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Full paper

The Class I PI3K inhibitor S14161 induces autophagy in malignant blood cells by modulating the Beclin 1/Vps34 complex

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ABSTRACT

S14161 is a pan-Class I PI3K inhibitor that induces blood cancer cell death, but its mechanism is largely unknown. In the present study, we evaluated the role of S14161 in autophagy, an emerging event in cell destination. Multiple myeloma cell lines RPMI-8226, OPM2, KMS11 and leukemia cell line K562 were treated with S14161. The results showed that S14161 induced autophagy as demonstrated by increased LC3-II and decreased p62, which were prevented by autophagy inhibitors including 3-methyladenine and bafilomycin A1. Mechanistic studies showed that S14161 had no effects on Vps34 expression, but increased Beclin 1 and decreased Bcl-2, two major regulators of autophagy. Furthermore, S14161 dissociated the Beclin 1/Bcl-2 complex and enhanced the formation of Beclin 1/Vps34 complex. Moreover, S14161 inhibited the mTORC1 signaling transduction. S14161 downregulated activation of mTOR and its two critical targets 4E-BP1 and p70S6K, suggesting S14161 inhibited protein synthesis. Taken together, these results demonstrated that Class I PI3K regulates autophagy by modulating protein synthesis and the Beclin 1 signaling pathway. This finding helps understanding the roles of PI3K in autophagy and cancer treatment.

1. Introduction

The small molecule chemical compound S14161, or 8-ethoxy-2-(4-fluorophenyl)-3-nitro-2H-chromene, is an inhibitor of pan-Class I phosphatidylinositol 3-kinases (PI3Ks) and displays potent anti-leukemia and anti-myeloma activities.[1] PI3Ks are a class of membranous lipid kinases that catalyze the production of PI(3,4,5)P3, a key second messenger, and regulate various cellular functions. Based on the structure and function, PI3Ks can be grouped into 3 classes, of which the Class I family is composed of both regulatory and catalytic subunits (PI3Kα, β, γ and δ) and promotes cell survival[2]; while the Class III family contains only single member, PI3KC3 or Vps34.[3] Vps34 is a major player in autophagy, a conserved survival manner of cells under stress, such as nutrient depletion.[4] Vps34 complexes with Beclin 1 (BENC) and PIK3R4/p150 to provide a PI(3)P-enriched domain for autophagosome formation. These core components bind different partners, including pro-survival protein Bcl-2, which inhibits autophagy by preventing Beclin 1 from binding to Vps34.[5] Thus, it is possible that the PI3K/AKT/mTOR pathway and the Vps34 complexes coordinate autophagy initiation but lack evidence. It is well known that inhibition of the Class I PI3Ks is a great strategy for cancer therapy and a specific inhibitor of PI3Kδ has been approved for the treatment of several subgroups of leukemia.[2] S14161 induced apoptosis in myeloma and leukemia cells by inhibiting the activities of PI3Kα, β,

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δ, and γ isoforms.[1] Because the PI3K signaling pathway is a critical regulator of the autophagy flux, we wondered whether S14161 also inhibited the autophagy in addition to its induction of apoptosis. Whether S14161 modulates the crosstalk between Class I PI3Ks and Vps34 thus inducing autophagy is not known. The present study showed that S14161 also induces autophagy by inhibiting the PI3K/AKT/mTOR signaling pathway and by promoting the formation of the Vps34/Beclin 1 complex.

2. Materials and methods

2.1. Cell culture

MM cell lines (RPMI-8226, OPM2, KMS11) and leukemia cell line (K562) were purchased from American Type Culture Collection or provided by Dr. Aaron Schimmer, the University of Toronto, Canada. MM and leukemia cells were maintained in Iscove's modified Dulbecco medium and RPMI-1640 medium (Invitrogen, CA, USA), respectively, in an incubator humidified with 95% air and 5% CO_2 at 37 °C. All the media were supplemented with 10% fetal bovine serum (HyClone, MA, USA), penicillin (100 units/ml) and streptomycin (100 μg/ml).

2.2. Chemicals and antibodies

S14161 was purchased from Maybridge Chemical Co., Ltd, Tintagel, UK. Bafilomycin A1 (BafA1) was purchased from Abcam Biochemicals (Cambridge, MA, USA); 3-methyladenine (3-MA) was obtained from Sigma–Aldrich (St. Louis, MO, USA); Wortmannin and idelalisib were purchased from Selleck Chemicals (Houston, TX, USA). The antibody against LC3 was purchased from Abcam. Antibodies against Beclin 1, Bcl-2, mTOR, p-mTOR (Ser2448), 4E-BP1, p-4E-BP1 (Ser65), p62 were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against PI3KC3/Vps34, p70S6K and p-p70S6K (Thr389) were purchased from Abgent (Suzhou, China). The Antibody against GAPDH was purchased from Sigma. Horseradish peroxidase-conjugate secondary antibodies, DAPI (4′,6-diamidino-2-phenylindole), Cy3-labeled goat anti-rabbit IgG were purchased from Beyotime Institute of Biotechnology (Nantong, China). Protein A/G agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). IRDye 680 goat anti-mouse and IRDye 800CW goat anti-rabbit antibodies were from Odyssey (San Ramon, CA, USA).

2.3. Cell viability assay by MTT

The MTT assay was performed as described previously.[6] Briefly, to evaluate cell viability, each well containing cells for analysis was added 10 μL of MTT dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma–Aldrich), cells were further incubated for 2–4 h until purple precipitates were visible. To evaluate cell viability, the precipitates were solubilized with 20 μL of 10% SDS (in 0.01 M HCl). The plates were then subjected to absorbance reading at 570 nm, the reference wavelength was 650 nm.

2.4. Western blot

Whole cell lysates were prepared from myeloma and leukemia cells as described previously.[7,8] Briefly, cells were washed with Tris-buffered solution (TBS) and re-suspended in a cell lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, Na_3VO_4 and leupeptin. Protein concentrations were determined by the BCA assay kit (Thermo Fisher, MA, USA). Equal amount (30 μg) of proteins were subjected to fractionation by SDS polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA) and probed with indicated antibodies.

2.5. Immunofluorescence

OPM2 and K562 (5×10^4 cells) were spread on glass slides by centrifugation at 500 rpm (400×*g*) for 5 min using a cytospin system (Thermo Fisher, MA, USA). Cells were fixed in cold 4% paraformaldehyde for 10 min. After fixation, cells were washed in phosphate buffer solution (PBS) and then blocked for 1 h with 1% bovine serum albumin containing 0.1% Triton X-100 at room temperature. Samples were incubated overnight with anti-LC3 antibody at 4 °C, followed by staining with Cy3-labeled goat anti-rabbit IgG and DAPI (5 μg/ml) as described previously.[9] All samples were analyzed on a Nikon confocal microscopy (EZ-C1 FreeViewer, Nikon).

2.6. Cycloheximide chase assay

Cycloheximide (CHX) was purchased from Sigma–Aldrich (USA). RPMI-8226 was pretreated with CHX, an inhibitor of protein *de novo* synthesis, followed by S14161 or DMSO treatment for 2–10 h and Western blotting to measure the protein level of p62.

2.7. Co-immunoprecipitation (Co-IP)

The process was described previously.[9] Briefly, cells were collected and washed with ice-cold PBS after treatment, then in lysis buffer for 20 min on ice, followed by clarification with high-speed centrifugation at 4 °C for 30 min. Supernatants were incubated overnight at 4 °C with specific primary antibodies as needed followed by incubation with 40 μl of a 50% slurry of protein A/G agarose beads with gentle rotation at 4 °C for 2 h. Agarose beads were collected and washed 5 times with lysis buffer, followed by re-suspension in 20 μl of 2 × SDS loading buffer. Samples were

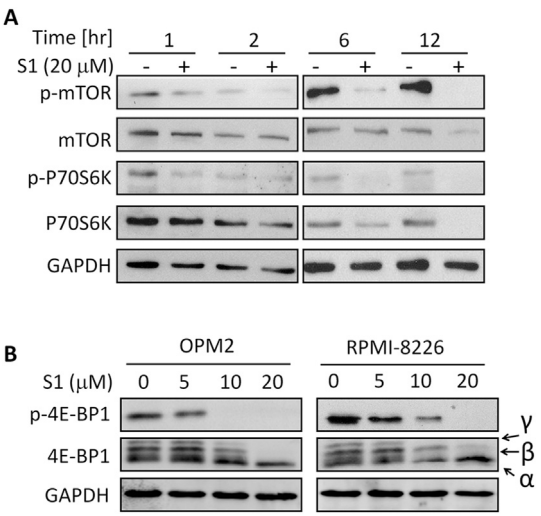

Fig. 1. S14161 inhibits the activation of the mTOR/p70S6K/4E-BP1 signaling pathway. A, S14161 inhibited the activation of mTOR and p70S6K in a time-dependent manner. RPMI-8226 cells were treated by S14161 (S1) at 20 μM for 1, 2, 6, or 12 h, followed by the analysis of the expression of p-mTOR and p-p70S6K. B, S14161 inhibited the activation of 4E-BP1 in a concentration-dependent manner. OPM2 and RPMI-8226 cells were treated with S14161 at increasing concentrations for 10 h. Expression of p-4E-BP1 and 4E-BP1 was measured by Western blot. GAPDH was used as a loading control.

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