Potassium Bisperoxo (1,10-phenanthroline) Oxovanadate (bpV(phen)) Induces Apoptosis and Pyroptosis and Disrupts the P62-HDAC6 Interaction to Suppress the Acetylated Microtubule-dependent Degradation of Autophagosomes

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*Running title: Impact of bpV(phen) on Autophagy and Cell Death

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Keywords: α-Tubulin, Apoptosis, Autophagy, bpV(phen), Caspase 1, histone deacetylase 6 (HDAC6), lactate dehydrogenase, microtubule, P62, Pyroptosis

Background: BpV(phen) is an insulin-mimetic small molecule.

Results: We have demonstrated that bpV(phen) reduces the stability of P62 in a proteasome-dependent way to activate HDAC6 to inhibit autophagy and induce apoptosis and pyroptosis.

Conclusion: We have proposed that bpV(phen) inhibits autophagy through P62.

Significance: We provide insights into a novel function of bpV(phen) and P62 in autophagy.

ABSTRACT

Autophagy is a cellular process that controls and executes the turnover of dysfunctional organelles and misfolded or abnormally aggregated proteins. Phosphatase and tensin homologue deleted on chromosome ten (PTEN) activates the initiation of autophagy. Autophagosomes migrate along acetylated microtubules to fuse with lysosomes to execute the degradation of the engulfed substrates that are usually binding with sequestosome 1 (SQSTM1) (P62). Microtubule-associated protein 1 light chain 3 (LC3) traces the autophagy process by converting from LC3-I to LC3-II isoform and serves as a major marker of autophagy flux. Potassium bisperoxo (1,10-phenanthroline) oxovanadate (bpV(phen)) is an insulin mimic and a PTEN inhibitor, and has the potential for treatment of different diseases. Here we show that bpV(phen) enhances the ubiquitination of P62, reduces the stability of P62, disrupts the interaction between P62 and histone deacetylase 6 (HDAC6), activates the deacetylase activity of HDAC6 on α-tubulin and impairs stable acetylated microtubules. Microtubular destabilization leads to blockade of autophagosome-lysosome fusion and accumulation of autophagosomes. Autophagy defects lead to oxidative stress and lysosomal rupture that trigger different types of cell death including apoptosis and pyroptosis. The consistent results from multiple systems including mouse and different types of...
mammalian cells are different from the predicted function of bpV(phen) as a PTEN inhibitor to activate autophagy flux. In addition, levels of P62 are reduced but not elevated when autophagosomal degradation is blocked, revealing a novel function of P62 in autophagy regulation. Therefore, it is necessary to pay attention to the roles of bpV(phen) in autophagy, apoptosis and pyroptosis when we develop it as a drug.

INTRODUCTION

Mammalian cells primarily use the autophagy-lysosome pathway to degrade dysfunctional organelles, misfolded/aggregated proteins and other macromolecules (1). Autophagy process is regulated by 1) a Class III phosphatidylinositol-3-kinase (PI3K) complex functioning in vesicle nucleation, 2) a serine-threonine kinase complex involved in autophagic induction, and 3) a pair of novel ubiquitin-like protein conjugating systems, the ATG12 and ATG8 systems, promoting the extension and completion of vesicles (2). Phosphatase and tensin homologue deleted on chromosome ten (PTEN), a phosphotyrosine phosphatase, activates autophagy initiation by inhibiting PI3K (3). Microtubule-associated protein 1 light chain 3 (LC3) is the mammalian homologue of ATG8, one of the key autophagy markers (4). LC3 precursor is truncated to form the cytosolic LC3-I and further conjugated with phosphatidylethanolamine to create the membrane-associated LC3-II with the assistance of ATG5 and ATG12 (5,6). The LC3-II-associated isolation membranes target to and then completely envelop individual organelles or macro-molecules that bind to polyubiquitin-binding protein p62/SQSTM1 (P62) to form autophagosomes (7,8). Autophagosomes migrate along acetylated microtubules to fuse with lysosomes to form autolysosomes in which substrates are degraded (9-11). Inhibition of autophagy initiation reduces the generation of autophagosomes so the levels of LC3-II and P62 are reduced when lysosomal activity is suppressed. However, inhibition of the degradation of autophagosomes leads to accumulation of LC3-II and P62.

Potassium bisperoxo (1,10-phenanthroline) oxovanadate (K[VO(O2)2C12H8N2]•3H2O) or bpV(phen) is a peroxovanadium (pV) small molecule with insulin-mimetic properties (12). It was demonstrated to activate insulin receptor kinase and to inhibit the dephosphorylation of autophosphorylated insulin receptors, and cause significant decreases of circulating insulin and plasma glucose levels (13). It was also characterized as a potent protein phosphotyrosine phosphatase inhibitor exhibiting anti-tumor activity (14). It inhibits PTEN to induce cardioprotection against ischemia–reperfusion injury (15).

Because of the potential of bpV(phen) for treatment of different diseases and its inhibition of PTEN that was suggested to regulate autophagy through PI3K, we were triggered to investigate whether bpV(phen) impact on autophagy. Here we reported that bpV(phen) promoted not only cell apoptosis but also pyroptosis, resulting in overall cell death. Surprisingly, it exhibited no impact on autophagy initiation but blocked the autophagosomal degradation by reducing the stability of P62 to activate HDAC6 to impair the fusion of autophagosome and lysosome supported by acetylated microtubules.

EXPERIMENTAL PROCEDURES

Antibodies, siRNAs, Plasmids and Other Reagents
Antibodies against human LC3 (NB 100-2331) and was purchased from Novus Biologicals. The IgG control antibody from rabbit (SC-2027), primary antibodies against PARP (sc-7150), PCNA (sc-9707), α-tubulin (SC-12462), β-actin (SC-47778), HDAC6 (sc-11420) and acetylated α-tubulin (SC-23950), random sequence control siRNA (sc-44234) and siRNA specific to P62 (sc-29679) were from Santa Cruz Biotechnology, Inc.. Antibody against P62 (SQSTN1, BWL-PW9860) was from Enzo Life Sciences International Inc. Horseradish peroxidase (HRP)-conjugated secondary antibodies against mouse or rabbit were from Bio-Rad. FITC goat anti-rabbit IgG (R6393 and A-21070) and Oligofectamine (12252-011) were from Invitrogen. RFP-LC3 was supplied by Dr. Mizushima (16). Antibody against caspase 1 (PRS3459), MG-132, Bafilomycin A1, bpV(phen), and Lactate Dehydrogenase Activity Assay Kit (MAK066-1KT) were from Sigma-Aldrich. The protein G beads were from Amersham Biosciences. Antibody against phospho-AKT (Ser473) (p-AKT) was from Cell Signaling.
Technology. CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) was from Promega, Biosciences, USA. Annexin V/FITC Apoptosis Detection kit was from BD Biosciences.

**Cell Culture, Cell Transfection with siRNA, Coimmunoprecipitation Assay, Immunoblot Analysis and Fluorescent Microscopy**

HepG2 cells, HeLa cells, HeLa cells stably expressing ERFP-LC3 and MEF cells were established and cultured as previously described (17,18). HeLa cells were transfected with random or P62-specific siRNA molecules packed with Oligofectamine in a similar way as we previously described (17,18). Cell lysates were prepared from attached cells and coimmunoprecipitation were performed as previously described (17,18). Cell lysates with the same amounts of total proteins were subjected to immunoprecipitation with equal amounts of specific antibodies and control antibodies (IgG) from the same species. Lysates and/or immunoprecipitates were subjected to immoblot analyses as previously reported (17,18). Immunofluorescent staining was performed and images were captured with the laser scanning microscope similarly as we previously described (17,18).

**Cell proliferation assay**

Cell proliferation rate was assessed using CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) (Promega, Biosciences, USA) according to the manufacturer’s protocols. Briefly, 10,000 cells were seeded in a well on 96-well plates (Corning, New York, USA) containing 100 µL culture medium plus different concentrations of bpV(phen) and grown at 37°C for 24 hrs. Then 15 µL Dye Solution was added to each well, plates were continuously incubated at 37°C for 4 hrs, reaction was terminated with 100 µL Stop Solution, and optical densities at 570 nm were determined.

**Detection of Apoptotic Cells**

Apoptosis was evaluated using the Annexin V/FITC Apoptosis Detection kit. At first, cells were treated with different concentrations of bpV(phen) for 48 hrs and harvested by twice centrifugation at 1000 rpm (5 min each spin). Cells were then washed twice (3 min each wash) in binding buffer, 1×10^6 cells were resuspended in 1 ml of binding buffer containing 1.25 µl of Annexin V-FITC (BD PharMingen, San Diego, USA) and 10 µl of PI, and incubated for 15 min at room temperature in the dark. Finally, cell cycle analysis was performed by flow cytometry (BD CALIBAR, USA). Scatter plots were performed against the intensities of the FITC fluorescence and PI fluorescence. The scatter plot was divided into four quadrants: the left lower quadrant (Annexin V-FITC (-) and PI (-)) representing viable cells, the left upper quadrant (Annexin V-FITC (-) and PI (+)) necrotic cells, right lower quadrant (Annexin V-FITC (+) and PI (-)) early apoptotic cells, and right upper quadrant (Annexin V-FITC (+) and PI (+)) late apoptotic cells.

**Lactate Dehydrogenase Release Assay**

To quantitate the extent of pyroptosis after bpV(phen) exposure, release of lactate dehydrogenase (LDH) in cell culture supernatants was determined using the Lactate Dehydrogenase Activity Assay Kit from Sigma-Aldrich according to the manufacturer’s instructions. Briefly, HeLa cells were cultured in the presence of different concentration of bpV(phen) for 48 hrs. Then 50 µl of cell culture supernatant was collected and added to the kit reagent and incubated for 10 min at room temperature. After addition of stopping solution, the fluorescence intensity was measured using the microplate reader at excitation wavelength of 560 nm and emission wavelength of 590 nm.

**Animal Experimentation.**

Animal protocols were approved by the Institutional Animal Care and Use Committee, Institute of Biosciences and Technology, Texas A&M Health Science Center. All animals received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). Three pairs of one-month old freely-fed female wild-type C57BL/6 mice were subjected to sub cutaneous injection of 2.5 µmol/30 g body weight in a total of 250 µl of saline (or saline only for control group) as described (19).

**RESULTS**

**Treatment with bpV(phen) Induces Cell Apoptosis and Pyroptosis.**
To test the function of Potassium Bisperoxo (1,10-phenanthroline) Oxovanadate (bpV(phen)), we treated MEF cells with increasing concentration of bpV(phen) for 48 hrs. As an PTEN inhibitor, bpV(phen) enhanced the levels of phosphorylated AKT (Fig. 1A). A dosage-dependent reduction of cell population and cell viability was detected (Fig. 1B,C). Examination of the treated cells by immunoblot revealed an even levels of PCNA and increasing levels of PARP 89 KD fragment (Fig. 1D,E). PCNA (proliferating cell nuclear antigen) is a cellular marker for proliferation. PARP (poly ADP ribose polymerase) is cleaved into an 89-KD fragment by the apoptosis-specific caspase-3 and levels of such fragment serve as a marker of apoptosis. These data suggest that the bpV(phen) exposure helps cell maintain a constant rate of cell proliferation but an increasing rate of apoptosis so that the cell population is reduced. Cell apoptosis was further confirmed by annexin-V flow cytometry (Fig. 1F). Pyroptosis is described as another type of cell death that is different from apoptosis and characterized by the activation of caspase 1 and release of cellular contents. Treatment with bpV(phen) led to dosage-dependent increases in cellular levels of active caspase 1 with molecular weight of 10 KD and levels of lactate dehydrogenase released in the media (Fig. 1G-I). Therefore, bpV(phen) induces both apoptosis and pyroptosis.

Treatment with bpV(phen) Results in Blockade of the Degradation of Autophagosomes.

Because of the close relationship between autophagy and cell death pathways of apoptosis and pyroptosis (20,21), we examined the impact of bpV(phen) on autophagy. We injected freely-fed wild-type mice with bpV(phen) and collected liver tissues for autophagy analysis. We found that bpV(phen) induced a significant increase in the levels of LC3-II (Fig. 2A,B), suggesting either an activation of autophagy initiation or an inhibition of autophagosomal degradation. To determine the exact mechanism, we treated MEF cells with bpV(phen) in the absence or presence of Bafilomycin A1 (BAF) to block the degradation of autophagosomes and observed the similar increases of LC3-II in the absence of BAF but no increase of LC3-II in the presence of BAF (Fig. 2C,D), indicating a blockade of the degradation of autophagosomes. The same trends were observed when either HeLa cells (Fig. 2E,F) or HepG2 cells (Fig. 2G,H) were exposed to bpV(phen). When HeLa cells stably expressing RFP-LC3 were exposed to bpV(phen), we detected a significant increase in the number of RFP-LC3 punctate foci in the absence of BAF but no increase in the presence of BAF (Fig. 2I,J). All results indicate that bpV(phen) induces a blockade of the degradation of autophagosomes.

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Treatment with bpV(phen) Reduces the Stability of P62 in a Proteasome-dependent Way. P62, another important marker of autophagy flux, was widely considered to act as a receptor to bring ubiquitinated proteins into autophagosome for degradation (7). It was expected to be elevated when the degradation of autophagosomes is inhibited. Surprisingly, we found a dosage-dependent decrease in the levels of P62 based on both immunoblot analysis (Fig. 3A,B) and immunofluorescent staining (Fig. 3C) of HeLa cells. Further analysis revealed that it was the stability of P62 that was significantly decreased in the presence of bpV(phen) (Fig. 3D,E). P62 was reported to bind with polyubiquitinated protein aggregates and help them be packed into autophagosomes and then degraded with the bound aggregates in lysosomes (7,22). Inhibition of lysosomal activity with Bafilomycin A1 (BAF) is predicted to increase the levels of P62. However, the decrease in levels of P62 in the presence of bpV(phen) was restored only partially by inhibition of lysosomal activity with BAF but more dramatically by inhibition of proteasomal activity of 26S complex with MG-132 (Fig. 3F,G). P62 was not associated with RFP-LC3 punctate foci in the presence of MG-132 but colocalized with the punctate foci in the presence of BAF (Fig. 3H). Thus, bpV(phen) exposure reduces the stability of p62 not through autophagy. Exposure to bpV(phen) led to a non-specific enhancement in the levels of total ubiquitinated proteins and a specific enhancement of P62 ubiquitination. Blocking proteasomal activity with MG132 caused an accumulation of polyubiquitinated P62 (Fig. 3I). Comparing to the control, MG132 treatment led to accumulation of more ubiquitinated P62 with low molecular weight, suggesting those ubiquitinated P62 with high molecular weight cannot be degraded through proteasomes (Fig. 3I). Thus, bpV(phen) treatment
promoted proteasomal degradation of poly-ubiquitinated P62.

**Suppression of P62 Causes Deacetylation of α-Tubulin and Inhibition of the Degradation of Autophagosomes.**

In addition to serving as a substrate receptor, P62 was recently reported to interact with histone deacetylase 6 (HDAC6) to suppress its deacetylase activity on α-tubulin (23). We detected no change in the levels of total α-tubulin but significant decreases in the levels of acetylated α-tubulin when the P62 levels were suppressed in HeLa cells with two different P62-specific siRNA molecules (Fig. 4A–C). Overexpression of P62 exhibited the opposite impact on the levels of acetylated tubulin (Fig. 4D). The levels of acetylated microtubules were dramatically reduced while the general microtubules remained constant (Fig. 4E). Acetylated microtubules are stable and required for the trafficking of autophagosomes to fuse with lysosomes (11). The reduction in levels of acetylated microtubules led to an increase of LC3-II in the absence of BAF but even levels of LC3-II in the presence of BAF (Fig. 4A–C). Overexpression of P62 accelerated the degradation of autophagosomes and led to reduction in LC3-II levels in the absence of BAF (Fig. 4D). Thus, degradation of P62 leads to a release of HDAC6 activity that causes deacetylation of α-tubulin, destabilization of microtubules and blockade of the degradation of autophagosomes.

**Treatment with bpV(phen) Leads to Reduction of P62 and Release of HDAC6 to Deacetylate the Acetylated α-Tubulin.**

To decipher how bpV(phen) impacts autophagy through P62, we tested the impact of bpV(phen) on the interaction of P62 with HDAC6. When HeLa cells were exposed to bpV(phen), the levels of HDAC6 were slightly reduced but the levels of acetylated α-tubulin were dramatically reduced although the total levels of α-tubulin were unchanged (Fig. 5A). The levels of acetylated microtubules in HeLa cells were also impaired in the presence of bpV(phen) (Fig. 5B). We detected much weaker interaction between P62 and HDAC6 in the presence of bpV(phen) than in the absence of bpV(phen) (Fig. 5C). We reasoned that such weak interaction might have been caused by reduced input levels of HDAC6 and P62. We treated the cells with BAF and MG-132 and restored the levels of both proteins to be close to the levels of untreated cells, the weak interaction was not improved at all (Fig. 5C). Therefore, bpV(phen) exposure has disrupted the interaction between P62 and HDAC6 and leads to release of HDAC6 to deacetylate α-tubulin, destabilize microtubules and impair the degradation of autophagosomes.

**DISCUSSION**

P62 protein contains multiple domains involving in different functions such as LC3-interacting region (LIR) and ubiquitin-associated (UBA) domain (24). It is generally considered as a substrate receptor that connects the polyubiquitinated protein aggregates and dysfunctional mitochondria to LC3-II and facilitates their being packaged into autophagosomes (7,8,25). Specific phosphorylation of the serine 403 in the UBA domain leads to enhancement of its association with the polyubiquitinated cargo for autophagosomal engulfment (24). The sequence 163-225 of P62 was identified as the HDAC6 interactive domain (23). The serine 207 in the HDAC6-interactive domain of P62 is highly phosphorylated in the presence of proteasomal inhibitor and inhibition of autophagy with Bafilomycin A1 was reported to show no accumulation of phosphorylated P62 at serine 207 (24). Treatment with bpV(phen) seems enhance the phosphorylation of P62 at serine 207, disrupt the interaction with HDAC6. Further analysis of the impact of bpV(phen) on the P62-HDAC6 interaction will clarified the mechanism in detail. Interestingly, proteasomal inhibition did not cause any alternation in the phosphorylation of tyrosine residue in P62 protein (24). Therefore, the impact of bpV(phen) on autophagy seems not through its PTEN inhibition because PTEN inhibition may activate autophagy initiation. P62 was reported to bind with ubiquitinated proteins and be engulfed and degraded in autophagosome. It is unknown if P62 is required to be ubiquitinated for lysosomal degradation (26). Cullin-3 (Cul-3) is an E3 ubiquitin ligase that catalyzes the ubiquitination of p62 and promote the degradation of P62 through proteasomes (27). Exposure to bpV(phen) may activate E3 ubiquitin ligases such as Cul-3 to promote the ubiquitination of P62, or promote the modification of P62 into a better substrate of ubiquitin ligase. The report that
proteasomal inhibition with MG132 did not cause any alternation in the ubiquitination of P62 protein (24) suggests the ubiquitination of P62 is a specific effect of bpV(phen) exposure.

Overactive autophagy leads to depletion of cellular contents and eventually cell death. In this sense, autophagy is classified as type II programmed cell death (28). Instead, autophagy has a pro-survival function when cells are under metabolic stress (29,30). Its dysfunction causes cells’ failure to sustain metabolic homeostasis and survive (31). If the autophagic process is blocked before autophagosomal formation, the fragmented mitochondria will release cytochrome c and other small molecules to induce conventional apoptosis that is usually associated with diverse forms of aggregation and perinuclear clustering of the dysfunctional mitochondria. The dead cell debris generated from apoptosis and autophagy is still contained in an intact plasma membrane and taken up by phagocytosis (32,33). If the process is blocked before the autolysosome formation or autophagosomes are not degraded efficiently, the accumulated mitochondria may become damaged by their own production of superoxide and start to leak electrons and lose their membrane potentials, and even further induce robust oxidative stress (1,34) or lysosomal rupture (35). Both oxidative stress and lysosomal rupture in turn activates NLRP3 inflammasome that results in direct activation of caspase-1 (36). Activation of caspase-1 subsequently induces secretion of potent pro-inflammatory cytokines and eventually an inflammatory form of cell death referred to as pyroptosis of the cell self and other cells in the environment (21,37-41). Although bpV(phen) has been suggested to applied as a drug for different types of diseases, it is necessary to consider the potential side effects caused by its impact on autophagy, apoptosis and pyroptosis.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHORS’ CONTRIBUTION

QC, JL, LL conceived and coordinated the study and wrote the paper. QC, TX, CH, YZ designed, performed and analyzed the experiments shown in Figures 1 and 2. QC, WL performed and analyzed the experiments shown in Figure 3. QC, FY performed and analyzed the experiments shown in Figure 4 and 5. JZ, KS, HH, GX, HH provided technical assistance and contributed to the preparation of the figures. All authors reviewed the results and approved the final version of the manuscript.

REFERENCES

FIGURE LEGENDS

**Figure 1.** Treatment with bpV(phen) (BPV) Induces Cell Apoptosis and Pyroptosis. **A,** Representative immunoblots showing the impact of bpV(phen) on the levels of phosphorylated AKT in HeLa cells treated with different concentration of bpV(phen) dissolved in DMSO for 48 hrs. **B,** Representative morphology of cultured MEF cells after treatment with different concentration of bpV(phen) for 48 hrs. **C,** Plots of viabilities (%) of HeLa cells incubated with increasing concentrations of bpV(phen) for 48 hrs by MTT assays. Data here or throughout are the average ± standard deviation of at least three repeats. **D,** Immunoblot analysis of the levels of PARP and PCNA in MEF cells treated with different concentration of bpV(phen) for 48 hrs. In immunoblot analyses shown here and later, cell lysates with the same amounts of total proteins were loaded and the level of β-Actin served as another loading control. **E,** Plots of relative intensities of the cleaved 89-KD PARP fragment to β-Actin as shown in panel (D). Plots here and shown later are the means ± S.D. of at least three repeats. **F,** Representative dot plots of apoptotic cells in HeLa cells treated with increasing concentrations of bpV(phen) by flow cytometry. **G,** Immunoblot analysis of the levels of caspase 1 in MEF cells treated with different concentration of bpV(phen) for 48 hrs. **H,** Plots of relative intensities of the cleaved 10-KD active caspase 1 to β-Actin as shown in panel (G). **I,** Plots of LDH activity released in medium from cultured HeLa cells.

**Figure 2.** Treatment with bpV(phen) Results in Blockade of the degradation of autophagosomes. **A,** Immunoblot analysis of the impact of bpV(phen) on the levels of autophagy markers LC3-II in the liver tissues of wild-type mice. **B,** Plots of relative intensities of LC3-II to β-Actin as shown in panel (A). **C-H,** Immunoblot analysis (C,E,G) and plots (D,F,H) of the impact of bpV(phen) on the levels of autophagy markers LC3-II in wild-type MEF (D,E), HeLa (E,F) or HepG2 cells (G,H) treated with different concentration of bpV(phen) in the absence (ctrl) or presence of Bafilomycin A1 (BAF). **I,** Fluorescent images of HeLa cells stably expressing RFP–LC3 treated with 20 µm bpV(phen) for 48 hrs. **J,** Quantification of GFP–LC3-labelled autophagosomes as shown in panel (I). The data were the average number of RFP–LC3 punctate foci ± S.D. for ten randomly selected images in a field with size of 512 pixels×512 pixels.

**Figure 3.** Treatment with bpV(phen) Reduces the Stability of P62 in a Proteasome-dependent Way. **A,** Immunoblot analysis of the impact of bpV(phen) on the levels of P62 in HeLa cells. **B,** Plots of relative intensities of P62 to β-Actin as shown in panel (A). **C,** Immunostaining analysis of P62 levels in HeLa cells untreated or treated with 20 µm bpV(phen) for 48 hrs with anti-P62 antibody. **D,** Immunoblot analysis of the impact of bpV(phen) on the stability of P62. HeLa cells were grown to confluent and protein translation was inhibited with cycloheximine (CHX) in the absence (Ctrl) or presence of Bafilomycin A1 (BAF). Cells were collected at different time points and the same amounts of total proteins were loaded and β-Actin served as another loading control. **E,** Plots of relative intensities of P62 to β-Actin as shown in panel (D). **F,** Immunoblot analysis of the impact of MG-132 and Bafilomycin A1 on the bpV(phen)-reduced stability of P62. HeLa cells were grown to confluent and treated with 20 µm bpV(phen) and cycloheximine in the absence or presence of MG-132 and/or BAF. Cells were collected at different time points and analyzed as in panel (D). **G,** Plots of relative intensities of P62 as shown in panel (F). The initial intensities of P62 at time zero under different conditions were set to be 1. **H,** Colocalization analysis of P62 with RFP–LC3 punctate foci in untreated (Ctrl), MG-132 or BAF-treated HeLa cells stably expressing RFP–LC3 in the presence of bpV(phen). P62 was visualized by staining with anti-P62 antibody. **I,** Immunoblot analysis of the impact of bpV(phen) on the levels of poly-ubiquitinated P62. The 293T cells overexpressing P62 and His-Ubiquitin were cultured in the absence or presence of 10 µM bpV(phen) for 30 hrs and untreated or treated with MG-132 for 6 hrs before cells were harvested. Cell lysates were subjected to immunoprecipitation with anti-P62 antibody and immunoblot with anti-P62 and Ubiquitin antibodies. Two panels of P62 immunoblot results were generated by long and short exposure. IgG and P62 were overlapped with each other.
Figure 4. Suppression of P62 Causes Deacetylation of α-Tubulin and Inhibition of the degradation of autophagosomes. **A,** Representative immunoblot results showing the impact of P62 suppression with a P62-specific siRNA on the levels of P62, HDAC6, total and acetylated α-Tubulin and LC3-II in the absence or presence of Bafilomycin A1. **B,** Plots of relative intensities of acetylated α-Tubulin (Ac-Tubulin) or LC3-II as shown in panel **(A).** **C,** Representative immunoblot results showing the impact of P62 suppression with another P62-specific siRNA. **D,** Representative immunoblot results showing the impact of P62 overexpression. **E,** Immunofluorescent analyses of the impact of P62 suppression on the acetylated microtubules and general microtubules visualized with antibody against acetylated α-Tubulin or α-Tubulin, respectively.

Figure 5. Treatment with bpV(phen) Leads to Reduction of P62 and Release of HDAC6 to Deacetylate the Acetylated α-Tubulin. **A,** Immunoblot analysis of the impact of bpV(phen) on the levels of P62, HDAC6 and total and acetylated α-Tubulin HeLa cells untreated or treated with 20 µm bpV(phen) for 48 hrs in the absence or presence of both MG-132 and Bafilomycin A1. **B,** Immunofluorescent analyses of the impact of bpV(phen) on the acetylated microtubules visualized with antibodies against acetylated α-Tubulin (Ac-Tubulin, green) and total α-Tubulin (red). **C,** Immunoblot analyses of interaction between HDAC6 and P62. HeLa cells were untreated or treated with 20 µm bpV(phen) for 48 hrs in the absence or presence of both MG-132 and Bafilomycin A1. Equal amounts of cell lysates were subjected to immuneprecipitation with equal amounts of antibodies against P62 or control IgG and immunoblot with respective antibodies as shown.
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B

Ac-Tubulin

Ctrl

BPV

20 μm

20 μm

20 μm

Merge

20 μm

20 μm

20 μm

C

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HDAC6

P62

62

20 μm

20 μm

20 μm

Merge

20 μm

20 μm

20 μm
Potassium Bisperooxo (1,10-phenanthroline) Oxovanadate (bpV(phen)) Induces Apoptosis and Pyroptosis and Disrupts the P62-HDAC6 Interaction to Suppress the Acetylated Microtubule-dependent Degradation of Autophagosomes
Qi Chen, Fei Yue, Wenjiao Li, Jing Zou, Tao Xu, Cheng Huang, Ye Zhang, Kun Song, Guanqun Huang, Guibin Xu, Hai Huang, Jun Li and Leyuan Liu

J. Biol. Chem. published online September 11, 2015

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