Autophagy Is a Survival Mechanism of Acute Myelogenous Leukemia Precursors during Dual mTORC2/mTORC1 Targeting

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Abstract

Purpose: To examine whether induction of autophagy is a mechanism of leukemic cell resistance to dual mTORC1/mTORC2 inhibitors in acute myelogenous leukemia (AML) leukemic progenitors.

Experimental Design: Combinations of different experimental approaches were used to assess induction of autophagy, including immunoblotting to detect effects on LC3II and p62/SQSTM1 expression and on ULK1 phosphorylation, immunofluorescence, and electron microscopy. Functional responses were assessed using cell viability and apoptosis assays, and clonogenic leukemic progenitor assays in methylcellulose.

Results: We provide evidence that treatment of AML cells with catalytic mTOR inhibitors results in induction of autophagy, which acts as a regulatory mechanism to promote leukemic cell survival. Such induction of autophagy by dual mTORC1/mTORC2 inhibitors partially protects primitive leukemic precursors from the inhibitory effects of such agents and limits their activities. Simultaneous blockade of the autophagic process using chloroquine or by knockdown of ULK1 results in enhanced antileukemic responses.

Conclusions: Dual targeting of mTORC2 and mTORC1 results in induction of autophagy in AML cells. Combinations of catalytic mTOR targeting agents and autophagy inhibitors may provide a unique approach to target primitive leukemic precursors in AML. Clin Cancer Res; 20(9); 1–10. ©2014 AACR.

Introduction

The mTOR pathway plays a central role in the regulation of mRNA translation of genes whose protein products promote cell proliferation and survival (1–3). There is emerging evidence that inhibition of both mTORC1 and mTORC2 complexes by catalytic targeting of mTOR may provide a powerful approach for the treatment of malignancies (1–5) and aging-related pathologies (6, 7). Beyond the classic mTOR inhibitors, the rapalogs, catalytic mTOR inhibitors have been recently developed or are in early clinical trials (8, 9). Such catalytic inhibitors of mTOR have emerged as potentially superior therapeutic options to rapalogs (rapamycin, temsirolimus, everolimus, and ridaforolimus), as the clinical utility of rapalogs is limited by the inability of these agents to fully block mTOR activation in neoplastic cells. So far, two distinct complexes have been described in living mammalian cells, mTORC1 and mTORC2. mTORC1 complexes are composed of Raptor, mLST8, Pras40, Deptor, and mTOR (1–3). These complexes are key and essential regulators of cellular pathways that control initiation of mRNA translation and ribosome biogenesis and exhibit important monitoring effects on cell metabolism, lipolysis, and autophagy (1–3). mTORC2 complexes are composed of mTOR, Rictor, Deptor, mLST8, Sin1, and mTOR (1–3). These complexes regulate downstream engagement of members of the AGC family of kinases, which account for prosurvival signals and control effector elements that regulate cell cycle progression and anabolism (1–3).

Acute myelogenous leukemia (AML) is a heterogeneous group of malignancies with diverse molecular pathogenic lesions, characterized by an aggressive, life-threatening, clinical course if left untreated (10–13). Despite extensive efforts over the years to improve survival and cure rates for this fatal disease, the treatment options remain relatively limited. As the mTOR pathway plays a central role in the survival and proliferation of malignant cells and there is evidence that it is dysregulated in AML (14–18), it provides an attractive molecular therapeutic...
Targeting the autophagic machinery may provide an approach to overcome relative resistance of acute myelogenous leukemia (AML) precursors to dual mTORC1/mTORC2 inhibitors. We demonstrate that catalytic mTOR inhibitors, such OSI-027 and AZD-2014, are potent inducers of autophagy in AML cells, resulting in regulatory effects on their inhibitory activities on primitive AML leukemic precursors. Concomitant inhibition of the autophagic process by pharmacologic means or by molecular targeting of elements of the autophagic machinery enhances induction of antileukemic responses. Taken together, our findings suggest that combinations of mTOR targeting agents with pharmacologic agents that inhibit autophagy may provide an approach to enhance antileukemic responses in AML.

**Materials and Methods**

**Cells and reagents**

The U937, HEL, Kasumi 1, and Kasumi 3 acute leukemia cell lines were obtained from American Type Culture Collection (ATCC). U937 and HEL were cultured in RPMI 1640 supplemented with 10% FBS. Kasumi 1 and Kasumi 3 were cultured in ATCC-modified RPMI 1640 supplemented with 20% FBS. OSI-027 was purchased from ChemieTek and AZD-2014 was purchased from Santa Cruz Biotechnology. All antibodies were purchased from Cell Signaling Technologies except the antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was purchased from Millipore, and p62/SQSTM1, which was purchased from Santa Cruz Biotechnology.

**Cell lysis and immunoblotting**

For the immunoblotting experiments, cells were treated with the indicated inhibitors or dimethyl sulfoxide (DMSO; used as control for untreated cells) for the indicated times and lysed in phosphorylation lysis buffer (27–29). Unless otherwise indicated, the final concentrations of the pharmacologic agents used in these experiments were as follows: OSI-027 (5 μmol/L), rapamycin (20 nmol/L), chloroquine (6 μmol/L), and AZD-2014 (0.1 or 1 μmol/L). DMSO (0.1%) was used as control treatment. Immunoblotting using an enhanced chemiluminescence (ECL) method was performed as in our previous studies (27–29).

**Evaluation of apoptosis**

Apoptosis of leukemic cells after various treatments was evaluated by flow cytometric analysis for Annexin V/Propidium Iodide (PI) staining as in our previous studies (30, 31).

**Clonogenic assays in methylcellulose to assess leukemic progenitor colony formation**

Peripheral blood or bone marrow samples were obtained from patients with AML after obtaining informed consent approved by the Institutional Review Board of Northwestern University. Cells were separated over Ficoll–Hypaque and cultured with the indicated concentrations of the indicated inhibitors and leukemic progenitor (CFU-L) colony formation was assessed in clonogenic assays in methylcellulose (24, 32). Experiments to assess CFU-L colony formation derived from different AML lines were performed, as indicated. The final concentrations used were OSI-027 (10 μmol/L), AZD-2014 (0.5 μmol/L), and chloroquine (2.5 μmol/L), unless otherwise indicated.

**RNAi targeting**

Nontargeting control and ULK1 siRNA was purchased from Santa Cruz Biotechnology. siRNA-mediated knockdown of ULK1, using Amaxa nucleofector kits from Lonza, and assessment of the effects of such knockdown on leukemic progenitor colony formation were performed as in previous studies (33, 34).

**Immunofluorescence**

Immunofluorescence was performed as in our previous studies (33). Briefly, human leukemic cells were collected and fixed with periodate/lysine/paraformaldehyde solution before staining. Fluorescence was detected using a Zeiss LSM 510 META, confocal microscope system and images were analyzed with ImageJ.

**Electron microscopy**

Such studies were performed as previously described (33, 35). Briefly, fixed samples were sectioned to 70-nm thin and poststained with 3% uranyl acetate and Reynolds staining.
lead citrate. The samples were examined under FEI Tecnai G2 Transmission Electron Microscopy (TEM) and images were obtained on an FEI Eagle camera. Samples were processed for TEM by the Cell Imaging Facility at Northwestern University, Feinberg School of Medicine.

**WST-1 assays**

Cell viability/proliferation was assessed as previously described, using the WST-1 reagent (Roche; ref. 36). IC\textsubscript{50} values were calculated by a sigmoidal dose–response curve fit using Prism Graphpad 6.0 for Windows.

**Results**

In initial studies, we sought to determine whether treatment of AML cells with mTOR inhibitors results in induction of autophagy. When U937 cells were treated with either the catalytic mTOR inhibitor OSI-027 or the selective mTORC1 inhibitor rapamycin, we found induction of autophagy as assessed in immunoblotting experiments demonstrating increased expression of LC3II (Fig. 1A and B), consistent with formation of autophagosomes. Such increase in LC3II expression was clearly more pronounced in response to treatment with OSI-027 (Fig. 1A) and was further increased by cotreatment of cells with chloroquine (Fig. 1C), an indication of autophagic flux. Induction of autophagy in response to treatment of cells with OSI-027 was further demonstrated using confocal microscopy, by monitoring leukemic cells for the presence of punctate LC3 (34; Fig. 1D).

In subsequent studies, we sought to directly establish whether there is induction of autophagic flux (37) in AML cells treated with mTOR inhibitors, by assessing the expression of p62/SQSTM1, an LC3-interacting protein degraded in the autolysosomes (38). Although treatment with either OSI-027 or rapamycin resulted in a decrease in p62/SQSTM1 protein levels, the effects of OSI-027 were more...
pronounced (Fig. 2A–C). Suppression of p62/SQSTM1 protein levels, consistent with autophagic flux, was also seen when HEL cells were used (Fig. 2D). In other studies, we used the catalytic mTOR inhibitor AZD-2014 (39), which as in the case of OSI-027 (24) inhibits proliferation of U937 cells (IC_{50} 45 nmol/L; Fig. 2E). AZD-2014 also suppressed p62/SQSTM1 expression (Fig. 2F and G). Notably, catalytic mTOR inhibition resulted in suppression of phosphorylation of ULK1 on serine 757 in different AML cell lines (Fig. 2H–J), a site in which phosphorylation inhibits activation of ULK1 (40), suggesting a mechanism for the induction of autophagy during treatment of AML cells with dual mTORC1/2 inhibitors. Formation of autophagic structures was also documented using electron microscopy (EM; Fig. 2K), definitely establishing induction of an autophagic state during treatment of AML cells with catalytic mTOR inhibitors. There was further increase in the number of autophagic structures when cells were treated concomitantly with OSI-027 and the autophagy inhibitor chloroquine (Fig. 2K), consistent with arrest of the autophagic process at the autophagic stage and lack of progression to autolysosome formation (38).

To define the functional consequences of induction of autophagy during inhibition of the mTOR pathway in AML...
cells, experiments were performed in which the effects of OSI-027, alone or in combination with chloroquine, on leukemia colony formation from different AML cell lines were examined. As shown in Fig. 3, concomitant treatment with chloroquine significantly enhanced the suppressive effects of OSI-027 on CFU-L colony formation from several AML lines, including the U937 (Fig. 3A), HEL (Fig. 3B), Kasumi 3 (Fig. 3C), and Kasumi 1 (Fig. 3D) leukemia cell lines.
lines. Similar suppressive effects on CFU-L colony formation were observed in AZD-2014-treated U937 cells (Fig. 3E). To determine whether the augmenting effects of chloroquine on OSI-027–dependent suppression of leukemic progenitors reflect enhanced programmed cell death, experiments were performed to examine induction of apoptosis. As shown in Fig. 3F, the addition of chloroquine promoted induction of apoptosis by OSI-027, suggesting a mechanism for the enhanced antileukemic effects.

Altogether, these studies suggested that autophagy is inducible during treatment of AML cells with catalytic mTOR inhibitors and may act as a survival mechanism during targeting of the mTOR pathway. To determine whether induction of autophagy occurs in primary leukemia cells, studies were performed using primary circulating peripheral leukemic blasts from patients with AML. Induction of autophagy in response to OSI-027 treatment of the cells was observed, reflected by the increase in punctate LC3 in primary leukemic cells (Fig. 4A). Formation of autophagic structures was also documented using EM (Fig. 4B), establishing induction of an autophagic state during treatment of cells derived from patients with AML with catalytic mTOR inhibitors.

To further determine the significance of OSI-027- or AZD-2014–induced autophagy in a pathophysiologically relevant system, we performed experiments aimed to determine the role of autophagy in the inhibitory effects of OSI-027 on primitive leukemic precursors from patients with AML. As expected (24), treatment with OSI-027 (Fig. 5A) or AZD-2014 (Fig. 5B) suppressed CFU-L colony formation from

Figure 4. Induction of autophagy by OSI-027 in primary AML leukemic blasts. A, circulating leukemic cells from a patient with AML were treated with OSI-027 for 24 hours. After collection, cells were stained with anti-DAPI (blue) or anti-LC3 (green) and signals were detected by confocal microscopy. Merged panels, areas of overlapping images of the two fluorescing signals. B, circulating leukemic cells from a patient with AML were treated for 24 hours with OSI-027 and/or chloroquine, as indicated. EM was used for analysis of autophagic compartments. Arrows, autophagic compartments.
primary AML cells in clonogenic assays in methylcellulose. Importantly, such effects were strongly enhanced by the combination of OSI-027 with chloroquine (Fig. 5A) or AZD-2014 with chloroquine (Fig. 5B). Notably, when ULK1 was targeted using specific siRNA-mediated knockdown (Fig. 5C), we also found enhanced OSI-027–mediated suppression of primitive leukemic precursors from patients with AML (Fig. 5D). This finding underscores the significance of autophagy as a survival mechanism during treatment of primary leukemia cells with mTOR catalytic inhibitors.

Discussion

mTOR is a key integrator of signals that regulate mRNA translation and metabolism and plays important roles in proliferation and survival of malignant cells (3, 4). mTORC1 and mTORC2 are attractive targets for the treatment of AML, as there is extensive evidence for dysregulation of mTOR effector pathways in AML blasts and leukemic precursors (41–43). Although selective mTORC1 inhibitors have shown some activity in AML in vitro and in vivo (19–23, 44, 45), these agents do not inhibit mTORC2 complexes, which are the complexes responsible for the activation of survival pathways downstream of AKT in malignant cells (3, 16). There is now increasing preclinical evidence suggesting that catalytic mTOR inhibitors may exhibit activity in AML and other myeloid malignancies (24–26, 29, 46–48). However, despite the recent emergence of catalytic mTOR inhibitors (8) and their introduction in clinical trials (49), there are potential limitations on the use of these agents as well. A key driver for the development of catalytic mTOR inhibitors was the anticipation that such compounds would be able to induce programmed cell death of neoplastic cells by inhibiting phosphorylation of AKT on serine 473 (8, 16). Indeed catalytic mTOR inhibitors have been found to induce apoptosis of some malignant cell lines and primary neoplastic cells (46, 50–52), but such effects are not always consistent. There is now evidence that in certain cases apoptosis is not seen or is minimal (46, 52), and the mechanisms of such resistance to cell death of...
malignant cells remain to be precisely defined. The occurrence of such resistance raises the possibility that escape mechanisms or feedback loops may be activated in malignant cells that prevent induction of programmed cell death during dual mTORC1/2 inhibition.

Autophagy is a lysosomal degradation process important for cellular homeostasis and adaptation to metabolic changes (53). Depending on the context and stimulus, autophagy can lead to divergent outcomes in the neoplastic process. It can be a caspase-independent death mechanism of malignant cells. On the contrary, it can act as an escape, and thus, protective mechanism for malignant cells during challenges by various antineoplastic agents and processes. Although the autophagic process generally impedes tumor initiation, under certain conditions it is also required for Ras-transformation (53). Autophagy has been shown to be essential for the generation of the antileukemic responses by certain agents such as arsenic trioxide and all-trans-retinoic acid (33, 34, 54), whereas it blocks or diminishes the antileukemic effects of other agents, such as histone deacetylase inhibitors (55). The role of autophagy in promoting or preventing cell death depending on the context underscores the complexity of the system and the fine balance between opposing cellular regulatory responses.

In the present study, we provide evidence that during treatment of AML cells with dual mTORC1/mTORC2 inhibitors, there is induction of cellular autophagy. Our studies definitively demonstrate induction of the autophagic process in response to treatment with OSI-027 or AZD-2014 in several AML cell lines with diverse phenotypes and molecular abnormalities, indicating that it is a universal leukemic cell response to catalytic mTOR inhibitors. Consistent with our observations, another study recently provided some evidence for induction of autophagy by another catalytic mTOR inhibitor, AZD-8055, although in that study, the role of autophagy in the generation of responses on primary leukemic progenitors from patients with AML was not addressed (25). We also provide important information on the mechanistic process that regulates autophagy by catalytic mTOR inhibitors, as our studies demonstrate that such agents block phosphorylation of the inhibitory site serine 757 on the kinase ULK1 in AML cells, a key regulator and inducer of autophagy (3, 53). Using a combination of methodologic approaches, we also demonstrate that such autophagy is inducible in primary leukemic blasts from patients with AML. Importantly, our studies establish that the pharmacologic or molecular inhibition of autophagy promotes the suppressive effects of OSI-027 and AZD-2014 on primitive leukemic precursors from patients with AML, suggesting that the autophagic process is an important survival mechanism for leukemic AML precursors during catalytic mTOR targeting. We have previously shown that catalytic mTOR inhibitor OSI-027 that inhibits both mTORC1 and mTORC2 is a potent suppressor of leukemic progenitor colony formation (24). Such effects may reflect the ability of dual mTORC1/2 inhibitors to block mRNA translation of mitogenic genes, such as cyclin D1, in leukemic cells (24) and/or induction of apoptosis. The enhanced antileukemic effects during combined inhibition of autophagy and dual mTORC1/2 inhibition reflect induction of apoptosis and promotion of targeted cell death. It is interesting that similar targeting of autophagy seems to also promote leukemic cell apoptosis in the context of BCR–ABL leukemic transformation (46).

These findings have important implications on our overall understanding of the mechanisms of survival of leukemia cells during complete suppression of the mTOR pathway. A reasonable hypothesis is that during disruption of mTOR regulatory networks that control metabolism in leukemia-initiating stem cells (LIC), ULK1-dependent induction of autophagy allows LICs to survive. This has important clinical implications and provides a rationale for the study of unique combination approaches to target early leukemic precursors. It should also be noted that catalytic mTOR inhibitors can enhance the activities of inhibitors of other cellular pathways in AML, such as inhibitors of Aurora kinase–dependent pathways (56) and chemotherapeutic agents (24), and have been shown to disrupt leukemia/stroma interactions (26). Thus, it is possible that autophagy modulators may also be useful as components of multiagent regimens for the treatment of AML, such as combinations including dual mTORC1/2 inhibitors, chemotherapy drugs, and/or other targeted therapies. Future studies should investigate the potential of such approaches, as they may provide a way to overcome resistance of primitive AML progenitors and LICs.

Disclosure of Potential Conflicts of Interest

J.K. Altman is a consultant/advisory board member of Astellas, Teva, BMS, Novartis, Ariad, and Foundation Medicine. No potential conflicts of interest were disclosed by the other authors.

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