3-Anhydro-6-hydroxy-ophiobolin A, a fungal sesterterpene from Bipolaris oryzae induced autophagy and promoted the degradation of α-synuclein in PC12 cells

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Autophagy is defined as an evolutionarily conserved process responsible for degradation of the cytoplasmic components including protein aggregates via the lysosomal machinery. Increasing evidence has linked defective autophagic degradation of protein aggregates with the pathogenesis of neurodegenerative disorders, and it is suggested that promotion of autophagy is regarded as a potential therapeutic discovery strategy. However, the narrow pore of the proteasome barrel precludes the clearance of oligomeric and aggregated form of α-synuclein. This may explain that drugs targeting of ubiquitin proteasome pathway are not effective for degradation of α-synuclein and subsequent for PD treatment.

Autophagy is defined as an evolutionarily conserved catabolic pathway responsible for bulk degradation of intracellular components including miss-folding protein, protein aggregates, and damaged organelles. It is characterized by a double-membrane structure called autophagosome surrounding to-be-digested cellular components. The autophagosome fuses with lysosome for subsequent degradation. As the crucial role in cell survival, cell growth and differentiation, disorder of autophagy regulation is associated with human pathogenesis. Additionally, modulating of autophagy activity has been recognized as a potential drug discovery strategy. Autophagy is more recently identified to be responsible for clearance of oligomeric and aggregated form of α-synuclein, as well as the A53T and A30P α-synuclein mutants, allowing autophagy as an alternative strategy for clinical application of PD treatment. Promotion of autophagic degradation of toxic protein aggregates has potential therapeutic benefits. To search for new bioactive molecules, we carried out a high-throughput screening of natural compounds that specifically induced autophagy.

Protein turnover is crucial for maintaining the integrity and normal functionality of cells, and increasing evidence has enlightened a close link between disorder of protein turnover and accumulation of pathogenic proteins leading to human pathogenesis, including Parkinson’s disease (PD), Huntington’s disease (HD) and other neurodegenerative diseases. PD is characterized by the loss of dopaminergic neurons in the substantia nigra and formation of aggregates (Lewy bodies) in neurons. α-Synuclein is the major component in Lewy bodies and the aggregation of α-synuclein is hallmark lesion of degenerating neurons in the brains of patients with PD. Mutation of α-synuclein acts a causal role in the pathogenesis of familial PD, and the single-nucleotide polymorphisms in SNCA gene, encoding for α-synuclein, are associated with increased susceptibility to sporadic PD via genome-wide association studies. Earlier studies demonstrated that ubiquitin proteasome pathway is partially responsible for the degradation of α-synuclein. However, the narrow pore of the proteasome
LC3, a soluble protein with ubiquitous expression in mammalian tissues and cultured cells, shows a dot-like distribution during autophagy, as the transformation from a cytosolic form of LC3 (LC3-I) to a lipidosed form of LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes. The autophagy-inducing activity can be evaluated via quantification the number of GFP-LC3 dots. Starvation (serum deprivation, amino acid deprivation, or both) is the most extensively studied condition that induces autophagy, and the autophagy is induced to generate amino acids, glucose or other biosynthesis blocks. We used the serum deprivation as a positive control in the screening assay. Taking advantage of this strategy, over 200 different kinds of fungal secondary metabolites were screened using GFP-LC3 stable HeLa cell. As a result, 3-anhydro-6-hydroxy-ophiobolin A (X15-2) isolated from the culture of Bipolaris oryzae was found to possess strong autophagy-inducing activity (Fig. 1A and B). 3-Anhydro-ophiobolin A (X15-6), that is, closely related to X15-2 in chemical structure did not show activity in inducing autophagy in GFP-LC3 stable HeLa cell (Fig. 1A and B). As described in our early work, X15-2 and X-15-6 were purified from the liquid culture of the fungus B. oryzae in a large scale fermentation. The structures of X15-2 and X-15-6 were confirmed by 1H and 13C NMR spectra.

To further confirm the autophagy-inducing activity of X15-2, we took advantage of confocal microscope to evaluate the bioactive activity of X15-2. As a result, X15-2, but not X15-6, can strongly induce autophagy in GFP-LC3 stable HeLa cells, shown as the increasing number of GFP-LC3 dots (Fig. 1C). In addition, western blotting analysis revealed obvious increasing quantity of the LC3-II form in X15-2-treated HeLa cells (Fig. 1D). Similarity, X15-2 could also induce autophagy both in U2OS and SH-SY5Y cells (Fig. 2). Collectively, our results clearly demonstrated that X15-2 induced an autophagic response. Confocal microscopic analyses showed that X15-2 induced the increasing number of punctuate GFP-LC3 dots in a time- and dose-dependent manner (Fig. 3A and B). Biochemical analysis revealed the steady increase of the LC3-II level, the biochemical hallmark for autophagy, in X15-2-treated HeLa cells, in a time- and dose-dependent manner (Fig. 3C). As increased punctuate GFP-LC3 dots and LC3-II levels can occur either due to the enhanced autophagy flux or defective of autolysosome formation, we used Baflomycin A1 (BA), an inhibitor of autophagy that acts by blocking the activity of the H+-ATPase responsible for acidification of autophagolysosomes, for evaluation of autophagic flux. BA could both significantly increase the number of punctate GFP-LC3 dots (Fig. 3D and E) and LC3-II level (Fig. 3F) in X15-2 exposed HeLa cells, suggesting that X15-2 induced autophagy is due to the enhanced autophagic flux rather than a defect of autolysosomal digestion.

To determine the potential role of core autophagy component in X15-2 induced autophagy, we constructed an autophagy-related protein 5 (ATG5) KO cell line via CRISPR/Cas9 system. The sequence 5' AACTTGTTTCACGCTATATC 3' reported by Shalem et al. was sub-cloned into the lenti-CRISPR plasmid, and the stable ATG5

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**Figure 1.** High-throughput screening of small compounds that induce autophagy. (A) GFP-LC3 stable HeLa cells were treated by over 200 different kinds of fungal secondary metabolites isolated from the PDB culture of a phytopathogenic fungus Bipolaris oryzae. Images were collected with an ArrayScan HCS 4.0 Reader, and the number of GFP-LC3 dots per cell was quantified. Data represent mean ± SEM of three independent experiments (n > 10,000 cells per condition in each experiment). NO.45 is X15-2, NO.49 is X15-6, and NO.197 is serum deprivation as a positive control. (B) The structures of ophiobolin derivative X15-2 and X15-6. (C) To confirm X15-2 triggering autophagy, GFP-LC3 stable cells were treated by X15-2, X15-6 or vehicle control for 6 h, respectively. Then the cells were fixed and images were captured by confocal microscope. The representative images are shown. Scale bar 10 µm. (D) HeLa cells were treated by 10 µM X15-2, X15-6 or vehicle control for 6 h. The whole cell lysates were analyzed by immunoblotting with anti-LC3 antibody. Actin was used as loading control.
KO cells were sorting using puromycin. We found that ATG5 KO almost completely suppresses X15-2 induced autophagy as showed by the absence of punctate GFP-LC3 dots formation and increasing of LC3-II level in X15-2 treated ATG5 KO HeLa cells (Fig. 4A and B). In addition, Beclin1 is one of the key mediators involved in the initial step of autophagosome formation. Unlike to ATG5, Beclin1 KO alone fails to block X15-2 induced autophagy (Fig. 4C). More recently, Beclin2 was identified as a Beclin1-like protein to function in autophagy, as the similar interactome profile. Interestingly, knockdown of Beclin2 in Beclin1 KO cells with specific siRNA oligo almost completely blocked X15-2 induced autophagy (Fig. 4C), demonstrating that both Beclin1 and Beclin2 are required for X15-2 induced autophagy.

Previous studies have shown that ophiobolin A can lead to substantial mitochondrial swelling and paraptosis-like cell death in glioblastoma multiforme cells, and also depolarize the mitochondrial membrane of the sperm cells. Here we found that X15-2 could induce collapse of mitochondrial network, from elongated linear network to fragmented short and punctate form (Fig. 5A). X15-2 fails to induce specific mitochondrial autophagy, as shown by the mitochondrial mass analysis by immunoblotting of mitochondrial marker proteins, Tom20 and Tim23 (Fig. 5B), as well as GFP-LC3/mitochondria co-localization analysis (Fig. 5C). In addition, X15-2 can injure mitochondrial and induce loss of mitochondrial membrane potential (Fig. 5D and E). A further investigation showed that X15-2 can induce accumulation of both cellular reactive oxygen species (ROS) and mitochondrial ROS, using cellular ROS and mitochondrial ROS probe DCF and Mito-SOX, respectively (Fig. 6A and B). Increasing evidence has shown that ROS and oxidative stress are responsible for autophagy and we found that N-acetyl-L-cysteine (NAC), the ROS scavenger, can strongly block the GFP-LC3 puncta formation and the increase of LC3-II level (Fig. 6A–D), suggesting that ROS is crucial for X15-2 inducing autophagy. c-Jun N-terminal kinase (JNK) signaling is reported to act a central role in ROS and oxidative stress inducing autophagy. To further investigate the role of JNK pathway in X15-2 induced autophagy, the activity of JNK pathway was checked. As a result, X15-2 could strongly activate JNK signaling, as shown the increased phosphorylated JNK'level in X15-2 exposed cell (Fig. 6F). SP600125, the JNK inhibitor, could almost completely block X15-2 inducing JNK activation, accompanying with blocking GFP-LC3 puncta formation and the increase of LC3-II level (Fig. 6E and F). These data demonstrate that mitochondrial oxidative stress activated JNK pathway is involved in X15-2 induced autophagy. JNK signaling modulates autophagy mainly through disruption the interaction between Bcl-2/Bcl-xL and Beclin1 via alteration of Bcl-2/Bcl-xL phosphorylated status. X15-2 also induces decrease of the interaction between Bcl-2/Bcl-xL and Beclin1 (Fig. 4D), as that of starvation treatment.

Induction of autophagy by small natural compounds is currently thought to be one potential strategy for fighting neurodegenerative diseases via autophagic degradation of pathogenic protein aggregates. α-Synuclein, the major constituent of Lewy bodies, is hallmark lesion of degenerating neurons in the brains of patients with PD. To investigate X15-2 induced autophagy in stability of α-synuclein, we used PC12 cells which are dopaminergic and can be differentiated into a neuron-like phenotype with nerve growth factor. Upon X15-2 treatment, α-synuclein forms a dot-like distribution co-localized with GFP-LC3 puncta, and the protein levels decreased as showed by Western blotting (Fig. 7A and B). The autophagy inhibitor, BA, prevented the degradation of α-synuclein mediated by X15-2, suggesting the degradation of α-synuclein is dependent on autophagic machinery mediated by X15-2 (Fig. 7C). To confirm this, we established the α-synuclein tetracycline-in-

Figure 2. X15-2 induced autophagy in U2OS and SH-SY5Y cell. GFP-LC3 stable U2OS and SH-SY5Y cell were treated by 10 μM X15-2 for 6 h. Then the cells were fixed and images were captured by confocal microscope. The representative images are shown. Scale bar 10 μm.
Figure 3. Small compound X15-2 increased autophagic flux. (A) GFP-LC3 stable HeLa cells were treated by 5 μM or 10 μM X15-2 for indicated time. Then the cells were fixed and images were captured by confocal microscope. The representative images are shown. Scale bar 10 μm. (B) Quantification of number of GFP-LC3 dots per cell, data represent mean ± SD of at least three independent experiments. (C) HeLa cells were treated by 0–20 μM X15-2 for indicated time, and the whole cell lysates were analyzed by western blotting with anti-LC3 antibody. Actin was used as loading control. (D) GFP-LC3 stable HeLa cells were treated by 10 μM X15-2 with or without autophagy inhibitor bafilomycin A1 (BA) for 6 h, respectively. Then the cells were fixed and images were captured by confocal microscope. The representative images are shown. Scale bar 10 μm. (E) Quantification of number of GFP-LC3 dots per cell, data represent mean ± SD of at least three independent experiments. (F) HeLa cells were treated by 10 μM X15-2 with or without autophagy inhibitor bafilomycin A1 (BA) for 6 h, respectively, and the whole cell lysates were analyzed by western blotting with anti-LC3 antibody. Actin was used as loading control.

Figure 4. X15-2 induced autophagy is ATG5 and Beclin1/Beclin2 dependent. (A) ATG5 KO HeLa cell and wild type HeLa cell were treated by 10 μM X15-2 for 6 h, and the whole cell lysates were analyzed by western blotting with anti-LC3 antibody. Actin was used as loading control. (B) ATG5 KO HeLa cell and wild type HeLa cell were transfected of GFP-LC3 plasmid. After 24 h of transfection, cells were treated by 10 μM X15-2 for 6 h. Then the cells were fixed and images were captured by confocal microscope. The representative images are shown. Scale bar 10 μm. (C) Beclin1 KO HeLa and wild type HeLa cell were transfected of Beclin2 siRNA or the scramble siRNA. After 48 h of transfection, cells were treated by 10 μM X15-2 for 6 h. Then the whole cell lysates were analyzed by immunoblotting with the indicated antibodies. (D) Tet inducible Bcl-xL HeLa cells were added with Tet to induce Bcl-xL expression or not firstly, following with treatment by 10 μM X15-2 for 6 h or 12 h, respectively. The Earle’s balanced salts solution (EBSS) starvation was used as a positive control. After the certain treatment, cells lysates were immunoprecipitated with anti-Beclin1 antibody. The indicated proteins were analyzed.
Figure 5. X15-2 induced mitochondrial dysfunction. (A) HeLa cells were treated by 10 μM X15-2 for 6 h. Then the cells were fixed and immunostained with anti-Tim23 antibody, and images were captured by confocal microscope. The representative images are shown. Scale bar 10 μm. (B) HeLa cells were treated by 10 μM X15-2 for indicated time. Then the cell lysates were harvested and analyzed by western blotting with anti-Tom20 and anti-Tim23 antibodies to evaluate mitochondrial mass. (C) GFP-LC3 stable HeLa cells were treated by 10 μM X15-2, following with immunostaining with anti-Tim23 antibody. The representative images are shown that no mitochondria were surrounded by GFP-LC3 positive autophagosome puncta. Scale bar 10 μm. (D) HeLa cells were treated by 10 μM X15-2 or mitochondrial uncoupler FCCP (carbonylcyanide-p-trifluoromethoxyphenylhydrazone). After the treatment, cells were stained by mitochondrial dye TMRE (tetramethylrhodamine ethyl ester) and the fixed cells were observed with confocal microscope. The representative images are shown to evaluate the mitochondrial membrane potential. Scale bar 10 μm. (E) HeLa cells were treated by 10 μM X15-2 or mitochondrial uncoupler FCCP. After the treatment, cells were stained by mitochondrial dye TMRE, following analysis by flow cytometry.

Figure 6. The elevated ROS is critical for X15-2 inducing autophagy. (A) HeLa cells were treated by X15-2 with or without ROS scavenger NAC for 24 h, respectively. Then the cells were digested and stained by ROS indicator DCF, following analysis by flow cytometry. (B) HeLa cells were treated by X15-2 with or without ROS scavenger NAC for 24 h, respectively. Then the cells were digested and stained by mitochondrial ROS indicator Mito-SOX, following analysis by flow cytometry. (C) GFP-LC3 stable HeLa cells were treated by X15-2 with or without ROS scavenger NAC for 6 h, respectively. Then the cells were fixed and images were captured by confocal microscope. The representative images are shown. Scale bar 10 μm. (D) HeLa cells were treated by X15-2 with or without ROS scavenger NAC for 6 h, respectively. Then the whole cell lysates were analyzed by western blotting with anti-LC3 antibody. Actin was used as loading control. (E) GFP-LC3 stable HeLa cells were treated by X15-2 with or without JNK inhibitor SP600125 for 6 h, respectively. Then the cells were fixed and images were captured by confocal microscope. The representative images are shown. Scale bar 10 μm. (F) HeLa cells were treated by X15-2 with or without JNK inhibitor SP600125 for 6 h, respectively. Then the whole cell lysates were analyzed by western blotting with anti-LC3, anti-JNK, and anti-JNK-P antibodies. Actin was used as loading control.
ducible PC12 cell lines, with or without ATG5 knockout via CRISPR/Cas9 system. We found that ATG5 KO completely block the X15-2 mediated degradation of α-synuclein [Fig. 7D]. Collectively, these data demonstrate that a small natural molecule, X15-2, induces autophagic degradation of α-synuclein.

As the major component in Lewy bodies, α-synuclein has been proved to act a causal role in the neurodegeneration and the pathogenesis of familial PD. Increasing studies have shown removal of α-synuclein as a therapeutic target of PD and related disorders. In current study, we have identified a small natural molecule, X15-2, as a small autophagy modulator via high-throughput image-based screening assay. We clearly showed that X15-2 could strongly induce autophagic flux to mediate autophagic clearance of α-synuclein, allowing it as a potential leading compound for the development of clinical drugs in PD treatment, which targets to autophagy. The fact that compound X15-6 showed no effect in inducing autophagy indicated that the hydroxyl group attached at C-6 greatly influenced the autophagy-inducing activity. In conclusion, the current report highlights the potential bioactivity of X15-2 to overcome PD through induction of autophagy to degrade α-synuclein.

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Supplementary data

Supplementary data (experimental procedures) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.02.030.

References and notes