specific C481S BTK Mutation Revealed by Longitudinal Functional Genomics in Mantle Cell Lymphoma

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ABSTRACT

Despite the unprecedented clinical activity of the Bruton tyrosine kinase (BTK) inhibitor ibrutinib in mantle cell lymphoma (MCL), acquired resistance is common.

By longitudinal integrative whole-exome and whole-transcriptome sequencing and targeted sequencing, we identified the first relapse-specific C481S mutation at the ibrutinib binding site of BTK in MCL cells at progression following a durable response. This mutation enhanced BTK and AKT activation and tissue-specific proliferation of resistant MCL cells driven by CDK4 activation. It was absent, however, in patients with primary resistance or progression following transient response to ibrutinib, suggesting alternative mechanisms of resistance. Through synergistic induction of PI3K/AKT and inhibition of PI3K-AKT activation, prolonged early G1 arrest induced by PD 0332991 (palbociclib) inhibition of CDK4 sensitized resistant lymphoma cells to ibrutinib killing when BTK was unmutated, and to PI3K inhibitors independent of C481S mutation. These data identify a genomic basis for acquired ibrutinib resistance in MCL and suggest a strategy to override both primary and acquired ibrutinib resistance.

SIGNIFICANCE: We have discovered the first relapse-specific BTK mutation in patients with MCL with acquired resistance, but not primary resistance, to ibrutinib, and demonstrated a rationale for targeting the proliferative resistant MCL cells by inhibiting CDK4 and the cell cycle in combination with ibrutinib in the presence of BTKWT or a PI3K inhibitor independent of BTK mutation. As drug resistance remains a major challenge and CDK4 and PI3K are dysregulated at a high frequency in human cancers, targeting CDK4 in genome-based combination therapy represents a novel approach to lymphoma and cancer therapy. Cancer Discov; 4(9): 1022–35. © 2014 AACR.

INTRODUCTION

Mantle cell lymphoma (MCL), a non-Hodgkin lymphoma of pregerminal center mature B cells, remains incurable despite the development of drug resistance (1). Bruton tyrosine kinase (BTK) is a TEC family cytoplasmic tyrosine kinase required for the development, activation, and differentiation of B cells (2), as shown by the development of X-linked agammaglobulinemia when BTK is genetically inactivated (3). By mediating B-cell receptor (BCR) signaling, BTK is also indispensable for the survival of B cells and lymphoma cells (4). Targeting BTK with the irreversible, orally bioavailable inhibitor ibrutinib (PCI 32765; ref. 5) achieved an unprecedented objective response rate of 68% in a phase II single-agent clinical trial in patients with relapsed/refractory MCL (6), and was similarly efficacious in chronic lymphocytic leukemia (CLL; ref. 7). On this basis, ibrutinib was approved by the FDA in late 2013 for treatment of patients with recurrent MCL and in 2014 for patients with CLL (8).

However, disease progression while on ibrutinib treatment is frequent in MCL. It was reported as the principal reason for discontinuation of ibrutinib in 50 of 65 patients in the phase II trial, and has been associated with a highly proliferative state and poor clinical outcomes (ref. 6; data not shown). Although the mechanism is unknown, the unrestrained proliferation of MCL cells at relapse suggests that targeting the cell cycle in combination therapy may delay the expansion of resistant clones or override some mechanisms of ibrutinib resistance. Because a hallmark of MCL is cell-cycle dysregulation (9) due to aberrant cyclin D1 expression from a t(11;14)(q13;q32) chromosomal translocation and activation of CDK4, targeting cyclin D1 or CDK4 represents a rational approach to controlling the cell cycle in MCL. Supporting this possibility, targeting CDK4/CDK6 with the selective and potent oral inhibitor PD 0332991 (palbociclib; ref. 10) in the first single-agent clinical trial in patients with recurrent MCL effectively arrested MCL cells in early G1, resulting in a durable clinical response with an excellent toxicity profile (11).

CDK4 is overexpressed or activated at a high frequency in both hematologic malignancies and solid tumors, and PD 0332991 is now a breakthrough therapy for breast cancer owing to its ability to more than double the progression-free survival (PFS) of patients with metastatic breast cancer when combined with letrozole (12). The clinical efficacy of PD 0332991 may stem from induction of prolonged early G1

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arrest (pG1) beyond the scheduled early G1 transit time by sustained CDK4/CDK6 inhibition (13), which reprograms cancer cells for killing by diverse clinically relevant agents (14). These include dexamethasone (13) and the proteasome inhibitor bortezomib in primary myeloma cells ex vivo and in animal models (14, 15), and PI3K inhibitors in primary MCL cells ex vivo (16).

Here, we demonstrate by longitudinal functional genomics and targeted sequencing a relapse-specific C481S missense mutation at the ibrutinib binding site of BTK in both patients who progressed on ibrutinib after a durable response, but not in patients (n = 6) with a transient response or primary resistance to ibrutinib. A further analysis of 1 patient revealed that the BTK<sup>C481S</sup> mutation is associated with heightened BTK and AKT activation, exacerbated genomic instability, and preferential CDK4-driven proliferation of resistant MCL cells in the spleen. Induction of pG1 by selective inhibition of CDK4 reprogrammed lymphoma cells for killing by ibrutinib when BTK is unmutated, and by selective PI3K inhibitors regardless of the presence of the BTK<sup>C481S</sup> mutation, suggesting a novel strategy to override ibrutinib resistance by targeting CDK4 in genome-based combination therapy.

**RESULTS**

**Relapse-Specific BTK<sup>C481S</sup> Mutation in MCL**

To elucidate the mechanism of acquired resistance to ibrutinib, we investigated the dynamic tumor evolution and discerned mutations that were expressed in MCL tumors by longitudinal integrative analysis of whole-exome sequencing (WES) and whole-transcriptome sequencing (WTS) of five serial biopsies of a representative male patient with MCL (Patient 1). This patient achieved a partial response (PR; equal to or greater than 50% reduction of tumor mass) for 14 months before progression with mild lymphadenopathy and massive splenomegaly (see Methods).

Single-nucleotide variant (SNV) analysis of serial WES and Sanger sequencing detected a dinucleotide substitution of G1442C and C1443T in BTK in MCL cells at relapse in both the bone marrow (r_IbBM, 74% of the reads) and the spleen (r_IbSP, 83% of the reads). This substitution resulted in a cysteine-to-serine missense mutation at residue 481 (C481S), localized in the tyrosine kinase domain of BTK (Fig. 1A–C). Importantly, the C481S mutation was not detected in any of the three lymph node biopsies taken 8 months (p_Ib1 and p_Ib2) or immediately (p_Ib3) before initiating ibrutinib, or in the cheek swab germline control (Fig. 1A).

Longitudinal WTS analysis of serial biopsies corroborated the very high frequency (~80%) of C481S mutation in BTK exclusively at relapse in MCL cells in both the bone marrow (read depth, 129) and the spleen (read depth, 372; Fig. 1D). The abundance of BTK mRNA was increased at relapse (2-fold) in bone marrow MCL cells along with the selective elevation of mRNA expression from genes in the BCR signaling pathway (CD79A, LYN, PLCG2, and PRKCB; Fig. 1D). However, no SNV was identified in other genes of the BCR signaling pathway (Fig. 1D) or in genes frequently mutated in MCL such as ATM, NOTCH1, and UBR5, except for SNVs in the 5’ untranslated region (5’-UTR) of CCND1 (Fig. 1E; refs. 17, 18). BTK<sup>C481S</sup> has not been detected in ibrutinib-naïve primary MCL cells by WTS or WES by us and others (refs. 16, 18–21; data not shown). These data demonstrate the specificity of BTK<sup>C481S</sup> mutation at relapse from ibrutinib in MCL.

Further integrative WES and WTS analysis revealed 190 SNVs that were expressed in MCL cells but not present in the germline: 35 in coding sequences (CDS) and 155 in UTRs (Fig. 2A and Supplementary Tables S1 and S2). Sixteen of the CDS SNVs were nonsynonymous and predicted to be damaging at the protein level (Supplementary Table S3), of which 11 were constitutive and five increased in frequency with time in BRAP, RC3H1, C14orf5159, BTK, and TRAPPC10 (Fig. 2B–D). Only C481S in BTK and V600F in TRAPPC10 (22) were detected at a very high frequency exclusively at relapse in MCL cells in both the bone marrow and the spleen (Fig. 2B and Supplementary Table S3). The significance of the concurrent TRAPPC10<sup>V600F</sup> mutation is unknown. However, its unique association with BTK<sup>C481S</sup> in both bone marrow and splenic MCL cells at relapse implicates a clonal origin for ibrutinib-resistant MCL cells.

BTK<sup>C481S</sup> was identified in a second male patient with MCL (Patient 2), who progressed on ibrutinib after achieving a PR that lasted for 30 months (see Methods). By targeted and Sanger sequencing of cells present in the pleural effusion, which comprised 69% CD19<sup>+</sup> cells in resistant MCL cells.

**Absence of BTK<sup>C481S</sup> Mutation in Transient Ibrutinib Response and Primary Resistance**

BTK<sup>C481S</sup> was absent in serial peripheral blood or lymph node MCL cells of 6 patients with primary resistance to ibrutinib or acquired resistance following a transient (<5 months) PR, using cheek swab DNA as controls for individual patients (Supplementary Table S5). No gain-of-function R665W mutation in the downstream PLCγ2 observed in ibrutinib relapse in CLL (23) was detected (Supplementary Table S5). Thus, mechanisms other than BTK<sup>C481S</sup> mutation or PLCγ2 activation must have contributed to the rapid resistance to ibrutinib in patients with MCL who retain the wild-type (WT) BTK.

This led us to ask whether BTK<sup>WT</sup> was inhibited by ibrutinib in vivo in resistant MCL cells. BTK<sup>WT</sup> was autophosphorylated (pY223) in peripheral blood MCL cells before (p_Ib1) but not at 3 weeks of ibrutinib treatment (Ib1) in Patient 4, who had a PR for more than 10 months, or in Patient 8, who maintained stable disease (SD; <50% reduction of tumor mass) for more than 4 months (Fig. 3A). A prominent loss of AKT activation (pS473) by mTORC2 was concurrent with BTK inhibition, even taking into account the reduction in total AKT, whereas PLCγ2 activation (pY759) was diminished only modestly (Fig. 3A). Of interest, BTK<sup>WT</sup> was also
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inactivated in lymph node (Patient 6) and peripheral blood (Patient 9) MCL cells of primary ibrutinib-resistant patients while on ibrutinib treatment, in which AKT was expressed at a high level and activated.

Primary MCL cells predominantly express PI3Kδ (PIK3CD), the hematologic lineage-restricted PI3K isoform (24), whereas PI3Kα (PIK3CA) is expressed at a lower level in some cases of MCL. PI3Kβ (PIK3CB) and PI3Kγ (PIK3CG) were barely expressed, and the regulatory subunits PIK3R5 and PIK3R6 were undetectable (16). However, expression of PI3Kα was not associated with the ibrutinib response, as PI3Kα was expressed in MCL cells of Patient 4 (PR), Patient 1, and Patient 3 (progression following a transient PR) and all 3 primary resistant patients, but not in Patient 8 (SD) or Patient 5 (progression following transient PR; Fig. 3A and B). Primary ibrutinib resistance or transient response, therefore, seems not to stem from defective ibrutinib inhibition of BTK-WT in MCL cells but rather may involve sustained PI3K–AKT activation.
Activation of BTK and AKT at Relapse in MCL Cells Harboring BTK<sup>C481S</sup> Mutation

The functional consequence of C481S mutation in vivo was then characterized in MCL cells isolated from serial biopsies of Patient 1. Ibrutinib inhibits BTK activity irreversibly by covalently binding to cysteine 481 in vitro and in cell lines (5), and the C481S mutation has been shown to markedly reduce (~25-fold) the affinity of BTK for ibrutinib in a model chicken lymphoma DT40 cell line (23). Notably, BTK was constitutively active in lymph node MCL cells before ibrutinib therapy and in peripheral blood B cells (PBC) from healthy donors, presumably for their survival (Fig. 3C). Activation of BTK was increased in bone marrow MCL cells at relapse, concurrent with a modest increase in PI3Kα and PI3Kδ and activation of AKT (pS473; Fig. 3C).

Figure 2. Longitudinal integrative WES and WTS analysis of acquired resistance following a durable response in MCL harboring BTK<sup>C481S</sup>. A, SNVs (1,291,421) were identified in MCL cells from serial biopsies of Patient (Pt) 1 before ibrutinib treatment (p<sub>lb1</sub>, p<sub>lb2</sub>) and after relapse from ibrutinib (r<sub>lbBM</sub>, r<sub>lbSP</sub>) by WTS using the Illumina platform and Genesifter (Geospiza). After exclusion of SNVs detected in PBC libraries, 44% (n = 6,060) of the 13,776 SNVs were also detected by WES analysis. A threshold of 10<sup>x</sup> coverage was applied, which reduced the number of SNVs to 2,679. After exclusion of the germline SNVs present in the cheek swabs, 190 SNVs were specific to the MCL cells of this patient: 155 detected in UTRs and 35 in CDS, of which 16 were predicted to be damaging at the protein level by SIFT, PROVEAN, or PolyPhen-2. Among them, 5 were detected at increasing frequency in serial biopsies but only BTK<sup>C481S</sup> and TRAPPC1D<sup>G115E</sup> mutations were detected at relapse exclusively (B and C), and 11 were present in all biopsies (D). The percentage of alternative allele (red) and mRNA abundance (RPKM) of the indicated genes are shown. Mutations are shown in red text.
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The increase in PI3Kα and PI3Kδ proteins mirrored the selective increases in the abundance of PIK3CD and PIK3CA mRNA in MCL cells in the bone marrow and the spleen, suggesting that it stems, in part, from transcriptional activation of PI3Kα and PI3Kδ (data not shown). Collectively, these data suggest that the C481S mutation relieves BTK from ibrutinib inhibition, leading to heightened activation of BTK and AKT-pS473 in resistant bone marrow MCL cells in vivo.

Relapse from ibrutinib was also associated with a preferential increase in CXCR4, but not CXCR5, mRNA in bone marrow MCL cells; and induction of CXCL12, but not CXCL13, mRNA in MCL cells in both the bone marrow and the spleen and CCL3, CCL4, TNF, and IL10 in the spleen (Fig. 3D). Because the expression of these chemokines and cytokines has been shown to promote proliferation and survival of MCL cells and to be inhibited by ibrutinib ex vivo (25), the tissue-specific upregulation at relapse provides additional functional evidence for ibrutinib resistance in vivo.

Tissue-Specific Cell-Cycle Control of Proliferation of BTKC481S MCL Cells at Relapse

Unrestrained tumor cell proliferation is characteristic of ibrutinib relapse in MCL. Progression of Patient 1 was associated with massive splenomegaly and mild lymphadenopathy, suggesting a tissue-specific response. Longitudinal WTS analysis of cell-cycle genes showed that, as expected, most cell-cycle genes were expressed at a higher level in MCL cells than in resting PBCs from normal donors (Fig. 4A). However, MKI67 (Ki67) mRNA was markedly repressed in the bone marrow at relapse, concurrent with selective elevation of CDK4 mRNA among genes scheduled for early G1 in both bone marrow and splenic MCL cells, and CCNA2, CCNB1, and CDK1 mRNAs (S and G2-M) in splenic MCL cells (Fig. 4A). These genes were expressed correspondingly at the protein level in bone marrow and splenic MCL cells, along with a reduction of p27 protein and increases in cyclin A and cyclin B in the splenic MCL cells (pretreatment MCL cells were no longer available for protein analysis; Fig. 4B). Surprisingly, the MYC mRNA level was lower in the MCL cells of Patient 1 than in the PBCs, and the c-MYC protein was undetectable despite a striking (>200-fold) increase in MYC mRNA in splenic MCL cells (Fig. 4B). Collectively, these data indicate that preferential proliferation of MCL cells in the spleen at relapse in this patient is independent of c-MYC, and partly driven by selective upregulation of CDK4, which cooperates with cyclin A, cyclin B, and CDK1 to accelerate the cell cycle in MCL cells.

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**Figure 3.** Concurrent inactivation of BTKWT and AKT by ibrutinib in responding patients and AKT activation independent of BTKWT inactivation in ibrutinib-resistant patients. A and B, immunoblotting of indicated proteins in MCL cells isolated from responding Patient (Pt) 4 before (Pt 4 p Ib1) and on day 21 of ibrutinib treatment (Pt 4 lB1); Pt 8 on day 21 of ibrutinib treatment (Pt 8 lB1); primary resistant Pt 6 and Pt 9 on day 21 of ibrutinib treatment, and at relapse (Pt 3 rB) after 3 months of ibrutinib response (See Supplementary Table S5 for details). *denotes a nonspecific signal in A, C, immunoblotting of MCL cells from serial lymph node biopsies of Pt 1 before ibrutinib treatment (p_Ib1, p_Ib2) and from the bone marrow at relapse from ibrutinib (r_IbBM). CD19+ B cells isolated from peripheral blood of healthy donors (PBCs) and JEKO-1 cells were used as controls. D, WTS analysis of mRNA abundance (RPKM) of indicated genes expressed in serial biopsies of Pt 1 and PBCs, as shown in Fig. 1.
Cyto genetic and FISH analyses further revealed a complex karyotype in proliferating splenic MCL cells at relapse. Multiple numerical and structural abnormalities were observed, most notably the hemizygous deletion of chromosome 13q that encompasses the RB1 gene (Fig. 4C). Copy-number variation (CNV) analysis of serial WES of purified MCL cells independently identified the hemizygous 13q deletion at relapse in bone marrow MCL cells (Fig. 4D), and discovered two additional relapse-specific hemizygous deletions in 21q that included the TRAPPC10 gene (data not shown). However, RB1 expression was unabated at the mRNA level in MCL cells at relapse (Fig. 4A), presumably through a dosage compensation mechanism to ameliorate the haploinsufficiency. Relapse from ibrutinib in Patient 1 is, thus, associated with exacerbated genomic instability of MCL cells in the presence of sustained RB1 expression.

Further evaluation of proliferation of MCL cells in situ in serial biopsies by IHC demonstrated that more MCL cells (PAX5+) were cycling (Ki67+) in the spleen at relapse (r_IbSP) than in the lymph nodes before ibrutinib therapy (p_Ib1; Fig. 4E). This was apparently driven by accelerated progression through early G1, given the >3-fold increase in CDK4/CDK6–specific phosphorylation of Rb (pSRb; Fig. 4E). In contrast, few bone marrow MCL cells cycled or expressed pSRb at relapse despite the common BTK<sup>C481S</sup> mutation (Fig. 4E). These results confirm that in Patient 1, MCL cells harboring the C481S mutation preferentially proliferate in the spleen due to CDK4 activation but rarely in the bone marrow at relapse.

**pG1 Sensitizes Resistant BTK<sup>WT</sup> MCL Cells to Ibrutinib via Synergic Induction of PIK3P1**

Previously, we have shown that induction of pG1 by sustained inhibition of CDK4 with the selective inhibitor PD 0332991 reporgrams cancer cells for killing by diverse cytotoxic partners (14, 16). Given that BTK<sup>WT</sup> was inhibited by ibrutinib in highly proliferative primary resistant MCL cells in vivo (Fig. 3A), induction of pG1 may lower the threshold...
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We tested this hypothesis in four RB1-positive MCL cell lines harboring no mutations in BTK or PLCG2 (WES data not shown) by first confirming that PD 0332991 induced pG1, which maintained cyclin D1 and CDK4 expression, elevated p27 protein levels, and prevented the expression of cyclin A, cyclin B, CDK1, and c-MYC protein as expected for pG1 (Fig. 4B). Ibrutinib (0.01–1 μmol/L) was not toxic to JEKO-1, MAVER-1, or Mino MCL cells, although it moderately inhibited S phase entry in Mino cells and potently induced apoptosis in SP53 cells during G1–S transition by 48 hours, as shown by BrdUrd/PI analysis (Supplementary Fig. S1). pG1 did not augment ibrutinib killing of SP53 cells, but profoundly inhibited the expansion of live cells through cell-cycle control (Fig. 5B). Importantly, induction of pG1 both prevented the ibrutinib-resistant JEKO-1, MAVER-1, and Mino cells from replicating and sensitized them to ibrutinib-induced apoptosis (Annexin V+/PI+), resulting in a 50- to 100-fold reduction of live cells by 96 hours of ibrutinib treatment (Fig. 5B).

We have recently uncovered that synergistic induction of PIK3IP1, a negative regulator of PI3K (26), mediates pG1 reprogramming of MCL cells for durable and enhanced killing by selective PI3K inhibition (16). PIK3IP1 mRNA expression in MCL cells from all serial biopsies of Patient 1 was 10- to 30-fold lower than in PBCs (Fig. 5C), consistent with previous observations in primary MCL cells (16). It was induced by pG1 and by ibrutinib modestly, but by ibrutinib and pG1 synergistically (~50 fold) in JEKO-1 and MAVER-1 cells (Fig. 5D). Knocking down PIK3IP1 by two independent shRNA–lentivirus constructs blunted the enhancement of ibrutinib killing in pG1, but not the death induced by pG1 alone in JEKO-1 or MAVER-1 cells (Fig. 5E). Thus, induction of pG1 by selective CDK4 inhibition not only prevents refractory MCL cells that are expressing the wild-type BTK from proliferating, but also reprograms them for ibrutinib killing through synergistic induction of PIK3IP1.

**pG1 and Ibrutinib Cooperatively Inactivate BTK, AKT, and NF-κB in BCR Signaling**

BTK and AKT were coordinately inactivated on ibrutinib treatment in MCL cells of a responding patient (Patient 4). However, AKT activation (pS473) was maintained despite inactivation of BTKWT in MCL cells of primary resistant patients (Patient 6 and Patient 9; Fig. 3A). Because pG1 inactivates AKT in primary MCL cells ex vivo (16), it may sensitize BTKWT-resistant MCL cells to ibrutinib through inactivation of AKT. Indeed, activation of BTK (pY223) and AKT (pS473) in BCR signaling stimulated by anti-IgM was partially neutralized by ibrutinib and in pG1, but completely abolished by ibrutinib in pG1-reprogrammed JEKO-1 cells (Fig. 6A). This finding was reminiscent of synergistic and durable inhibition of AKT activation by a PI3K inhibitor in pG1, which led to enhanced killing of primary MCL cells and MCL cell lines by PI3K inhibitors (16), suggesting that inactivation of PI3KAKT mediates pG1 reprogramming of MCL cells for killing by both ibrutinib and PI3K inhibitors.

Primary resistance to ibrutinib despite inactivation of BTKWT suggests that activation of distal BCR signaling may contribute to ibrutinib resistance. The classic (pS0/RELA) of ibrutinib killing in addition to inhibiting the cell cycle (Fig. 5A).

Figure 5. pG1 sensitizes resistant BTKWT MCL cells to ibrutinib via synergistic induction of PIK3IP1. A, schema for dual targeting of BTK with ibrutinib and CDK4 with PD 0332991 in MCL cells expressing BTKWT, pG1, prolonged early G1 arrest that exceeds the schedule early G1 transit time (16–20 hours in MCL cells) by selective and sustained inhibition of CDK4. B, schema for sequential incubation with PD 0332991 (0.5 μmol/L) and ibrutinib (left), total viable cells (×10,000 cells/mL) at 48 and 96 hours of ibrutinib treatment (1 μmol/L for JEKO-1, MAVER-1, and Mino and 0.1 μmol/L for SP53, middle); FACS analysis of apoptotic (Annexin V+PI+), resulting in a 50- to 100-fold reduction of live cells (right). C, WTS analysis of PIK3IP1 mRNA abundance in serial biopsies of Patient (Pt) 1 and PBCs. D, qRT-PCR analysis of relative PIK3IP1 mRNA levels in MCL cells cultured with PD and ibrutinib for 48 hours as indicated. E, live cells (percentage of untreated cells) and cell death (Topro-3+ cells) in MCL cell lines infected with PIK3IP1 shRNA or LacZ shRNA lentivirus and treated with PD and ibrutinib as in D. Error bars, SD. *, P < 0.05; **, P < 0.01; calculated using the Student t test. Data are representative of four independent experiments.
NF-κB pathway mediates BCR signaling for B-cell proliferation and survival (4), further indicating that pG1 may reprogram MCL cells by attenuating NF-κB activation. To address this possibility, we showed by immunofluorescence staining (IMF) that nuclear localization of RELA (p65) indicative of NF-κB activation in response to anti-IgM was undetectable in some JEKO-1 cells by 24 hours of PD 0332991 treatment and completely absent by 48 hours (Fig. 6B). Thus, in a time-dependent manner, pG1 reestablishes the rapid (within 5 hours) inactivation of the classic NF-κB pathway by ibrutinib in BCR signaling (Fig. 6B and C). The alternative (p52/RELB) NF-κB pathway has been shown recently to contribute to ibrutinib resistance in MCL cell lines (20). p52 was expressed in the ibrutinib-resistant JEKO-1 cells used in this study but not appreciably inhibited by ibrutinib in pG1, along with invariable expression of NIK (MAP3K14) required for the processing of p100 to p52 (Supplementary Fig. S2).

Longitudinal WES/WTS analysis of serial biopsies of Patient 1 further revealed an association of ibrutinib relapse with upregulation of selective genes of the classic NF-κB pathway such as NFKB1 and RELA as well as NFKB2 and RELB of the alternative NF-κB pathway in bone marrow MCL cells, although MAP3K14 expression did not vary (Fig. 6D). Taken together, these data suggest tissue-specific upregulation of genes in the NF-κB pathways at relapse in a patient with BTKC481S mutation, and that pG1 reprograms MCL cells for cytotoxic killing, in part by selective inactivation of the classic, but not the alternative, NF-κB pathway.

**pG1 Sensitizes Resistant Lymphoma Cells Independent of BTKC481S Mutation to PI3K Inhibitors**

PI3K is required for B-cell development and proliferation (27), and acts upstream of BTK for BCR-dependent survival in mature B cells (28). Expression of PI3K and AKT was sustained in all patients with MCL, whereas activation of ATK (pS473) was reduced in an ibrutinib-responder patient, sustained in BTKWT MCL cells of primary resistant patients, and enhanced in BTKC481S MCL cells in a patient at relapse from a durable ibrutinib response (Fig. 3). Because pG1 reprograms primary MCL cells for PI3K inhibitor killing *ex vivo* through durable inhibition of AKT activation (16), it may sensitize ibrutinib-resistant lymphoma cells to PI3K inhibitors independent of the BTKC481S mutation (Fig. 7A).

To test this hypothesis, we reconstituted the expression of V5-tagged BTKWT and BTKC481S in equal molar in the DT40 BTK−/− chicken lymphoma cell line, in which the BTKC481S has been shown to attenuate ibrutinib-mediated inhibition of BTK (ref. 29; Fig. 7B and C). The expression of p110α, p110β and AKT and AKT activation in DT40 BTK−/− cells were not altered by stable expression of BTKWT, BTKC481S, or the GFP-V5 vector (Fig. 7C). Sustained inhibition of CDK4 by PD 0332991 led to pG1 in DT40 cells expressing either BTKWT or BTKC481S (Fig. 7D), which were then reprogrammed for killing by three clinically relevant selective PI3K inhibitors independent of the BTKC481S mutation. pG1 conferred sensitivity to the PI3K-δ-specific inhibitor GS-1101 (idelalisib; refs. 29–31), which alone did not kill DT40 cells expressing BTKWT or BTKC481S, presumably due to functional compensation by the PI3Kα expressed in these cells. Thus, it seems that when combined with inhibition of PI3Kδ and ATK activation, pG1 bypasses PI3Kα. pG1 also profoundly enhanced killing by the dual PI3Kα/δ inhibitors BAY 80-6946 (copanlisib; ref. 32) and GDC-0941 (pictilisib; ref. 33; Fig. 7E; Supplementary Fig. S3). Sensitization by pG1 led to virtual eradication of live cells by all PI3K inhibitors characterized, even when BTKC481S was expressed at a 5-fold excess of BTKWT (Fig. 7E and Supplementary Fig. S3). Induction of pG1 by sustained CDK4 inhibition thus profoundly reprograms ibrutinib-resistant lymphoma cells for PI3K inhibitor killing independent of the BTKC481S mutation.
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**DISCUSSION**

Acquired resistance to therapy remains a formidable challenge in human cancer. It is particularly urgent in MCL, which remains incurable due to the development of drug resistance even to the novel BTK inhibitor ibrutinib, despite its exceptional single-agent activity. By longitudinal integrative WES and WTS analysis of MCL cells from serial biopsies of a patient with MCL who progressed following a durable PR, we have now identified the first relapse-specific C481S missense mutation at the ibrutinib-binding site of BTK in MCL. The heightened activation of BTK and AKT,
Second, the gain-of-function R665W mutation in PLCγ in proliferative lymphomas may be less well controlled by ibrutinib. Follow-up of 20.9 months; ref. 7), suggesting that more pro-

First, disease progression on ibrutinib is much more frequent in MCL is distinguishable from that in CLL in other aspects. From this common genomic aberration, ibrutinib resistance 13 CLL patients who relapsed while on ibrutinib (23). Apart from this common functional evidence in vivo for impaired BTK inhibition caused by a BTK mutation in acquired ibrutinib resistance.

The acquired resistance to ibrutinib could be treatment-related or due to clonal evolution, which are not mutually exclusive. Although no mutations in BTK have been identified by WES or WTS in ibrutinib-naïve MCL patients so far, the existence of a BTKC481S-mutant clone at a very low frequency before treatment cannot be excluded, as demonstrated by <0.01% of cells becoming the predominant clone in relapsed leukemia (34). In addition, the BTKC481S is likely to confer a selective advantage over MCL cells expressing BTKWT and responding to ibrutinib-mediated killing. Consistent with a clonal origin, ibrutinib-resistant MCL cells at relapse in bone marrow and spleen of Patient 1 have in common: (i) identical dinucleotide substitution in BTK; (ii) unique concurrent high-frequency TRAPPC10 stop mutation; (iii) exacerbated genomic instability in the absence of mutations in ATM, the most frequently mutated gene in MCL (35), and hemizygous 13q deletion (Fig. 4).

This raises the question of whether other nonsynonymous potentially damaging mutations, identified by WES and WTS analysis, contribute to the development of acquired ibrutinib resistance in MCL. Of particular interest is the increasing frequency of the N80Y mutation in BRAP, a BRC2-binding protein; the constitutive R273C mutation in TP53 and S295N mutation in GTSE1, a p53-binding protein and G2–M checkpoint regulator and the K23R mutation in MEF2B, a transcriptional activator frequently mutated in MCL (19) and in diffuse large B-cell lymphoma, which led to deregulation of the target oncogene BCL6 (Fig. 2; ref. 36).

Detection of BTKC481S in a second patient with MCL who progressed following an even more durable PR (30 months), but not in any of the 6 patients with MCL who progressed on ibrutinib within 5 months or failed to respond, suggests that the C481S mutation in MCL is specific to acquired ibrutinib resistance after a durable response. Although this possibility is being addressed in a larger MCL patient population, alternative mechanisms must have contributed to primary or rapid resistance to ibrutinib.

The BTKC481S mutation has been identified by WES in 5 of 13 CLL patients who relapsed while on ibrutinib (23). Apart from this common genomic aberration, ibrutinib resistance in MCL is distinguishable from that in CLL in other aspects. First, disease progression on ibrutinib is much more frequent in MCL (50 of 115 with a median follow-up of 15.3 months; ref. 6) than in the more indolent CLL (11 of 85 with a median follow-up of 20.9 months; ref. 7), suggesting that more proliferative lymphomas may be less well controlled by ibrutinib. Second, the gain-of-function R665W mutation in PLCγ2 identified in CLL (23) has not been detected in MCL (Supplementary Table S5). Third, BTK was inactivated by ibrutinib in BTKWT MCL cells of patients with primary resistance (Fig. 3), suggesting that dysregulation of distal BCR signaling other than through an activating PLCγ2 mutation contributes to rapid ibrutinib resistance. Fourth, the enhanced BTK and PI3K–AKT activation at relapse in BTKC481S MCL cells provided the first evidence for divergent functional consequences caused by BTKC481S mutation in vivo and reinforces the critical importance of developing genome-based combination therapy in MCL.

Relapse from ibrutinib is often characterized by aggressive proliferation of resistant MCL cells and poor clinical outcomes. Here, we show that it was also associated with sustained AKT activation (pS473) in BTKWT MCL cells with primary resistance and enhanced AKT activation (pS473) in BTKC481S MCL cells at relapse (Fig. 3). These findings demonstrate a pivotal role of PI3K–AKT activation in ibrutinib resistance and provide a strong rationale to override ibrutinib resistance by targeting the cell cycle in combination with inhibition of BTK or PI3K. Indeed, induction of pG1 by PD 0332991 inhibition of CDK4 reprograms MCL cells expressing BTKWT for killing by ibrutinib, which, together with inhibition of cell proliferation, resulted in a dramatic reduction of live cells (Fig. 5). Furthermore, pG1 sensitizes ibrutinib-resistant MCL cell lines (16) and DT40 lymphoma cells independent of BTKC481S mutation to killing by clinically relevant PI3K inhibitors (Fig. 7), which has important mechanistic and clinical implications.

Previously, we have shown that induction of pG1 by selective CDK4/CDK6 inhibition leads to restricted expression of genes scheduled for early G1 only, thereby forcing an imbalance in gene expression that reprograms cancer cells for killing by diverse cytotoxic partners (14). PIK3IP1 was synergistically induced by a PI3K inhibitor (GS-1101 or GDC-0941) and pG1 in MCL cells for enhanced killing through sustained inhibition of pAKT (16). Here, we demonstrated that PIK3IP1 was also synergistically induced by ibrutinib and pG1 in MCL cells to mediate pG1 enhancement of ibrutinib killing, most likely by abrogating the activation of BTK and AKT in BCR signaling (Figs. 5 and 6). PIK3IP1 thus plays a pivotal role in pG1 sensitization of MCL cells to killing by either PI3K or BTK inhibitor. As such, it represents a novel molecular therapeutic biomarker for pG1 combination therapy.

In addition, we have now discovered that pG1 selectively antagonizes the activation of the classic NF-κB pathway in BCR signaling, but not the alternative NF-κB pathway (Fig. 6). Inactivation of NF-κB in pG1 is redundant with inhibition of BTK by ibrutinib, but it may cooperate with inactivation of PI3K–AKT in pG1-reprogrammed MCL cells to enhance PI3K inhibitor killing independent of the BTKC481S mutation. By inactivating NF-κB, pG1 antagonizes BCR signaling downstream of PKCβ, and could also bypass various gain-of-function upstream mutations in the BCR signaling pathway (Fig. 5A). Given the importance of NF-κB in transcriptional regulation, inactivation of NF-κB may, in fact, mediate the imbalance in gene expression in pG1, thereby playing a broader role in pG1 reprogramming.

Targeting CDK4 with PD 0332991 in combination with PI3K or BTK inhibition is poised for clinical validation in MCL as well. PD 0332991 had already achieved a durable clinical response with a favorable toxicity profile in the first single-agent clinical trial in patients with recurrent MCL (11). It is now a breakthrough therapy for metastatic breast cancer owing to its exceptional clinical activity when combined with letrozole. Although pG1 reprogramming by PD 0332991...
requires Rb, the substrate of CDK4 and CDK6 (14, 16), it is independent of ATM and TP53, the most frequently mutated genes in MCL (17).

As for targeting PI3K, the novel PI3Kδ-specific inhibitor GS-1101 is a breakthrough therapy for CLL that exhibits its transient clinical activity in MCL. We showed that pG1 reprogrammed primary MCL cells for sustained and enhanced growth inhibition by GS-1101 via inhibition of AKT activation, and by the dual PI3Kα/δ inhibitor GDC-0941 (16) and BAY 80-6946 (Fig. 7). CDK4 and PI3K are dysregulated at a high frequency in human cancers that develop resistance to therapy (37). To overcome drug resistance by targeting the cell cycle, one of the critical next steps is to define the mechanism by which pG1 inactivates PI3K–AKT. Given that the carboxyl-terminus of AKT is directly phosphorylated by CDK2/cyclin A (38) and that induction of pG1 suppresses cyclin A synthesis (14), it seems likely that pG1 may inactivate AKT via cyclin A suppression (Fig. 4). This exciting possibility is amenable to investigation in targeting CDK4 in clinical studies by longitudinal integrative WTS.

Our unbiased, longitudinal integrative WES and WTS analysis of individual patients represents the first such undertaking in targeted lymphoma therapy. It has provided new insight into the genomic basis and mechanism for acquired resistance in MCL. In conjunction with functional genomics, the availability of exciting new targeting agents offers a unique opportunity to overcome acquired resistance by selective targeting of CDK4 in genome-based combination therapy in lymphoma, which has implications for other human cancers as well.

METHODS

Additional methods are detailed in the Supplementary Data.

Patients and Isolation of Primary MCL Cells

Tissue biopsies from lymph node, bone marrow, and spleen, and peripheral blood samples (Supplementary Table S5) were obtained from 9 patients with MCL at the New York-Presbyterian Hospital (New York, NY). Patient 2 samples were obtained at the Willamette Valley Cancer Institute and Research Center (Springfield, OR). All samples were collected after informed consent as part of a study approved by the local Institutional Review Boards. All patients were treated with ibrutinib after prior therapies. Patient 1 had received four therapies before starting single-agent ibrutinib and achieved a PR that lasted 14 months before disease progression marked by a rapidly enlarging spleen and spontaneous splenic hematoma requiring urgent surgery. Patient 2 achieved a PR lasting 30 months before progression marked by progressive adenopathy and a pleural effusion. Time-to-progression and best response of other patients are indicated in Supplementary Table S5. Primary MCL cells were purified using MACS CD19 MicroBeads (Miltenyi Biotec) at 4°C, and the percentage of MCL tumor cells (CD19+/CD5+) was determined to be >90% by flow cytometry. PBCs were isolated from healthy volunteers using the same protocol.

MCL Cell Lines

JEKO-1 cells were obtained from DSMZ, and MAVER-1 and MINO from ATCC. SP53 cells were kindly provided by Dr. Jiangao Tao (Moffit Cancer Center, Tampa, FL) and the chicken DT40 lymphoid cells deficient in BTK were described previously (39). No authentication of these cell lines was done by the authors. Cell lines were cultured in the presence of PD 0332991, GS-1101, GDC-0941, and BAY 80-6946 (all from Selleck Chemicals) or ibrutinib (Pharmacyclics) at concentrations and for times indicated.

WES

For each experimental condition, 100 ng of high-quality total RNA (RNA integrity number > 8 on the Agilent Bioanalyzer 2100) was isolated using the RNeasy kit according to the manufacturer’s instructions (Qagen). All RNAs were converted to cDNA with the SuperScript III First-Strand Synthesis kit (Life Technologies), isolated with the TruSeq mRNA prep kit (v2; Illumina), and then ligated to Illumina adapters, as per the standard TruSeq Illumina protocol. Using these multiplexed cDNA libraries, we generated clusters on the Illumina cBot station and paired-end sequenced each sample to 50 × 50 bp on the Illumina HiSeq2000 at the Weill Cornell Medical College (WCMC) Genomics Core. Cluster generation, sequencing, and processing of the images were done using the Real-Time Analysis (RTA) software on the HiSeq2000 and after processing with CASAVA (v1.8.2). To optimize library preparation, we used a TACON high-throughput RNA prep station. Raw data were filtered for high median quality (Q-value > 20) and then sent to Cornell’s High Performance Computing (HPC) cluster, to be run through our RNA sequencing (RNA-Seq) analysis pipeline. The RNA-Seq data presented in this study, which include libraries from serial biopsies of MCL tumors before ibrutinib treatment (p_Ib1, p_Ib2, p_Ib3) and after ibrutinib relapse (r_IbBM, r_IbSP), and PBCs from 3 healthy volunteers, have been deposited in the Gene Expression Omnibus (GEO).

Disclosure of Potential Conflicts of Interest

P. Martin has received an honorarium from Janssen. J. Shorman has received a commercial research grant from Pharmacyclics. S. Ali is an employee of Foundation Medicine and has ownership interest in the same. B. Chang is employed by Pharmacyclics. J.P. Leonard is a consultant/advisory board member of Pharmacyclics. No potential conflicts of interest were disclosed by the other authors.

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REFERENCES


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