

Critical roles for mTORC2- and rapamycin-insensitive mTORC1-complexes in growth and survival of BCR-ABL-expressing leukemic cells

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mTOR-generated signals play critical roles in growth of leukemic cells by controlling mRNA translation of genes that promote mitogenic responses. Despite extensive work on the functional relevance of rapamycin-sensitive mTORC1 complexes, much less is known on the roles of rapamycin-insensitive (RI) complexes, including mTORC2 and RI-mTORC1, in BCR-ABL-leukemogenesis. We provide evidence for the presence of mTORC2 complexes in BCR-ABL-transformed cells and identify phosphorylation of 4E-BP1 on Thr37/46 and Ser65 as RI-mTORC1 signals in primary chronic myelogenous leukemia (CML) cells. Our studies establish that a unique dual mTORC2/mTORC1 inhibitor, OSI-027, induces potent suppressive effects on primitive leukemic progenitors from CML patients and generates antileukemic responses in cells expressing the T315I-BCR-ABL mutation, which is refractory to all BCR-ABL kinase inhibitors currently in clinical use. Induction of apoptosis by OSI-027 appears to negatively correlate with induction of autophagy in some types of BCR-ABL transformed cells, as shown by the induction of autophagy during OSI-027-treatment and the potentiation of apoptosis by concomitant inhibition of such autophagy. Altogether, our studies establish critical roles for mTORC2 and RI-mTORC1 complexes in survival and growth of BCR-ABL cells and suggest that dual therapeutic targeting of such complexes may provide an approach to overcome leukemic cell resistance in CML and Ph+ ALL.

mRNA translation | cell proliferation | cellular signaling | kinase | OSI-027

The hallmark of chronic myeloid leukemia (CML), the BCR-ABL oncoprotein, has been heavily exploited over recent years as a therapeutic target for the treatment of CML and Ph+ acute lymphoblastic leukemia (ALL) (1, 2). Extensive previous work has firmly established that BCR-ABL results from reciprocal translocation involving chromosomes 9 and 22 and plays critical and essential roles in the pathogenesis of CML (3–7). Identifying BCR-ABL as the major molecular abnormality in CML had major therapeutic implications, as it ultimately led to the identification and clinical development of the ABL kinase inhibitor imatinib mesylate. Inhibition of the kinase activity and transforming capacity of BCR-ABL with imatinib mesylate results in long-lasting remissions in CML patients and this pharmacological agent has had a dramatic impact in the natural history of this disease (reviewed in refs. 8 and 9). Beyond remarkable therapeutic results, the introduction of imatinib mesylate in the treatment of BCR-ABL expressing malignancies has also provided an important model for the development of other specific therapies against distinct molecular targets.

Targeted therapies against BCR-ABL have further evolved in recent years with the development of second-generation BCR-ABL kinase inhibitors, such as nilotinib and dasatinib, which are clinically active in resistant Ph+ leukemias associated with BCR-ABL mutations (10–13). However, certain BCR-ABL

mutations such as T315I, are refractory to all known BCR-ABL kinase inhibitors in vitro and in vivo (14, 15). The realization of emerging resistance to second-generation BCR-ABL kinase inhibitors has led to intense efforts to design and develop new specific inhibitors that can block the activity of the T315I BCR-ABL mutant. Recent studies have suggested that targeting the myristate binding site of BCR-ABL may be an approach to overcome such resistance (16, 17), whereas combinations of allosteric BCR-ABL inhibitors with ATP-binding site inhibitors are effective in preclinical models of T315I-resistant leukemia (16). Although selective targeting of BCR-ABL with new agents may be an approach to overcome resistance associated with BCR-ABL mutations, there is also evidence for the emergence of other forms of cellular resistance unrelated to mutations of the BCR-ABL oncoprotein (18–20). This suggests that targeting downstream effectors of BCR-ABL that mediate diverse cellular signals may provide an important and possibly more effective approach to reverse leukemic cell resistance in BCR-ABL malignancies.

The serine-threonine kinase mTOR (mammalian target of rapamycin) is a critical mediator of many cellular signals that promote mitogenic responses (reviewed in ref. 21). mTOR has been shown to participate in two signaling complexes with distinct cellular functions, mTORC1 and mTORC2 (reviewed in ref. 22). In the present study we demonstrate that rapamycin-insensitive (RI)—mTORC1 complexes are activated in BCR-ABL cells and play key roles in mRNA translation of gene products that mediate mitogenic responses. We also provide evidence for activation of the mTORC2 complexes in BCR-ABL expressing cells and demonstrate that such complexes play important roles in their growth and survival. Dual targeting of mTORC2/mTORC1 in leukemic cells with a unique pharmacological inhibitor, OSI-027, results in inhibition of polysomal assembly and potent suppressive effects on primitive leukemic progenitors from CML patients. Unlike allosteric inhibitors such as rapamycin, OSI-027 is a potent, selective small molecule inhibitor of the catalytic site of mTOR, thereby targeting both mTORC1 and mTORC2 (23). Importantly, OSI-027 potently inhibits proliferation and induces apoptosis in cells expressing the T315I-BCR-ABL mutation, indicating that dual mTORC2/mTORC1 target-

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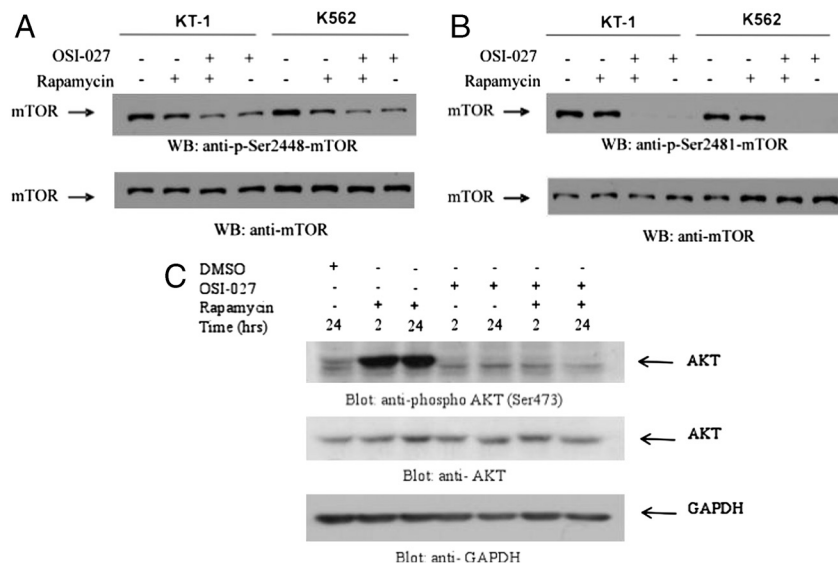


Fig. 1. Evidence for formation of mTORC2 complexes in BCR-ABL transformed cells and inhibition of their activities by OSI-027, but not rapamycin. (A) and (B) KT-1 or K562 cells were incubated with OSI-027 (10 μ M) or rapamycin (20 nM) for 90 minutes, as indicated. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with antibodies against the phosphorylated form of the mTOR on Ser2448 (A) or Ser2481 (B) or mTOR, as indicated. (C) K562 cells were treated the presence or absence of DMSO (control) or OSI-027 (10 μ M) or rapamycin (20 nM) for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with antibodies against the phosphorylated form of AKT on Ser473 or against AKT or GAPDH as indicated.

ing may provide an effective approach to overcome resistance in refractory Ph⁺ hematological malignancies.

Results

We sought to determine if beyond classic mTORC1 complexes (24–27), mTORC2 complexes are also present in BCR-ABL transformed cells and whether such complexes can be targeted with OSI-027, a unique dual mTORC1 and mTORC2 inhibitor (23). Recent studies have demonstrated that mTORC1 contains primarily mTOR phosphorylated on Ser2448, whereas mTORC2 contains mTOR phosphorylated on Ser2481 (28) and have demonstrated that phosphorylation of mTOR on Ser2481 is a marker for the presence of mTORC2 complexes (28). When we compared the phosphorylation of mTOR on Ser2448 versus Ser2481 in the CML-blast crisis KT1 and K562 cell lines, we found significant levels of phosphorylation on both sites, reflecting the presence of both mTORC1 and mTORC2 complexes (Fig. 1 A and B). Treatment of cells with either rapamycin or OSI-027 resulted in suppression of phosphorylation of mTOR on Ser2448 (Fig. 1A), consistent with inhibition of mTORC1 activity by both agents. However, only OSI-027 inhibited phosphorylation of mTOR on Ser2481 (Fig. 1B), demonstrating selective targeting of mTORC2 by OSI-027 (Fig. 1B). Similar results were obtained when the Ph⁺ ALL (preB ALL) cell line, BV173, was studied (Fig. S1). When the phosphorylation of AKT on Ser473, a marker of AKT activation and mTORC2 activity was also examined, we found that there was some baseline phosphorylation in K562 cells (Fig. 1C). Treatment with rapamycin resulted in strong enhancement of AKT phosphorylation/activation, reflecting potent induction of mTORC2 activity that was noticeable at 2 h and persisted after 24 h of treatment of the cells (Fig. 1C). Such mTORC2 activation was completely blocked by treatment of cells with OSI-027 (Fig. 1C). Thus, some baseline mTORC2 activity is present in BCR-ABL transformed cells, whereas treatment with the mTORC1 inhibitor rapamycin results in activation of mTORC2. On the other hand, the dual mTORC2/mTORC1 inhibitor, OSI-027, completely suppresses such mTORC2 activity.

We subsequently performed studies to compare the effects of OSI-027 and rapamycin on pathways activated downstream of mTORC1. Treatment of K562 or BV173 cells with OSI-027 resulted in complete suppression of phosphorylation of rpS6 on Ser235/236 and Ser240/244, as well as 4E-BP1 on Thr37/46, Ser65, and Thr70 (Fig. 2A). Rapamycin completely blocked rpS6 phosphorylation, consistent with suppressive effects on S6K activity, but had modest effects on 4E-BP1 phosphorylation on Thr70, and essentially no effects on 4E-BP1 phosphoryla-

tion on Thr37/46 and Ser65 (Fig. 2A). Consistent with the complete suppression of 4E-BP1 phosphorylation, OSI-027-treatment resulted in formation of 4E-BP1-eIF4E complexes (Fig. S2 A and B) that suppress cap-dependent translation and blocked formation of eIF4E-eIF4G complexes (Fig. S2 A and B), which are required for initiation of mRNA translation (21, 29). On the other hand, rapamycin had much weaker effects in promoting formation of 4E-BP1-eIF4E complexes, whereas it did not disrupt formation of eIF4E-eIF4G complexes (Fig. S2 A and B). Notably, even when used at very high, supranormal concentrations, rapamycin failed to block 4E-BP1 phosphorylation on Thr37/46 in K562 cells (Fig. 2B), establishing that such mTORC1 function is absolutely rapamycin insensitive. Similar results were seen when the effects of OSI-027 or rapamycin were examined on the phosphorylation of 4E-BP1 in primary leukemic cells from CML or Ph⁺ ALL patients (Fig. 2C).

In subsequent studies we sought to define the functional relevance of targeting mTORC2 and RI-mTORC1 complexes in BCR-ABL expressing cells. As our data demonstrated that formation of eIF4E-eIF4G complexes is relatively insensitive to rapamycin in these cells, we examined whether OSI-027 impairs mRNA translation in such cells. OSI-027 treatment resulted in suppression of mRNA recruitment to polysomes (Fig. 3 A and B), directly establishing suppressive effects on mRNA translation. Treatment of cells with OSI-027 also resulted in antiproliferative responses in the several BCR-ABL expressing cell lines (Fig. 3C). Studies were also performed in which the effects of OSI-027 on primitive leukemic progenitor colony formation from CML patients were examined in vitro in clonogenic assays in methylcellulose. OSI-027 exhibited potent dose-dependent, inhibitory effects on leukemic CFU-GM colony formation (Fig. 3D), establishing that dual mTORC2/mTORC1 inhibition results in potent suppressive effects on CML precursors.

There is emerging evidence for BCR-ABL mutations associated with clinical resistance to BCR-ABL kinase inhibitors. One BCR-ABL mutation (T315I) is refractory to all BCR-ABL kinase inhibitors currently used for the treatment of Ph⁺ hematological malignancies, in vitro and in vivo (14, 15). Treatment of Ba/F3 cells expressing T315I-BCR-ABL with OSI-027 resulted in inhibition of mTOR phosphorylation on Ser2481, whereas rapamycin had no effects (Fig. 4A). On the other hand both OSI-027 and rapamycin inhibited phosphorylation of mTOR on Ser2448 (Fig. 4B) and also suppressed phosphorylation of S6K and rpS6 (Fig. 4C). Consistent with inhibitory effects on RI-mTORC1 complexes, OSI-027-treatment of Ba/F3-T315I-BCR-ABL cells resulted in inhibition of phosphorylation of

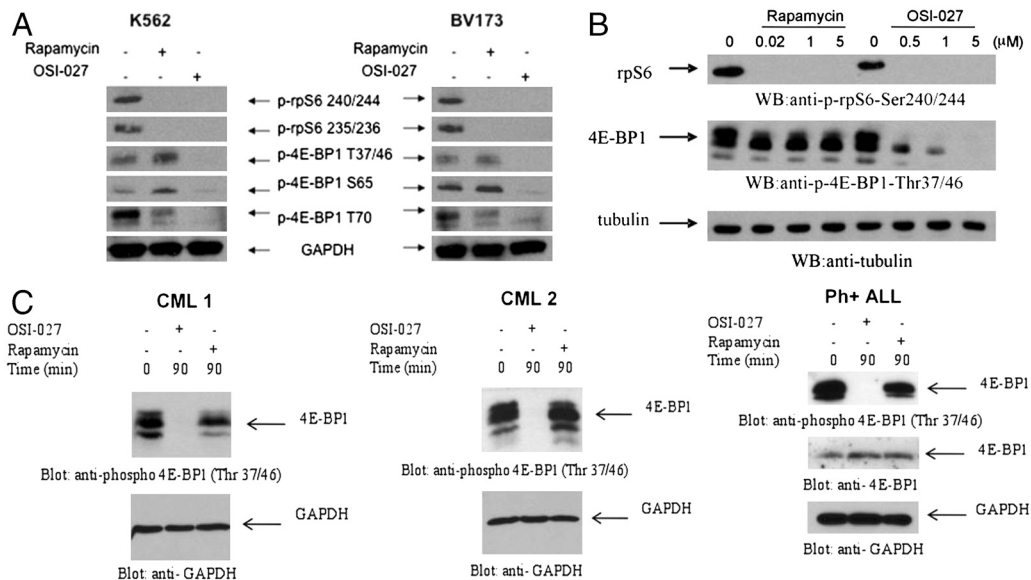


Fig. 2. Rapamycin-sensitive and -insensitive signaling events downstream of mTORC1 in BCR-ABL expressing cells. (A) K562 or BV173 cells were incubated with OSI-027 (10 μ M) or rapamycin (20 nM) for 16 h, as indicated. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. (B) K562 cells were incubated for 90 minutes with the indicated concentrations of rapamycin or OSI-027. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. (C) Primary peripheral blood leukemic cells from 2 different patients with CML or 1 patient with Ph+ ALL were treated with OSI-027 (10 μ M) or rapamycin (20 nM) for the indicated times in vitro. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with antibodies against the phosphorylated form of 4E-BP1 on Thr37/46 or GAPDH as indicated.

4E-BP1 on Thr37/46 (Fig. 4C), whereas rapamycin had no significant effects. Similar results were obtained when primary leukemic cells from a patient with CML expressing the T315I mutation were examined (Fig. 4D). Importantly, treatment of Ba/F3-T315I-BCR-ABL expressing cells with OSI-027 resulted in potent induction of apoptosis, whereas nilotinib had no effects (Fig. 4E). We also examined whether dual mTORC2/mTORC1 inhibition induces apoptosis of the BV173R mutant cell line, which expresses T315I-BCR-ABL (30). Treatment of BV173R cells with OSI-027, but not rapamycin, blocked phosphorylation of mTOR on Ser2481 (Fig. 4F). Similarly, OSI-027 blocked phosphorylation of 4E-BP1 on Ser 65 (Fig. 4F) and Thr37/46 (Fig. 4G), whereas rapamycin had no significant effects. On the other hand, phosphorylation of mTOR on Ser2448 (Fig. 4H) as well as phosphorylation of S6K and rpS6 on various sites, were

blocked by both OSI-027 and rapamycin (Fig. 4F–H). Importantly, OSI-027- but not rapamycin-treatment induced apoptosis of these cells (Fig. 4I), further establishing that dual mTORC2/mTORC1 inhibition can overcome leukemic cell resistance associated with expression of the T315I mutation.

Recent work has shown that during treatment of BCR-ABL cells with imatinib or other BCR-ABL kinase inhibitors, there is induction of autophagy associated with endoplasmic reticulum stress (31, 32). Such induction of autophagy acts as a protective mechanism for leukemic cells; and pharmacological inhibitors of autophagy or siRNA-mediated knockdown of key components of the autophagic machinery enhance imatinib- or nilotinib-dependent apoptosis and antileukemic responses in vitro and in vivo (31, 32). As the mTOR pathway is an important regulator of autophagy (33), we examined whether there is OSI-027-dependent induction of autophagy in

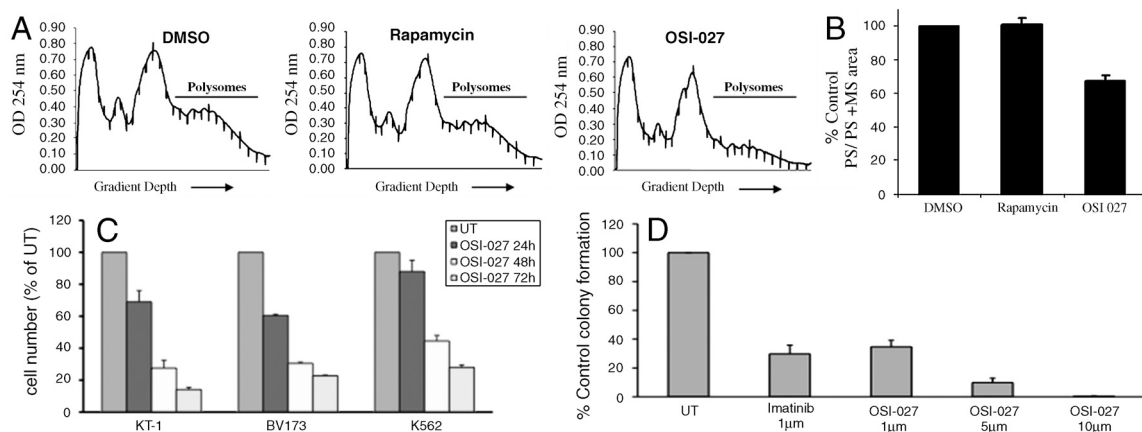


Fig. 3. OSI-027-dependent suppression of polysomal assembly and induction of antileukemic responses. (A) BV173 cells were treated with DMSO (control), rapamycin (20 nM), or OSI-027 (10 μ M) for 24 h. Cellular extracts were fractionated over a 10–50% sucrose gradient and absorbance of monosomal and polysomal fractions were continuously monitored at 254 nm. The optical density (OD) 254 nm is shown as a function of gradient depth for each treatment. (B) The areas under the polysome (PS) and monosome (MS) peaks were quantified using Image J software and ratios of PS over total (PS + MS) areas were calculated. Data are expressed as % control (DMSO) and represent means $\pm$ SE of 2 independent experiments. (C) Cells were treated with OSI-027 (10 μ M) for the indicated times and cells were counted at the various time points. Data are expressed as % untreated samples, and represent means $\pm$ S.E. of 4 experiments. (D) Effects of different concentrations of OSI-027 on primary leukemic progenitor colony formation (CFU-GM) from different CML patients in clonogenic assays in methylcellulose. Data are expressed as percent control leukemic CFU-GM colony formation from untreated samples and represent means $\pm$ S.E. of 8 experiments.

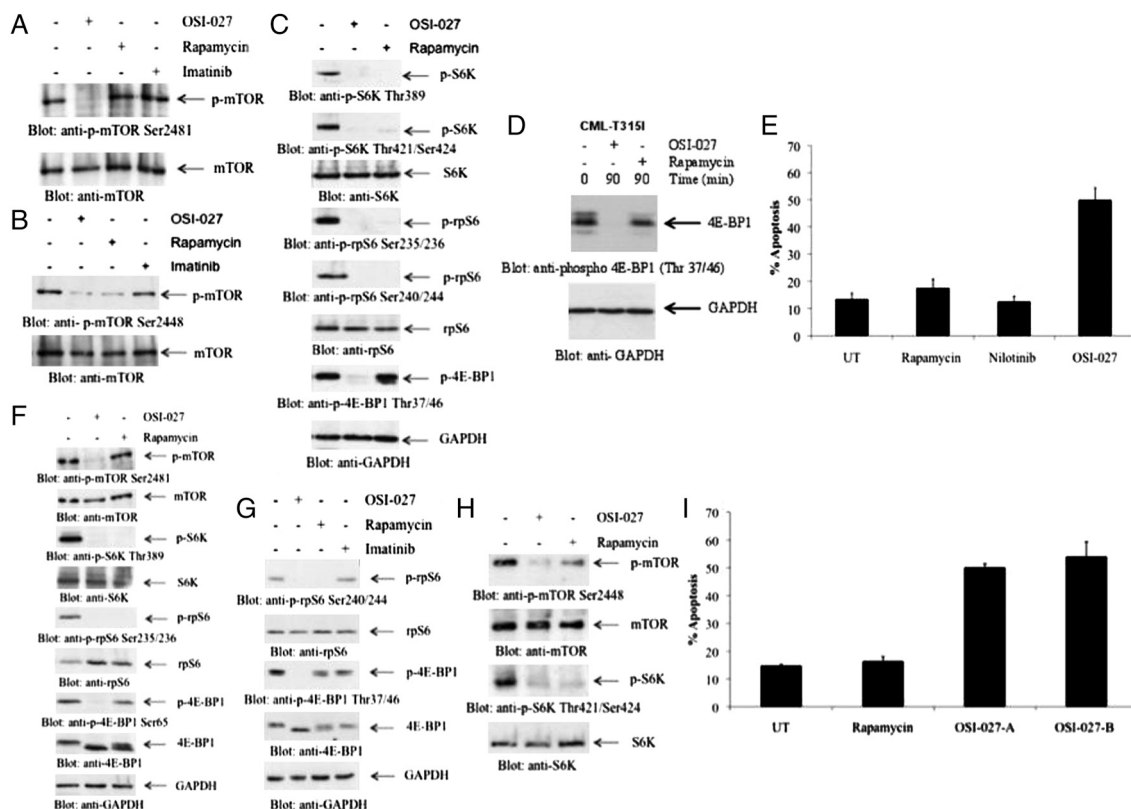


Fig. 4. Dual mTORC2/mTORC1 targeting results in potent antileukemic responses in T315I-BCR-ABL expressing cells. (A) T315I-BCR-ABL expressing Ba/F3 cells were incubated with OSI-027 (10 μ M), rapamycin (20 nM), or imatinib (1 μ M) for 90 minutes, as indicated. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with antibodies against the phosphorylated form of mTOR on Ser2481 or against mTOR, as indicated. (B) T315I-BCR-ABL expressing Ba/F3 cells were incubated with OSI-027 (10 μ M), rapamycin (20 nM), or imatinib (1 μ M) for 180 minutes, as indicated. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with antibodies against the phosphorylated form of mTOR on Ser2448 or against mTOR, as indicated. (C) T315I-BCR-ABL expressing Ba/F3 cells were treated with OSI-027 (10 μ M) or rapamycin (20 nM) for 90 minutes, as indicated. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. (D) Primary peripheral blood mononuclear cells from a CML patient with the T315I mutation were treated with OSI-027 (10 μ M) or rapamycin (20 nM) as indicated. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. (E) T315I-BCR-ABL expressing Ba/F3 cells were treated with OSI-027 (10 μ M), rapamycin (20 nM), or nilotinib (100 nM) for 72 h and apoptosis was assessed by annexin V/PI staining. Means $\pm$ SE of 10 experiments are shown. (F) BV173R cells were incubated with or without OSI-027 (10 μ M) or rapamycin (20 nM) for 90 minutes, as indicated. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. (G) BV173R cells were incubated with or without OSI-027 (10 μ M), rapamycin (20 nM), or imatinib mesylate (5 μ M) for 90 minutes, as indicated. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. (H) BV173R cells were incubated with or without OSI-027 (10 μ M), rapamycin (20 nM) for 90 minutes. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. (I) BV173R cells expressing the T315I mutation were treated with OSI-027 (A, 5 μ M, B, 10 μ M) or rapamycin (20 nM) for 72 h, as indicated. Apoptosis was assessed by annexin V/PI staining. Means $\pm$ SE of 5 experiments are shown.

BCR-ABL expressing cells. Treatment of K562 cells with OSI-027 induced autophagy, as reflected by the increasing levels of LC3 II after OSI-027 treatment (Fig. 5A), as well as by the presence of punctate GFP-LC3, a characteristic of formation of autophagosomes, in cells transfected with a GFP-LC3 expressing vector (Fig. 5B). Importantly, when K562 cells were treated simultaneously with OSI-027 and the autophagy inhibitor chloroquine (CQ), there was strong induction of apoptosis as assessed by annexin V/PI staining, whereas OSI-027 alone had minimal effects on these cells (Fig. 5C). Thus, autophagy may be a key defensive mechanism that limits the extent of proapoptotic responses by OSI-027 in some cells, and combined use of OSI-027 with autophagy inhibitors may provide an approach to enhance OSI-027-dependent leukemic cell death in BCR-ABL transformed cells.

Discussion

Extensive work over many years has led to important information and understanding on the mechanisms by which BCR-ABL transforms cells and promotes leukemic cell growth. Beyond dramatically advancing our overall understanding of leukemogenesis and neoplastic transformation, such work had important translational implications. The introduction of imatinib mesylate in the treat-

ment of CML and Ph+ ALL was a major breakthrough that had a dramatic impact in the management of patients suffering from such leukemias (reviewed in ref. 2). The rational identification and targeting of the BCR-ABL kinase translated to remarkable clinical results that have changed the natural history of BCR-ABL expressing malignancies. Nevertheless, despite the long-lasting hematological and cytogenetic responses seen in patients with CML who undergo treatment with imatinib, minimal residual disease is detectable in significant numbers of patients (2, 34), demonstrating a need for the development of novel approaches to target CML stem cells.

The emergence of several BCR-ABL mutant forms that are resistant to imatinib mesylate in vitro and in vivo led to the development of second-generation BCR-ABL kinase inhibitors, such as nilotinib and dasatinib (35). These pharmacological inhibitors are active against various imatinib-resistant BCR-ABL kinase mutants in vitro and in patients with resistant CML in vivo (13, 36–39). However, resistance to nilotinib or dasatinib, associated with BCR-ABL mutations also develops, and one mutant, T315I, is completely refractory to all kinase inhibitors (imatinib mesylate, nilotinib, dasatinib) currently available for the treatment of CML and Ph+ ALL (14, 15, 35). Remarkably, there is also

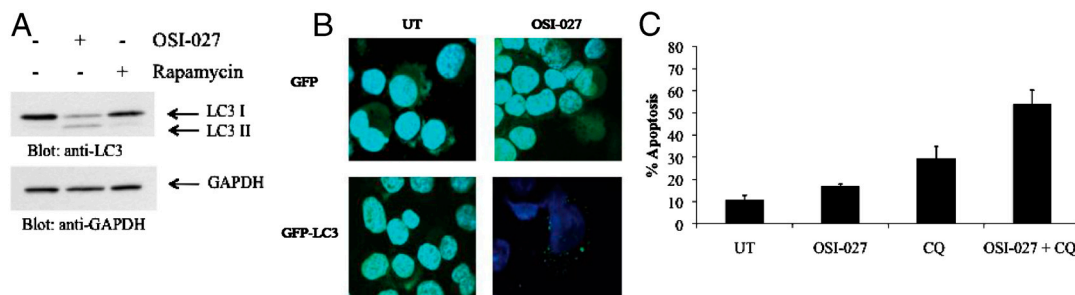


Fig. 5. Induction of autophagy by OSI-027 and enhanced proapoptotic effects by combining OSI-027 with CQ. (A) K562 cells were treated for 24 h with OSI-027 (10 μ M) or rapamycin (20 nM). Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with antibodies against total LC3, detecting both forms (I and II) of LC3. (B) K562 cells were either transiently transfected with GFP-LC3 (lower right and left panels) or with control empty vector (Upper Right and Left). Samples were either not treated or treated with OSI-027 (10 μ M) for 24 h. Cells were stained with anti-GFP antibody and signals were detected by confocal microscopy. (C) K562 cells were pretreated for 2 h with chloroquine (6 μ M) and then treated with OSI-027 (10 μ M) for 48 h, in the continuous presence or absence of chloroquine and apoptosis was assessed by annexin V/PI staining. Means $\pm$ SE of 4 experiments are shown.

emerging evidence for differential resistance of distinct BCR-ABL mutations to second-generation BCR-ABL kinase inhibitors. For instance, although relatively sensitive to dasatinib, the E255V BCR-ABL mutation is completely refractory to nilotinib and imatinib (35, 36). On the other hand, the V299L BCR-ABL mutation is resistant to dasatinib, but sensitive to nilotinib and imatinib (35, 36). It should be also noted that beyond BCR-ABL mutations, a variety of BCR-ABL-independent cellular mechanisms have been implicated in the development of leukemic cell resistance, including changes in the P-glycoprotein (Pgp) efflux pump, epigenetic modulation, alterations of the function of the organic cation transporter hOCT1, and activation of various alternative signaling cascades (35). Such studies suggest that beyond selective targeting of BCR-ABL kinase mutations, development of other means to target leukemic cells may be necessary to effectively overcome resistance to kinase inhibitors.

The Akt/mTOR signaling cascade regulates downstream cellular events required for mRNA translation and plays critical roles in neoplastic cell growth (21, 40). Because of its critical importance in leukemogenesis, this pathway has been the focus of extensive investigations as a therapeutic target in hematological malignancies (41). Previous work has established that BCR-ABL-mediated engagement of the PI 3'-kinase is essential for BCR-ABL leukemogenesis (42), whereas there has been also evidence that mTOR pathways are engaged in BCR-ABL expressing cells (20, 24–27, 43). Rapamycin was previously shown to enhance the antileukemic effects of imatinib mesylate on primary committed leukemic progenitors from CML patients (20, 43), raising the potential that combinations of rapamycin with BCR-ABL kinase inhibitors may be an approach to enhance generation of antileukemic responses in CML. However, a substantial limitation in the clinical use of rapamycin and other related rapalogs has been the selective targeting of mTORC1, but not mTORC2, complexes.

mTORC1 and mTORC2 are distinct complexes that share mTOR as their catalytic subunit (44). mTORC1 is formed by mTOR, Raptor and mLST8; whereas mTORC2 includes mTOR, Rictor, mLST8, and SIN1 (44). Rapamycin and other clinically approved rapalogs (temsirolimus, everolimus) are allosteric inhibitors of mTORC1, but not mTORC2. This is highly relevant, as engagement of mTORC2 during inhibition of mTORC1 leads to increased AKT activity and activation of antiapoptotic pathways (44, 45). Such effects reflect to a large extent rapamycin-mediated reversal of the suppressive effects of mTORC1 on AKT, mediated by the S6K-IRS negative feedback loop (45, 46). Thus, targeting mTORC2 complexes may provide an approach to overcome the AKT-mediated antiapoptotic signals in malignant cells and elicit apoptosis and antitumor effects in vitro and in vivo.

In the present study we provide evidence that the mTORC2 and RI-mTORC1 complexes play critical roles in cell proliferation and survival of BCR-ABL transformed cells. Our data show that a

unique dual mTORC2/mTORC1 inhibitor, OSI-027, exhibits potent antileukemic effects on CML cells. OSI-027 inhibits the growth of several BCR-ABL expressing myeloid and lymphoid cell lines and acts as a potent suppressor of mTORC2-associated AKT activity in such cells. In contrast, treatment of BCR-ABL transformed cells with rapamycin can result in mTORC2-mediated activation of AKT and induction of an antiapoptotic state. Beyond targeting mTORC2, our studies establish that OSI-027 inhibits activation of RI-mTORC1 complexes, which appear to be the primary complexes responsible for phosphorylation/deactivation of the translational repressor 4E-BP1 and control of cap-dependent mRNA translation. Moreover, our studies establish a critical role for such complexes in mRNA translation, as evidenced by the OSI-027-, but not rapamycin-mediated inhibition of polysomal assembly in BCR-ABL transformed cells. The functional consequences of mTORC2 and RI-mTORC1 complexes in BCR-ABL cells are major, as reflected by the very potent inhibitory responses elicited by OSI-027 on primary leukemic CML progenitors.

In other studies, we found that OSI-027 induces apoptosis of different types of cells transformed by the T315I-BCR-ABL mutation, which confers resistance to imatinib mesylate, nilotinib, and dasatinib. OSI-027 is currently under clinical development in phase I studies for the treatment of solid tumors and lymphomas and, based on our data, its use may provide a unique approach to overcome resistance in patients with CML or Ph+ ALL expressing T315I or other imatinib mesylate-resistant BCR-ABL mutations. As the mechanism by which OSI-027 inhibits growth and/or induces apoptosis of BCR-ABL expressing cells is unrelated to direct targeting of BCR-ABL, it is also possible that it may be effective against BCR-ABL expressing cells with other, BCR-ABL-unrelated, mechanisms of resistance to ABL kinase inhibitors. Notably, a very recent study that was published while this work was near completion demonstrated that PP242, a drug that blocks both TORC2 and TORC1, also exhibits potent antileukemic effects on wild type and mutant BCR-ABL-transformed cells (47). That study also demonstrated potent in vivo antileukemic effects of that inhibitor (47). Taken together with that study, the results of our work establish a critical role for mTORC2 complexes in survival of BCR-ABL leukemic cells and provide a firm basis for the ultimate development of clinical trials using dual mTORC2/mTORC1 inhibitors for the treatment of BCR-ABL expressing malignancies. Finally, our data demonstrating enhancement of the proapoptotic effects of OSI-027 by chloroquine, suggest that combinations of dual mTORC2/mTORC1 inhibitors with autophagy inhibitors should be also exploited as a therapeutic approach for Ph+ leukemias.

Materials and Methods

Cells and Reagents. K562, KT1, BV173 cells, and Ba/F3 cells stably expressing a T315I-BCR-ABL mutant were grown in RPMI medium 1640 supplemented with 10% fetal bovine serum and gentamicin. Antibodies against the phosphory-

lated forms of AKT, mTOR, S6 kinase, ribosomal protein S6, and 4E-BP1 were purchased from Cell Signaling Technology, Inc. Rapamycin was purchased from Calbiochem. Imatinib mesylate and nilotinib were purchased from ChemieTek. Peripheral blood or bone marrow aspirates from patients with CML or Ph⁺ ALL were collected after obtaining informed consent approved by the Institutional Review Board of Northwestern University.

Cell Lysis, Immunoprecipitations, and Immunoblotting. Cell lysis, immunoprecipitation, and immunoblotting were performed as in previous studies (26, 27, 48).

Evaluation of Apoptosis. Apoptosis was evaluated by flow cytometry for annexin V/PI staining as in our previous studies (49).

Human Hematopoietic Progenitor Cell Assays. Clonogenic hematopoietic progenitor assays in methylcellulose to assess primary leukemic CFU-GM progenitor colony formation were performed as in previous studies (26).

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