Autophagy in the pharmacological activities of celastrol (Review)

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Abstract. Celastrol, a natural compound extracted from the traditional Chinese medicinal herb *Tripterygium wilfordii* Hook F, possesses broad-spectrum pharmacological properties. Autophagy is an evolutionarily conserved catabolic process through which cytoplasmic cargo is delivered to the lysosomes for degradation. Autophagy dysregulation contributes to multiple pathological processes. Therefore, targeting autophagic activity is a promising therapy for various diseases, as well as a drug-development strategy. According to previous studies, autophagy is specifically targeted and may be altered in response to celastrol treatment, highlighting that autophagy modulation is an important mechanism underlying the therapeutic efficacy of celastrol for the treatment of various diseases. The present study summarizes the currently available information regarding the role of autophagy in the effect of celastrol to exert anti-tumor, anti-inflammatory, immunomodulatory, neuroprotective, anti-atherosclerosis, anti-pulmonary fibrosis and anti-macular degeneration activities. The diverse signaling pathways involved are also analyzed to provide insight into the mechanisms of action of celastrol and thereby pave the way for establishing celastrol as an efficacious autophagy modulator in clinical practice.

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Abbreviations: Hsp90, heat shock protein 90; mTOR, mammalian target of rapamycin; AMPK, AMP-activated protein kinase; ER, endoplasmic reticulum; LC3, microtubule-associated protein light chain 3; Atgs, autophagy related genes; ULK, unc-51-like kinase; PI3K, phosphatidylinositol 3 kinase; Rab, member RAS oncogene family protein; MDR, multidrug resistance; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; ROS, reactive oxygen species; NLRP3, NOD-like receptor family pyrin domain containing 3; ASC, apoptosis associated speck-like protein containing a CARD; LPS, lipopolysaccharide; IL, interleukin; DSS, dextran sodium sulfate; AD, Alzheimer's disease; PD, Parkinson's disease; VSMCs, vascular smooth muscle cells; miRNAs/miRs, microRNAs; AR, androgen receptor; TFEB, transcription factor EB; mTORC1, mammalian target of rapamycin complex 1; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; Wnt, wingless/integrase 1; SIRT3, sirtuin 3; JNK, c-Jun NH2-terminal kinase; HIF-1α, hypoxia-inducible factor-1α; SR, sarcoplasmic reticulum; Nur77, nuclear receptor-77; LXRα, liver X receptor α; TLR2, toll-like receptor 2; HMGB1, high mobility group box 1

Key words: celastrol, autophagy, anti-tumor, anti-inflammatory, immunomodulatory, neuroprotective, anti-atherosclerosis, anti-pulmonary fibrosis, anti-macular degeneration

Contents

1. Introduction

The bioactive constituents from traditional Chinese medicine are valued as promising novel drug discovery sources owing to their unique chemical structures and potent efficacy in treating human diseases. One such constituent of considerable interest is celastrol. Celastrol (tripterine) is a pharmacologically active pentacyclic triterpene extracted from *Tripterygium wilfordii* Hook F. Celastrol has been reported to exhibit anti-tumor, anti-inflammatory, anti-obesity and anti-oxidant activities. In addition, celastrol provided substantial therapeutic efficacy against infectious, neurodegenerative, cardiovascular and metabolic diseases (1). However, its clinical translation has yet to make substantial progress owing to its poor aqueous solubility, low bioavailability and narrow therapeutic window. Various strategies have been investigated so far to deal with these challenges, including the usage of water-soluble analogs, combination therapy and nanotechnology-based formulations (1,2). In fact, celastrol's underlying mechanisms of action have been vigorously investigated in several disease models and various molecular targets or signaling pathways responsible for these activities have been identified, including heat shock protein 90 (Hsp90), heat shock factor 1, proteasomes, NFκB, protein

kinase B (AKT)/mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) pathways (2). In addition, it has been reported that the pharmacological activities of celastrol are attributable to its ability to influence autophagy (3).

Autophagy is a highly conserved intracellular catabolic process that transports cytoplasmic materials to lysosomes for degradation. Mammalian cells may undergo various types of autophagy, including chaperone-mediated autophagy, microautophagy and macroautophagy (4,5). Current research has mainly focused on macroautophagy (hereafter referred to as 'autophagy' for simplicity). To maintain normal cellular metabolism, autophagy occurs at a basal level to eliminate damaged organelles and harmful protein aggregates. Autophagy contributes to cell survival when environmental stresses are encountered, such as nutrient deprivation, oxidative stress and microbial infection. By contrast, prolonged constitutive defective or excessive autophagy eventually causes autophagic cell death induction (type II programmed cell death) (6). Thus, autophagy has a crucial role in cellular physiology and dysregulations of this process may lead to the pathogenesis of diverse human diseases (7). For instance, autophagy alteration has been observed in the affected neurons of patients with neurodegenerative disorders (8); the essential autophagy gene Beclin1 was mono-allelically deleted in human breast cancers and autophagy deficiency in mice made them susceptible to benign hepatomas (9).

Based on these observations, autophagy modulation has emerged as an attractive therapeutic strategy for diverse diseases and the pharmacological functions of several drugs relate to potent autophagic-regulatory effects. The present article provides an overview of autophagy regulation by celastrol and discusses the underlying molecular mechanisms that define celastrol's effect on autophagy to outline the critical roles of autophagy in the pharmacological activities of celastrol.

2. An overview of autophagy

Autophagy begins at the proposed site for autophagosome formation termed phagophore assembly site in the cytoplasm. Following initiation, the membranes possibly derived from endomembrane compartments, such as endoplasmic reticulum (ER), Golgi complex and mitochondria, begin to expand and form the cup-shaped autophagic precursor structure called the phagophore, which sequentially engulfs a portion of the cytoplasm and elongates, resulting in the formation of double-membrane spherical autophagosomes (10). Ultimately, autophagosomes fuse with lysosomes to form autolysosomes, where the cytoplasmic materials and the inner membranes are degraded by lysosomal hydrolases and the breakdown products are released into the cytoplasm to be recycled as an energy source or new macromolecule building blocks (11).

Autophagy was once characterized as a bulk, nonselective, degradative process. However, the identification of multiple autophagy adaptor proteins, such as p62 and neighbor of Brca1 gene 1 (NBR1), which simultaneously bind ubiquitinated substrates and autophagy-specific ubiquitin-like system modifiers on the surface of autophagosomes, has unraveled the selectivity in autophagy (12). The selective elimination of damaged or redundant mitochondria via autophagy is termed as mitophagy. The interaction between ubiquitin kinase phosphatase and tensin homolog-induced kinase 1 (PINK1) and E3 ubiquitin ligase Parkin activates mitophagy, leading to the translocation of Parkin to the damaged mitochondrial membrane. Subsequently, Parkin-mediated poly-ubiquitination on mitochondrial outer-membrane proteins recruits autophagy adaptor protein p62 for the clearance of mitochondria (13). Furthermore, adipocyte triglyceride lipase on the lipid droplets acts as a selective autophagy receptor associated with the autophagic component microtubule-associated protein light chain 3 (LC3), culminating in lipophagy activation and degradation of lipid droplets (14). Based on the type of the digested organelle, other forms of selective autophagy have been named, such as ER-phagy, ribophagy, perophagy and nucleophagy. Selective autophagy greatly contributes to the efficient removal of organelles or macromolecules and is deemed essential for organelle quality control and homeostasis regulation (15).

A series of autophagy-related genes (Atgs) is involved in autophagy regulation. The unc-51-like kinase (ULK) complex, containing various Atg proteins, is required for autophagy induction. The class III phosphatidylinositol 3 kinase (PI3K)-Atg14 complex has important roles in autophagosome expansion and maturation, while also requiring two ubiquitin-like proteins (Atg12 and Atg8/LC3 conjugation systems). The member RAS oncogene family protein 7 (Rab7), homotypic fusion and protein sorting protein and soluble N-ethylmaleimide-sensitive factor attachment protein receptors contribute to the fusion of autophagosomes with lysosomes (16). Multiple signaling pathways have been associated with autophagy, including the mTOR, PI3K/AKT, AMPK and Hedgehog signaling pathways (17). Furthermore, there is strong evidence supporting epigenetic modifications of both Atgs and signaling molecule genes by DNA methylation and histone modifications, as well as non-coding RNAs, which impact their transcription and subsequently manage the autophagic process (18).

3. Autophagy regulation by celastrol

Extensive experimentation has indicated that autophagy modulation may be an important mechanism underlying the anti-tumor therapeutic effect of celastrol (19-30). Celastrol also alleviates inflammatory reactions and restrains the immune response through autophagic pathways (31-40). Furthermore, autophagy has a role when celastrol exerts neuroprotective, anti-atherosclerosis, anti-pulmonary fibrosis and anti-macular degeneration effects (41-48). Further details on the autophagy modulation involved in the multiple pharmacological activities of celastrol *in vitro* and *in vivo* are listed in Tables I and II, respectively.

Autophagy is involved in the anti-tumor activities of celastrol. Autophagy modulation is intimately related to the anti-tumor effects of celatrol; however, the role of autophagy in tumors is complex and may either act as a tumor suppressor or display opposing pro-oncogenic functions in a context and

Table I. Autophagy modulation by celastrol *in vitro*.

Cell lines	Celastrol dosage and treatment times	Autophagy status	Related biological effects	(Refs.)
Human prostate cancer cell line LNCaP	2 μmol/l, 3-24 h	↑	Atg5 and Atg7 mRNA↑; LC3II↑; p62↓; LC3 puncta↑; AR↓; miR-101↓; cell death↓	(19)
Human prostate cancer cell line LNCaP	2 μmol/l, 3-24 h	↑	LC3II↑; p62↓; LC3 puncta↑; miR-17-92a cluster↓; Atg7↑	(49)
Ox-LDL-treated human clear cell renal cell carcinoma cell line 786-O	0.25-1 μmol/l, 24 h	↑	LC3II↑; p62↓; p-mTOR↓; autophagic flux↑; LXRα↑; ABCA1↑; Vim↓; MMP2↓; lipophagic vesicles↑; lipid accumulation↓	(50)
Human glioblastoma cell line U251N	1-10 μmol/l, 3-24 h	↓	LC3II↑; p62↑; autophagic flux↓; lysosome number↑; lysosomal integrity↓; proteotoxic stress↑	(21)
Human glioma cell line U251	0.3-10 μmol/l, 3-24 h	↑	LC3 puncta↑; LC3II↑; Beclin1↑; p62↑; lysosomal degradation ↓; cell death↓; ROS/JNK signaling pathway↑; Akt/mTOR signaling pathway↓	(22)
Human osteosarcoma cell lines HOS, MG-63	2-3 μmol/l, 24 h	↑	LC3II↑; acidic vesicular organelles↑; autophagic vacuoles↑; cell death↑; ROS↑; p-JNK↑	(23)
Human primary osteosarcoma cell OS-718	1-1.5 μmol/l, 24 h	↑	LC3II↑; p-JNK↑; ROS↑; cell proliferation↓	(23)
Human primary osteosarcoma cell OS-1227	4-6 μmol/l, 24 h	↑	LC3II↑; p-JNK↑; ROS↑; cell proliferation↓	(23)
Human osteosarcoma cell line HOS	3 μmol/l, 24 h	↑	LC3II↑; p62↓; Bip↑; p-PERK↑	(24)
Human pancreatic ductal adenocarcinoma cell line MiaPaCa-2	0.5-4 μg/ml, 24-48 h	↑	LC3II↑, PTEN↑; LC3 puncta↑; autophagic vacuoles↑; acidic vesicular organelles↑; apoptosis↓	(25)
Human pancreatic ductal adenocarcinoma cell line CFPAC-1	0.5-2 μg/ml, 24-48 h	↑	LC3II↑; PTEN↑; LC3 puncta↑; autophagic vacuoles↑; acidic vesicular organelles↑	(25)
Human hepatocellular carcinoma cell line Bel7402	0.625-2.5 μmol/l, 24 h	↑	Autophagic vacuoles↑; LC3 puncta↑; LC3II↑	(26)
Human hepatocarcinoma cell line HepG2	4 μmol/l, 6-24 h	↑	LC3II↑; LC3 puncta↑; ROS↑; p-Akt↑; p-p70S6K↑; HIF-1a↑; BNIP3↑	(27)
Human stomach adenocarcinoma cell line AGS	0.25-1 μmol/l, 0.25-24 h	↑	LC3 puncta↑; LC3II↑; Atg5↑; Atg7↑; Beclin1↑; cell growth↓; p-AMPK↑; p-Akt↓; p-mTOR↓; and p-p70S6K↓	(28)
Human gastric carcinoma cell line YCC-2	0.25-1 μmol/l, 0.25-24 h	↑	LC3II↑; Atg5↑; Atg7↑; Beclin1↑; cell growth↓; p-AMPK↑; p-Akt↓; p-mTOR↓; p- p70S6K↓	(28)
Mouse pituitary ACTH-secreting adenoma cell line AtT20	0.5, 1 μmol/l, 48 h	↑	LC3 puncta↑; LC3II↑; p62↓; p-Akt↓; p-mTOR↓; cell viability↓; cell migration and invasion↓	(29)
Human colorectal cancer cell lines HCT-116, SW480	1.25-5 μmol/l, 24 h	↑	LC3II↑; p62↓; Beclin1↑; Beclin1-Bcl2 interaction↓; *Atg4B* and *Beclin1* mRNA↑; autophagic vesicles↑; Atg7↑; Nur77↓	(30)
Human cervical cancer cell line Hela	1 μmol/l, 0.5-4 h; 0.25-2 μmol/l, 24 h	↑	LC3II puncta↑; LC3II↑; autophagic flux↑; Ca^{2+} mobilization↑; cell death↑; CaMKKβ↑; p-AMPK↑; p-p70S6K↓; ER stress and unfolded protein response↑	(51)

Table I. Continued.

Cell lines	Celastrol dosage and treatment times	Autophagy status	Related biological effects	(Refs.)
Human cervical cancer cell line Hela	1.2 μmol/l, 12 h	↑	LC3II↑; LC3 puncta↑; autophagy flux↑; paraptosis-related cytoplasmic vacuolization↓; proteasome function↓; ER stress↑; Hsp90 function↓	(52)
Human non-small cell lung cancer cell lines H23, H292	0.8 μmol/l, combined with 10 μmol/l afatinib, 12-24 h	↓	LC3II/LC3I ratio↓; p62↑; NBR1↑; p62 aggregates↑; autophagic flux↓; lysosomal activity↓	(53)
Human lung cancer cell line A549	1-4 μmol/l, 12 h	↓	LC3II↑; p62↑; autophagy flux↓	(54)
Human non-small cell lung cancer cell line H1975	0.5-4 μmol/l, 24 h	↑	LC3 puncta↑; Ca^{2+} mobilization↑; EGFR↑; Akt↑; apoptosis↑	(55)
Human non-small cell lung cancer cell line H1650	1 μmol/l, 24 h	↑	LC3 puncta↑; Ca^{2+} mobilization↑; apoptosis↑	(55)
Human lung adenocarcinoma cell line HOP62	0.75 μmol/l, combined with 25 μmol/l ellagic, acid 24 h	↑	LC3II↑; CIP2A↓; cell proliferation↓	(56)
Human breast cancer cell line MCF-7	0.3 μmol/l, combined with 10 μmol/l tamoxifen, 6-24 h	↑	LC3II/LC3I ratio↑; p62↓; LC3 puncta↑; p-Akt↓; p-mTOR↑; cell death↑	(57)
Human non-small cell lung cancer cell line HCC827	1.25 μmol/l, combined with 2.5 μmol/l erastin, 24 h	↑	LC3 puncta↑; LC3II↑; autophagic flux↑; Beclin1↑; Atg5↑; Atg7↑; p62↑; ROS↑; cell death↑; colocalization of p62 with TOM20↑; PINK1 and Parkin↑; mitophagy↑; ubiquitinated mitochondrial proteins↑; p-DRP1↑; mitochondrial fission↑	(58)
TNFα-treated human cervical cancer cell line Hela	2-4 μmol/l, 1-6 h	↑	LC3II↑; LC3 puncta↑; IκBa↓; Nur77-p62 interaction↑; mitophagy↑	(31)
TNFα-treated mouse embryonic fibroblasts	2 μmol/l, 1 h	↑	LC3 puncta↑, mitophagy↑	(31)
LPS-primed mouse peritoneal macrophages	0.25 μmol/l, 0.5 h	↑	LC3II↑; IL-1β secretion↓; cleaved caspase-1↓; ASC oligomerization↓	(33)
High glucose-treated mouse podocytes	1.5 μmol/l, 5 h	↑	LC3II↑; p62↓; Beclin1↑; cell viability↑; proinflammatory cytokines (TNF-α, IL-1β and IL-6) ↓; glucose uptake↑; HO-1↑	(36)
Human rheumatoid arthritis fibroblast-like synoviocytes MH7A	1-2 μmol/l, 24 h	↑	LC3 puncta↑; autophagic flux↑; Ca^{2+} mobilization↑; p-AMPK↑; p-p70S6K↓; CaMKKβ↑; cell death↑	(37)
Human rheumatoid arthritis synovial fibroblasts	1-2 μmol/l, 24 h	↑	LC3 puncta↑; LC3 II↑; autophagic flux↑; p-AMPK↑; p-p70S6K↓; CaMKKβ↑	(37)
Primary cardiomyocytes from collagen induced rheumatoid arthritis rats	10 μmol/l, 48 h	↓	LC3II↓; Beclin1↓; p62↑; TLR2↓; HMGB↓	(38)
Primary SD rat chondrocytes	0.2 μmol/l, 24 h	↑	LC3II↑; p62↓; mTOR↓; caspase-12↓, DDIT3↓	(39)
IL-1β-treated primary SD rat chondrocytes	0.2 μmol/l, 24 h	↑	Autophagic vacuoles↑; LC3II/LC3I ratio↑; Beclin1↑; p62↓; LC3 puncta↑; apoptosis↓; pro-inflammatory cytokines (TNF-α and IL6) ↓; p-IκBα/IκBα and p-p65/p65↓; nuclear p65↓	(40)

Table I. Continued.

Cell lines	Celastrol dosage and treatment times	Autophagy status	Related biological effects	(Refs.)
Human cervical cancer cell line HeLa cells stably expressing 3XFlag-TFEB	0.25-1 μmol/l, 24 h	↑	LC3II↑; autophagy flux↑; lysosomal contents↑; LAMP1↑; autophagy-lysosome-related genes mRNA (Atg16L1, Atg12, Atp6V1H, CtsB, CtsD, CtsF, LAMP1, GLA, MAP1LC3B, MCOLN1, SQSTM1) ↑; nuclear TFEB↑	(59)
Mouse neuronal cell line N2a transiently expressing P301L Tau	0.05-0.2 μmol/l, 24 h	↑	Nuclear TFEB↑; LC3II↑; autophagy flux↑; p-Tau aggregates↓	(59)
Rotenone-treated human dopaminergic neuronal cell line SH-SY5Y	0.5 μmol/l, 1 h	↑	LC3II/LC3I ratio↑; autophagic vacuoles↑; α-synuclein aggregates↓; SOD and GSH↑; ROS↓; mitochondria membrane potential↑; cytochrome C release↓; mitophagy↑; cell death↓	(41)
α-synuclein-pulsed monocyte derived dendritic cells	0.25 μmol/l, 1 h	↑	Beclin1↑; LC3II/LC3I ratio↑; p62↓; Rab5/Beclin1/α-synuclein and Rab7/LC3/α-synuclein puncta↑; Th1 and Th17↓; Th17/Treg ratio↓	(42)
Human dopaminergic neuronal cell line SH-SY5Y	0.1-3 μmol/l, 2-24 h	↑	MPAK signaling pathways (p-p38, p-ERK1/2, p-Akt1/2/3, p-p65 and p-JNK1/2/3) ↑; LC3I↓; LC3II↑; p62↓; Beclin1↑; Ambra1↑; Vps34↑; Atg7↑; Atg12↑; PINK1↑; DJ-1↑; LRRK2↓; mitophagy↑; MPP+neurotoxicity↓; apoptosis↓	(43)
ox-LDL treated human vascular smooth muscle cells	0.2 μmol/l, 24 h	↑	LC3II/LC3I ratio↑; p62↓; autophagic flux↑; lipid accumulation↓	(45)
Angiotensin II-treated rat vascular smooth muscle cells	0.05 μmol/l, 12 h	↑	LC3 puncta↑; LC3 II↑, p62↓; ROS↓; p-PI3K, p-Akt, p-mTOR and p-p70S6K↓; senescence↓	(46)
PDGF-BB-stimulated human vascular smooth muscle cells	0.05-0.2 μmol/l, 24 h	↑	autophagic vacuoles↑; autophagic flux↑; LC3II/LC3I ratio↑; p62↓, Wnt5a↓, p-PKC↓; p-mTOR↓; c-Myc↓; cell proliferation↓	(47)
H_2O_2-treated human retinal pigment epithelial cell line ARPE-19	0.5-1 μmol/l, 0.5 h	↑	LC3II/LC3I ratio↑; Beclin1↑; SIRT3↑	(48)
CCCP-treated human cervical cancer cell line Hela	10 μmol/l, 1.5-12 h	↓	Parkin mitochondrial recruitment↓; PINK1↓; p-Parkin↓; mitophagy↓; mitochondrial clustering↓; PINK1-TOM20 interaction↓	(60)

↑, upregulation; ↓, downregulation; Atgs, autophagy related genes; LC3, microtubule-associated protein light chain 3; AR, androgen receptor; miRs, microRNAs; ox-LDL, oxidized low-density lipoprotein; mTOR, mammalian target of rapamycin; LXRα, liver X receptor α; ABCA1, ATP-binding cassette transporter A1; Vim, vimentin; MMP2, matrix metalloproteinase 2; ROS, reactive oxygen species; JNK, c-Jun NH2-terminal kinase; Akt, protein kinase B; Bip, glucose-regulated protein 78; PERK, double-stranded RNA-dependent protein kinase (PKR)-like ER kinase; PTEN, phosphatase and tensin homolog; p70S6K, p70 ribosomal S6 kinase; HIF-1α, hypoxia-inducible factor-1α; BNIP3, Bcl2/adenovirus E1B 19kD-interacting protein3; AMPK, AMP-activated protein kinase; Nur77, nuclear receptor-77; CaMKKβ, Ca2+/calmodulin dependent protein kinase kinase β; ER, endoplasmic reticulum; Hsp90, heat shock protein 90; NBR1, neighbor of Brca1 gene 1; CIP2A, cancerous inhibitor of protein phosphatase 2A; DRP1, dynamin-related protein 1; IκBa, NFκB inhibitor α; TNFα, tumor necrosis factor-α; LPS, lipopolysaccharide; IL, interleukin; ASC, apoptosis associated speck-like protein containing a CARD; HO-1, heme oxygenase-1; TLR2, toll-like receptor 2; HMGB1, high mobility group box 1; DDIT3, DNA damage-inducible transcript 3; TFEB, transcription factor EB; SOD, superoxide dismutase; GSH, glutathione; Rab, member RAS oncogene family protein; Th1, T-helper 1; Treg, regulatory T; MPAK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; MPP+, 1-methyl-4-phenylpyridinium; PI3K, phosphatidylinositol 3 kinase; PDGF-BB, platelet-derived growth factor-BB; Wnt, Wingless/integrase 1; PKC, protein kinase C; PINK1, PTEN-induced kinase 1; SIRT3, sirtuin 3; CCCP, carbonyl cyanide m-chlorophenylhydrazone; TOM20, translocase of the outer membrane 20.

Table II. Autophagy modulation by celastrol *in vivo*.

Tissue type	Celastrol dosage and treatment times	Autophagy status	Related biological effects	(Refs.)
Xenografted human clear cell renal cell carcinoma from high-fat diet-fed BALB/c nude mice	1 mg/kg/d, 28 d	↑	Plasma TC, TG, LDL and VLDL↓; LC3↑; ABCA1↑; LXRα↑; E-cadherin↑; Vim↓; MMP2↓; p62↓; tumor growth↓; lipid droplet↓	(50)
Xenografted human glioma from BALB/c-nude mice	2-4 mg/kg/d, 14 d	↑	LC3↑; p-JNK↑; p-Akt↓; p-mTOR↓; tumor growth↓	(22)
Xenografted human osteosarcoma from BALB/c-nude mice	1-2 mg/kg/d, 7 d	↑	LC3II↑; p-JNK↑; tumor volume↓; cell viability↓	(23)
Xenografted pituitary ACTH-secreting adenoma from C57/B6J mice	2 mg/kg/2d, 14 d	↑	LC3II↑; tumor volumes and weights↓; cell viability↓; G0/G1 phase arrest↑	(29)
Xenografted human colorectal cancer from BALB/C nude mice	1.25-2.5 mg/kg/2d, 21 d	↑	LC3II↑; Atg7↑; Nur77↓; tumor growth↓	(30)
Xenografted human lung adenocarcinoma from BALB/C nude mice	1 mg/kg/d, combined with 40 mg/kg/d ellagic acid, 22 d	↑	LC3II↑; CIP2A↓; tumor growth↓	(56)
Xenografted human non-small cell lung cancer from BALB/c nude mice	1 mg/kg/d, combined with 5 mg/kg/d erastin, 21 d	↑	LC3II↑; Parkin↑; tumor volumes and weights↓	(58)
Liver tissue from LPS- and D-GalN-injected C57BL/6 mice	0.2 or 0.5 mg/kg, 12 h	↑	LC3II↑; LC3 puncta↑	(31)
Liver tissue from high-fat diet-fed C57BL/6 mice	0.1 mg/kg/d, 14 d	↑	LC3II↑; LC3 puncta↑	(31)
Serum of LPS-induced septic shock mouse model	1 mg/kg, 4 h	↑	IL-1β↓; NLRP3 inflammasome activity↓	(33)
Colon tissues of DSS-induced colitis mouse model	1.0 mg/kg/d, 7 d	↑	IL-1β↓; caspase-1↓; ASC↓; COX2↓; NLRP3 inflammasome activity↓; IL-1β, IL-6, TNF-α, IL-17A and IFNg mRNA↓; colitis↓	(33)
Colon tissues of dextran sodium sulphate-induced colitis rat model	1 mg/kg/d, combined with 20 mg/kg/d CP-456773, 16 d	↑	Beclin1↑; p62↓; p-AMPKα↑; p-mTOR↓; HSP-90↓; NLRP3 mRNA↓; TNF-α, IL-6, IL-1β and IL-18↓	(32)
Proximal colons of IL-10 deficient mice	2 mg/kg/d, 7 d	↑	LC3II/LC3I ratio↑; LC3 puncta↑; PI3K↓; Akt1↓; mTOR↓; p70S6K↓; colitis↓; proinflammatory cytokines (IL-1β, IL-17A, TNF-α and IFN-γ) and chemokines (CXCL-1 and CXCL-2) ↓	(34)
Renal tissues of diabetic nephropathy rat model	1.5 mg/kg/d, 28 d	↑	LC3II↑; mTOR↓; PI3K↓; Akt mRNA↓; p-Akt↓	(35)
Synovial joints of adjuvant-induced arthritis rat model	1 mg/kg/d, 36 d	↑	Ca^{2+}↑; LC3B↑; Vim↓; arthritic score and hind paw volume↓	(37)
Myocardium of collagen induced rheumatoid arthritis rat model	0.2 mg/kg/d, 6 d	↓	Inflammatory cytokines (TNF-α, IL-6 and IL-1β) ↓; apoptosis↓; Bcl-2↑; Bax↓; cleaved caspase-3↓	(38)
Articular cartilage of anterior cruciate ligament transection SD rat model	0.5-1 mg/kg/d, 84 d	↑	Beclin1↑; p62↓; apoptosis↓; articular cartilage degeneration↓; cleaved caspase-3↓; IL6↓; p-p65↓	(40)
Frontal cortex of C57 mice	1-2 mg/kg/d, 7 d	↑	LC3II↑; LAMP1↑; cathepsin B↑; cathepsin D↑; nuclear TFEB↑	(59)
Brain of P301S tau mice	1-2 mg/kg/d, 7 d	↑	LC3II↑; p62↓; cathepsin B↑; cathepsin D↑; nuclear TFEB↑; p-tau aggregates↓	(59)

Table II. Continued.

Tissue type	Celastrol dosage and treatment times	Autophagy status	Related biological effects	(Refs.)
Brain of 3xTg mice	1, 2 mg/kg/d, 270 d	↑	LC3II↑, p62↓; p-Tau aggregates↓	(59)
Striatum of PD mouse model	3 mg/kg/d, 3 d	↑	PINK1↑; DJ-1↑; dopaminergic nerve terminal degeneration↓	(43)
Lung tissue from bleomycin-induced pulmonary fibrosis male Wistar albino rat model	5 mg/kg/81 h, 28 d	↑	Beclin 1↑; Vps34↑; Atg5↑; Atg7↑; Atg 12↑; Atg16L↑; LC3II↑; Atg3↑; autophagic vacuoles↑; p62↓; PI3K↓, Akt↓; mTOR↓; fibrosis↓	(44)
Injured femoral artery of C57BL/6 mice	2 mg/kg/day, 28 d	↑	Autophagic vacuoles↑; LC3II/LC3I ratio↑; p62↓; Wnt5a↓; p-PKC↓; p-mTOR↓; c-Myc↓; neointimal hyperplasia↓	(47)

↑, upregulation; ↓, downregulation; TC, total cholesterol; TG, triglyceride; LDL, low density lipoprotein; VLDL, very low-density lipoprotein; LC3, microtubule-associated protein light chain 3; LXRα, liver X receptor α; ABCA1, ATP-binding cassette transporter A1; Vim, vimentin; MMP2, matrix metalloproteinase 2; JNK, c-Jun NH2-terminal kinase; Akt, protein kinase B; mTOR, mammalian target of rapamycin; Atgs, autophagy related genes; Nur77, nuclear receptor-77; CIP2A, cancerous inhibitor of protein phosphatase 2A; LPS, lipopolysaccharide; IL, interleukin; NLRP3, NOD-like receptor family pyrin domain containing 3; DSS, dextran sodium sulfate; TNFα, tumor necrosis factor-α; ASC, apoptosis associated speck-like protein containing a CARD; IFN, interferon; AMPK, AMP-activated protein kinase; Hsp90, heat shock protein 90; PI3K, phosphatidylinositol 3 kinase; p70S6K, p70 ribosomal S6 kinase; HIF-1α, hypoxia-inducible factor-1α; TFEB, transcription factor EB; PINK1, phosphatase and tensin homolog-induced kinase 1; Wnt, Wingless/integrase 1; PKC, protein kinase C.

cell type-dependent manner (61). In prostate cancer cells, celastrol treatment suppressed cell viability and upregulated autophagic activity, which was indicated by elevated Atg5 and Atg7 expression, increased autophagosome formation and promoted degradation of the canonical autophagy substrate p62. However, autophagy impairment significantly potentiated the cytotoxic effects of celastrol. These results demonstrate that autophagy is a cytoprotective mechanism for prostate cancer cell survival (19,49). Of note, a celastrol pyrazine derivative (11i) displayed potent anti-breast cancer activity and induced remarkable autophagy, whereas autophagy blockage markedly increased breast cancer cell viability, suggesting that induced autophagy may serve a pro-death function (20). As a defining morphological hallmark of clear cell renal cell carcinoma, excessive cytoplasmic lipid droplets were selectively delivered for lysosomal degradation via celastrol-induced lipophagy, thereby markedly facilitating cholesterol efflux, impairing the epithelial-mesenchymal transition process and finally contributing to the inhibition of clear cell renal cell carcinoma cell migration, invasion and tumor growth (50).

Through interference with both autophagic and proteasomal protein degradation quality-control pathways, celastrol promoted the accumulation of polyubiquitinated aggregates as well as p62 and exerted widespread proteotoxicity via a thiol-dependent mechanism in glioblastoma cells (21). However, the exposure of glioma cells to celastrol markedly increased LC3 puncta formation and improved LC3 and Beclin1 expression, while it also unexpectedly upregulated p62 expression. Application of autophagy inhibitor moderately reinforced the inhibitory effect of celastrol on cell viability. These results suggest that celastrol may induce autophagosome accumulation, accompanied by the partial blockage of lysosomal degradation function, and that celastrol-mediated autophagy has a role in promoting cell survival. In addition, celastrol simultaneously induced apoptosis in glioma cells, whereas apoptosis suppression strengthened autophagy and *vice versa*. Hence, the relationship between autophagy and apoptosis caused by celastrol may be antagonistic in glioma cells (22). Celastrol also triggered both autophagy and apoptosis in osteosarcoma cell lines and primary cells. Celastrol-induced apoptosis was moderately diminished by an autophagy inhibitor, whereas apoptosis blockage rendered the cells particularly susceptible to autophagic death. This observation indicates that autophagy aids the pro-death function and that the cells may switch between the two responses following celastrol treatment (23,24). Similarly, autophagy and apoptosis were simultaneously induced by celastrol in pancreatic ductal adenocarcinoma cells (25), hepatocellular carcinoma cells (26,27), gastric carcinoma cells (28), pituitary adrenocorticotropic hormone-secreting adenoma cells (29), and colorectal cancer cells (30). However, the celastrol dosage required to stimulate autophagy was lower than that for the apoptotic threshold in hepatocellular carcinoma cells, and autophagy occurred at an earlier phase than apoptosis (27). The celastrol-induced apoptosis in pancreatic cancer cells and colorectal cancer cells was upgraded with autophagy inhibition, which enhanced the therapeutic effect of celastrol. Thus, autophagy may be activated after celastrol treatment as an adaptive mechanism for cell survival to protect against apoptosis (25,30). However, autophagy induction by celastrol treatment likely sensitized the gastric cancer cells to apoptosis, thereby distinctly inhibiting cell proliferation and migration (28). Although the relationship between celastrol-induced autophagy and apoptosis was not fully addressed, autophagy and apoptosis may act synergistically to increase pituitary adenoma cell mortality rates (29).

Multidrug resistance (MDR) frequently develops in certain tumor cells after repeated chemotherapy treatments

and MDR tumor cells exhibit apparent insensitivity toward drug-induced apoptosis. Celastrol has the potency to effectively induce autophagic cell death as an alternative cell death mechanism, re-sensitize resistant cancer cells or produce a synergistic effect in combination with chemotherapeutic agents by either stimulating or inhibiting autophagy for the treatment of MDR cancer (51,53-55). Taxol-resistant cervical cancer cells displayed an Atg7-dependent increased autophagosome formation and enhanced autophagic flux on celastrol treatment. As autophagy inhibition completely abolished the celastrol-mediated cytotoxicity, it is indicated that autophagic cell death was ultimately induced. Concomitantly, celastrol produced obvious collateral sensitivity of MDR cells and reversed the taxol-resistant phenotype *in vitro*, as well as suppressed tumor growth and metastasis *in vivo* (51). In addition, another study indicated that treatment with celastrol simultaneously induced autophagy accompanied by apoptosis and an atypical cell death mode termed 'paraptosis' in cervical cancer cells, which was morphologically characterized by extensive cytoplasmic vacuolization derived from dilated ER and mitochondria (62). However, the fact that autophagy inhibition significantly enhanced rather than prevented paraptosis confirmed that autophagy was possibly a survival mechanism in celastrol-treated cells (52). In afatinib-resistant lung cancer cells, the combination of afatinib and celastrol impaired autophagic activity by inhibiting lysosomal enzymatic activities, leading to increased expression of autophagy substrates, such as LC3II, p62 and NBR1. Of note, the combination induced paraptosis and a subsequent cell death phenomenon, indicating a potential interdependent and interactive relationship (53). Autophagy flux suppression mediated by celastrol also restored sensitivity to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in TRAIL-resistant lung cancer cells. A combined regimen of celastrol and TRAIL markedly promoted the cytotoxic effects of TRAIL, which in turn yielded an improved outcome relative to that with celastrol or TRAIL treatment alone (54). On the contrary, celastrol was observed to act as an autophagy inducer to promote oncogenic epidermal growth factor receptor protein elimination, which is responsible for activating downstream cell survival signaling, thereby combating gefitinib-resistant lung cancer cells by inducing apoptosis (55). Furthermore, cotreatment with celastrol potentiated the inhibitory effects of ellagic acid against lung cancer cell proliferation and tumor growth by elevating autophagic cell death (56). Celastrol plus the ERα antagonist tamoxifen markedly upregulated the apoptotic rate when compared with tamoxifen alone in ER-positive breast cancer cells. Furthermore, increased protein expression of LC3II and decreased p62 levels were recorded, indicating an enhancement of autophagy. Although the relationship between increased apoptosis and induced autophagy by the combination treatment was not addressed, either a collaboration or a backup mechanism seems probable (57). In addition, cotreatment with celastrol and erastin, a ferroptosis inducer, initiated Atg5/Atg7-mediated autophagy, as well as significantly enhanced the expression and mitochondrial translocation of PINK1 and Parkin, thereby markedly inducing mitochondrial degradation by selective mitophagy and exerting a powerful anti-lung cancer effect (58).

Collectively, these findings provided substantial evidence that autophagy is a target of action of celastrol and that altered autophagic activities contribute to the anti-tumor properties of celastrol.

Autophagy is involved in the anti-inflammatory and immunomodulatory activities of celastrol. Regarding the anti-inflammatory functions, celastrol has the ability to impair B-cell development and suppress T-cell proliferation and T-helper 17-cell differentiation, while facilitating the generation of regulatory T cells (63,64). A connection between autophagy and anti-inflammatory activities of celastrol has been determined, as autophagy may affect the systemic inflammatory responses by modulating inflammatory cytokine secretion or directly removing the exogenous and endogenous inflammation sources, such as bacterial pathogens, protein aggregates or damaged organelles (65). Celastrol reversed the pathological states associated with inflammatory diseases, including acute hepatic inflammatory injury and obesity, by specifically triggering mitophagy (31). The damaged mitochondria release reactive oxygen species (ROS) and function as an important inflammatory source that initiates the assembly and activation of the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasomes, a high molecular protein complex consisting of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1, and is implicated in several inflammatory disorders (66). In lipopolysaccharide (LPS)-primed murine peritoneal macrophages, pretreatment with celastrol markedly promoted autophagy, thereby attenuating NLRP3 inflammasome activation by blocking ASC oligomerization and NLRP3-ASC interaction and subsequently interrupting interleukin (IL)-1β secretion and caspase-1 activation in a dose-dependent manner. *In vivo* experiments have demonstrated that autophagy inhibition ameliorated celastrol-mediated NLRP3 inflammasome suppression in LPS-induced septic shock or dextran sodium sulfate (DSS)-induced colitis mouse models, thereby indicating that autophagy may be responsible for the protection against NLRP3-related diseases exerted by celastrol (33). Another study validated that the dual administration of celastrol and NLRP3 inhibitor CP-456773 markedly inhibited Hsp90 expression to couple with NLRP3, which resulted in autophagy induction to increase NLRP3 degradation, which decreased caspase-1 activation and IL-1β and IL-18 release, restrained the pyroptosis process and provided significant therapeutic benefits for DSS-induced colon injury (32). Celastrol also markedly inhibited colonic proinflammatory cytokine and chemokine production, and decreased neutrophil infiltration of proximal colon tissues during experimental colitis in IL-10-deficient mice. The amelioration of inflammation was attributed to the upregulation of intestinal autophagy (34).

Podocyte injury is a primary feature of diabetic nephropathy and a critical role of the inflammatory mechanism was identified in driving the loss of kidney function during disease progression (67). With autophagy deficiency observed in high glucose-administered podocytes, as well as in the renal tissues of diabetic rats, it has been suggested that autophagy is implicated in the pathogenesis of diabetic nephropathy and is a potential therapeutic target for diabetic nephropathy treatment (35,36). Indeed, celastrol treatment restored blunted

autophagy and antagonized high glucose-invoked pro-inflammatory cytokine secretion, insulin resistance and cytotoxic damage in podocytes. However, the protective effect of celastrol on podocytes was abrogated when the autophagy pathway was inhibited (36). It was found that celastrol consistently and effectively promoted autophagy in diabetic nephropathy rats, thereby protecting them from glomerular basement membrane thickening and significantly relieving renal injury (35).

Rheumatoid arthritis is a chronic, progressive inflammatory autoimmune disease that predominantly affects the synovial joints (68). Of note, autophagy has a significant role in the mechanism of the anti-arthritis properties of celastrol. In fact, celastrol treatment triggered autophagic cell death to eliminate synovial fibroblast over-proliferation through the enhancement of autophagy flux, as it did in apoptosis-resistant fibroblasts. In the adjuvant-induced arthritis rat model, celastrol administration significantly induced autophagy, suppressed the proliferation and epithelial-mesenchymal transition of fibroblasts, downregulated a panel of inflammatory- and autoimmunity-associated genes and ameliorated the arthritis phenotype in the joint tissues (37). Evidence suggests that patients with rheumatoid arthritis have a higher cardiovascular disease risk than the general population and that systemic inflammation is closely linked to cardiac dysfunction (69). Autophagy was significantly upregulated in cultured primary cardiomyocytes as well as myocardial tissues of collagen-induced rheumatoid arthritis rat models, whereas celastrol treatment simultaneously ameliorated cardiomyocyte injury, suppressed the expression of inflammatory cytokines and inhibited cardiomyocyte autophagy at the same time. However, the autophagy inducer rapamycin attenuated the cardioprotective effect of celastrol toward rheumatoid arthritis-induced cardiotoxicity, highlighting that celastrol-mediated autophagy suppression accounts for the alleviation of cardiomyocyte injury (38). Osteoarthritis is another most common type of arthritis, mainly characterized by progressive articular cartilage degeneration. During this process, large amounts of inflammatory cytokines, such as IL-1β, are released into the osteoarthritis cartilage, which induces chondrocyte apoptosis (70). Autophagy deficiency is always accompanied by an increase in apoptotic chondrocytes, whereas celastrol treatment resulted in obvious reversal effects (39). By restoring autophagy activation, celastrol inhibited the IL-1β-stimulated inflammatory response, counteracted chondrocyte apoptosis and achieved a promising therapeutic effect against osteoarthritis *in vitro* and *in vivo* (40).

Taken together, these results suggested that autophagy regulation is a specific mechanism for celastrol to confer its anti-inflammatory and immunomodulatory effects.

Autophagy is involved in the neuroprotective activities of celastrol. As the two most common neurodegenerative diseases, Alzheimer's disease (AD) and Parkinson's disease (PD) are frequently pathologically defined by the abnormal accumulation of misfolded tau or α-synuclein protein aggregates in the affected neurons, respectively, which probably arises from autophagy impairment to a certain extent (71). Although much controversy remains regarding the role of these aggregates, it is well recognized that their appearance may alter neuronal function, cause neurotoxicity and ultimately lead to cell death (72). The accelerated autophagic removal of related toxic aggregates may potentially mitigate disease severity, considering that autophagy controls the degradation of protein aggregates and that autophagy failure represents the primary underlying pathology of neurodegenerative diseases. In a mouse model of AD, improved cognitive deficits and reduction of hyperphosphorylated tau aggregate in the brain tissues were observed after the oral administration of celastrol, which reveals the essential role of autophagy in its anti-AD effect. Furthermore, celastrol treatment specifically promoted autophagy flux and lysosomal biogenesis, accompanied by hyperphosphorylated Tau aggregate degradation in neuronal cell lines, exhibiting a significant increase in the expression of multiple autophagic and lysosomal associated genes. Of note, inhibition of autophagy and lysosomal pathways compromised celastrol-mediated tau reduction, which further verifies that autophagy is required for decreased hyperphosphorylated tau levels in response to celastrol (59). Similarly, celastrol activated autophagic pathways in a dose- and time-dependent pattern and markedly protected dopaminergic neurons from cell injury, with a particular focus on reducing rotenone-induced α-synuclein aggregation in a cellular model of rotenone-induced PD (41). In addition to inducing autophagy with upregulated Beclin1 expression, decreased p62 expression and enhanced LC3II conversion, celastrol was able to promote the interaction between autophagic pathway components and fibrillar α-synuclein for modulating the trafficking pathways in α-synuclein-pulsed monocyte-derived dendritic cells, which possibly facilitated α-synuclein processing, thereby minimizing the availability of antigenic peptides for antigen presentation and counteracting the specific T-cell immune response to α-synuclein in PD treatment (42). Mitophagy induction is probably another pathway of the neuroprotective effect of celastrol in PD. By clearing the dysfunctional mitochondria for quality control, celastrol was able to prevent the release of ROS and apoptosis-promoting factors caused by rotenone or 1-methyl-4-phenylpyridinium, thereby ameliorating oxidative stress and further blocking cell death (41,43). PD mouse model studies further confirmed the pivotal role of mitophagy in improving motor symptoms and diminishing dopaminergic neuronal degeneration (43).

Overall, these findings have demonstrated that the neuroprotective effects of celastrol are intimately associated with autophagy modulation.

Autophagy is involved in other pharmacological activities of celastrol. Overexpression of p62 protein in lung tissues represents insufficient autophagy during severe pulmonary fibrotic responses (73). Of note, celastrol was proven to be effective in the treatment of bleomycin-induced experimental rat pulmonary fibrosis via the induction of protective autophagy. Celastrol treatment significantly elicited the initiation, elongation and maturation of autophagosomes, resulting in enhanced p62 degradation, thereby reducing collagen deposition and slowing down pulmonary fibrosis progression (44).

Celastrol serves an anti-atherosclerosis role by triggering autophagy in vascular smooth muscle cells (VSMCs). On the one hand, celastrol-mediated autophagy significantly impeded lipid storage induced by oxidized low-density lipoprotein in VSMCs, thereby inhibiting VSMC-derived foam cell formation

and the resultant atherosclerotic plaque development (45). On the other hand, celastrol-stimulated autophagy functioned as an intracellular ROS scavenging system to specifically inhibit angiotensin II-mediated ROS production, which ultimately counteracted VSMC senescence (46). Furthermore, autophagy was found to be involved in alleviating the overgrowth and migration of VSMCs by celastrol. Celastrol treatment markedly activated VSMC autophagy in both *in vitro* and *in vivo* intimal hyperplasia models, as manifested by accumulated autophagosomes, promoted autophagic flux, increased LC3II/LC3I ratio and decreased p62 expression (47). C-myc, a key transcription factor that induces the continuous proliferation of VSMCs, combined with p62 to form a conjugate during celastrol-induced autophagy, which was then delivered to lysosomes for degradation, and thus exerted inhibitory effects on abnormal VSMC proliferation and vascular neointimal hyperplasia (47).

In a human retinal pigment epithelial cell line, incubation with hydrogen peroxide led to a significant induction of apoptosis and oxidative stress, as well as the inhibition of cell survival and autophagy, whereas this phenomenon was obviously antagonized upon celastrol cotreatment. Celastrol-induced autophagy markedly impeded ROS production and protected against hydrogen peroxide-induced cell damage by increasing the LC3II/LC3I ratio and Beclin1 expression, suggesting the therapeutic potential of celastrol in age-related macular degeneration via autophagy modulation (48).

Therefore, these findings confirm that autophagy modulation acts as a crucial pathway for celastrol to alleviate the pathogenic symptoms of multiple diseases, such as pulmonary fibrosis, atherosclerosis, neointimal hyperplasia and macular degeneration.

4. Mechanisms underlying autophagy modulation by celastrol

The autophagy regulation by celastrol includes epigenetic pathways through a series of microRNAs (miRNAs/miRs), which are short non-coding RNAs that bind to specific mRNAs, leading to their degradation or translational repression (74). By negatively regulating the expression of autophagy-related genes or regulators, such as Atg4D, Rab5A and Stathmin1, miR-101 functions as a potent autophagy initiation and maturation inhibitor (75). Decreased miR-101 expression was confirmed upon celastrol treatment, which rescued the suppressive effect on the dynamic process of autophagy in prostate cancer cells (19). Analogously, celastrol treatment led to downregulation of two miR-17-92a cluster members, miR-20a and miR-17a, which targeted Atg7 to inhibit autophagy, resulting in autophagy induction in prostate cancer cells (49). Furthermore, the ligand-regulated transcription factor androgen receptor (AR) that selectively transactivates the miR-101 and miR-17-92a cluster was degraded in the same sample mentioned above during celastrol-induced autophagy and AR knockdown decreased, whereas the ectopic expression of AR enhanced the expression of these miRNAs in the presence of celastrol (19,49). Therefore, downregulation of AR caused by celastrol was proposed to mediate the transcription reduction of its downstream target miRNAs, which subsequently abolished the negative regulation of autophagy, leading to autophagy activation.

Celastrol may regulate autophagy via a mechanism involving transcription factor EB (TFEB). TFEB, the transcription factor that binds to a promoter motif coordinating the transcription of multiple autophagic and lysosomal genes, is known as the key regulator of the autophagy pathway (76). Celastrol increased the nuclear translocation of TFEB from the cytosol in a neuronal cell line and in mouse brains. The participation of the mammalian target of rapamycin complex 1 (mTORC1) in the activation of TFEB by celastrol has been characterized. By inhibiting the activity of mTORC1 kinase, celastrol inhibited the phosphorylation of TFEB that caused the dissociation of TFEB with the tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein and the rapid transport of TFEB to the nucleus, consequently enhancing autophagy and lysosomal biogenesis (59,77). In the mTORC1, the highly conserved serine/threonine kinase mTOR is the central catalytic subunit of the multiprotein complex and may phosphorylate multiple autophagy proteins, which results in a blockade of autophagy-initiating kinase ULK1 and functions as a negative autophagy mediator (78). Transcriptomics data and network pharmacology analysis predicted that mTOR is the direct therapeutic target of celastrol against osteoarthritis (79), and a subsequent study validated that the promotion of chondrocyte autophagy by celastrol occurs as a result of the reduction in mTOR levels (39). In addition, numerous signaling pathways converging at mTOR involve celastrol-mediated autophagy. After treatment with celastrol, AMPK was phosphorylated by the upstream kinases, including liver kinase B1 complex, Ca^{2+}/calmodulin dependent protein kinase kinase 2 (CaMKK2) and TGF-β-activated kinase 1, leading to its activation and the subsequent inhibition of the phosphorylation of the downstream target mTOR, resulting in increased autophagic activity in gastric cancer cells and gastric tumors of xenografted mice (28,80). AMPK/mTOR pathway activation was also implicated in the pro-autophagic effects of the celastrol and CP-456773 combination on the colon tissues of the rat colitis model, as both increased phosphorylated AMPK and decreased phosphorylated mTOR were observed in comparison to the control (32). Furthermore, the effect of celastrol on autophagy is wingless/integrase 1 (Wnt)5α-dependent, which is a secretory glycoprotein belonging to the Wnt family. Celastrol significantly reduced Wnt5α expression and attenuated the phosphorylation and activation of protein kinase C and mTOR, thereby promoting autophagy of VSMCs (47). The activated form of AKT, phosphorylated AKT, was significantly suppressed in pituitary adenoma cells, breast cancer cells and glioma cells exposed to celastrol, which resulted in decreased expression of phosphorylated mTOR and induction of autophagy (22,29,57). However, in bleomycin-induced rat lung tissues, IL-10-deficient mouse colon tissues and diabetic nephropathy rat renal tissues, celastrol treatment reduced the expression of PI3K along with its downstream effectors, AKT and mTOR, and upregulated autophagy, suggesting that inactivation of the PI3K/AKT/mTOR pathway may represent a mechanism through which celastrol induces autophagy (34,35,44).

ROS may act as crucial upstream intracellular transducers that sustain autophagy (81). Previous evidence indicates that sirtuin 3 (SIRT3), a histone/protein deacetylase, contributes to the effect of celastrol on autophagy through the modulation

of ROS production. Celastrol treatment markedly promoted autophagy and impeded the expression of SIRT3 and ROS production in hydrogen peroxide-treated retinal pigment epithelial cells, whereas SIRT3 depletion reversed the effects of celastrol on ROS production and autophagy (48). The autophagy regulation by celastrol was also proceeded via the ROS/c-Jun N-terminal kinase (JNK) signaling pathway. A sharp decrease in the mitochondrial membrane potential due to mitochondrial dysfunction occurred after glioma and osteosarcoma cells were exposed to celastrol, which promoted the generation of an excessive amount of ROS. ROS phosphorylated the stress-activated signaling molecule JNK that positively regulated autophagy and mediated autophagy activation (22,23). Celastrol stimulated hepatocarcinoma cell autophagy by ROS to activate AKT/p70 ribosomal S6 kinase signaling, which further promoted hypoxia-inducible factor-1α (HIF-1α) translation and sequentially elevated the expression of the HIF-1α-target gene Bcl2/adenovirus E1B 19kD-interacting protein 3 (27). The damage to mitochondria in lung cancer cells was markedly augmented after cotreatment with celastrol and erastin, which resulted in marked ROS accumulation, followed by autophagy upregulation. In the meantime, high levels of ROS enhanced p38 phosphorylation and increased its interaction with dynamin-related protein 1, a protein that mediated mitochondrial fission, thereby promoting mitophagy induction (58).

In addition to ROS accumulation, increased cytoplasmic calcium release from the internal stores appeared in osteosarcoma cells and hepatocarcinoma cells following treatment with celastrol, with ER stress induction and subsequent unfolded protein response signaling activation to induce the autophagy-related protein expression and the assembly of autophagic structures (24,26). Celastrol also stimulated calcium-mediated autophagy via the activation of the CaMKKβ-AMPK-mTOR signaling cascade in cervical cancer cells and synovial fibroblasts. The calcium homeostasis perturbation by celastrol may be ascribed to its inhibitory activity toward the sarcoplasmic reticulum (SR)/ER Ca^{2+}-ATPase pump, which transports calcium ions back into the SR/ER lumen from the cytoplasm and is necessary for the release of calcium into the cytosol (37,51).

Other regulatory pathways of autophagy in response to celastrol have been explored. Celastrol obviously enhanced heme oxygenase-1 expression in podocytes under high-glucose conditions and antagonized high glucose-induced autophagy pathway deficiency (36). Celastrol-triggered autophagy in colorectal cancer cells was associated with the decreased expression of nuclear receptor-77 (Nur77), a transcription factor in the nucleus negatively regulating the transcription of Atg7 which was essential for autophagosome formation (30). Another study demonstrated that celastrol promoted the mitochondrial translocation of Nur77 and its ubiquitination by E3 ubiquitin ligase, named tumor necrosis factor receptor-associated factor 2. The subsequent interaction between Nur77 and p62 served to prime the ubiquitin-labeled mitochondria for autophagic degradation (31). However, celastrol was also identified as a mitophagy inhibitor in response to mitochondrial damage by blocking Parkin recruitment into the mitochondria and simultaneously by disrupting the association between PINK1 and TOM20, a component of the translocase of the outer mitochondrial membrane machinery (60).

In VSMCs and clear cell renal cell carcinoma cells, celastrol-mediated upregulation of autophagy involved the upstream activation of transcription factor liver X receptor α (LXRα), as LXRα reduction reversed the promotion of autophagic flux induced by celastrol (45,50). In addition, the participation of the toll-like receptor 2 (TLR2)/high mobility group box 1 (HMGB1) signaling pathway in the regulation of autophagy by celastrol has been confirmed. Through the attenuation of TLR2 and HMGB1 protein expression, celastrol was able to suppress rheumatoid arthritis-induced autophagy in cardiomyocytes (38). Overall, these results demonstrated the complexity through which celastrol regulated autophagy via multiple targets or signaling pathways (Fig. 1).

5. Conclusion and future perspectives

Celastrol has been regarded as one of the bioactive components identified from certain traditional Chinese herbs that is most likely to be developed as a modern drug thanks to its multiple biological activities and high efficacy in animal and cell culture disease models. Hence, elucidating the molecular mechanisms behind the action of celastrol is in high demand. Studies have indicated that celastrol alters autophagic activities through diverse mechanisms, which take part in an intricate network that modulates the crosstalk and interplay with other metabolic processes, highlighting that autophagy may be a specific action target for celastrol to accomplish its multiple pharmacological activities. Based on the available data, celastrol mostly functions as an autophagy inducer, except that celastrol was observed to suppress autophagy in lung cancer cells (53,54), glioblastoma cells (21), cervical cancer cells (60) and cardiomyocytes (38). This discrepancy may be explained by different cell phenotypes, pathological status and the particular stimulus of celastrol. To date, studies have mainly addressed the effects of celastrol on autophagy in certain cellular models with pharmacologic activators/inhibitors such as rapamycin, bafilomycin A1 and 3-methyladenine; however, more specific gene deletion or overexpression methods may confirm whether autophagy is the main mechanism of celastrol activity. Furthermore, the lack of relevant *in vivo* data has limited the determination of the exact role of autophagy. The systemic evaluation of the *in vivo* activities will further validate the conclusions. Furthermore, whether autophagy is implicated in other pharmacological activities of celastrol remains elusive. Although autophagy is a general house-keeping process to maintain cellular homeostasis, how to improve the specific targeting and selectivity of celastrol to pathological tissues while minimizing undesired negative effects on normal tissues will be a major issue when using celastrol as an autophagy modulator.

The ubiquitin-proteasome system and autophagy comprise the main proteolytic mechanisms in eukaryotic cells (82). The inhibitory effect of celastrol on the chymotrypsin-like activity of the purified 20S proteasome and 26S proteasome has been confirmed in several types of cultured tumor cells and tumor tissues (83). Autophagy is compensatorily activated with the inhibition of the ubiquitin-proteasome system, whereas the proteasomal flux is impaired if autophagy is

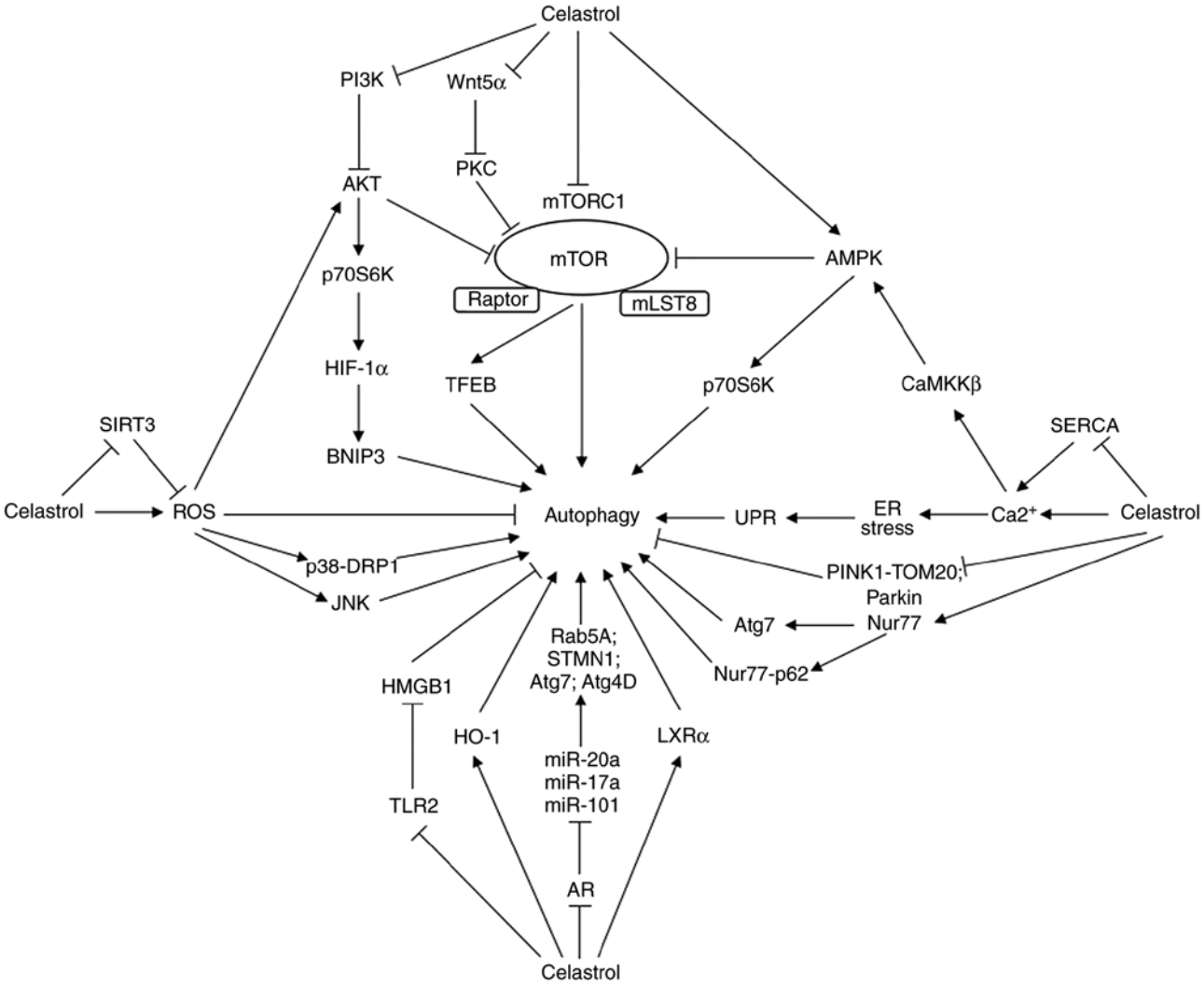

Figure 1. Schematic diagram depicting the potential mechanisms underlying celastrol modulating autophagy. Through either positive or negative regulation of a variety of effectors, including AR, mTOR, ROS, Ca^{2+}, Nur77, LXRα, TLR2, HO-1, Parkin and PINK1, celastrol influences the downstream molecular machineries or signaling pathways, resulting in autophagy activation or suppression. AR, androgen receptor; miRs, microRNAs; Rab, member RAS oncogene family protein; STMN1, stathmin1; Atg, autophagy-related gene; HO-1, heme oxygenase-1; TLR2, toll-like receptor 2; HMGB1, high mobility group box 1; ROS, reactive oxygen species; SIRT3, sirtuin 3; DRP1, dynamin-related protein 1; JNK, c-Jun NH2-terminal kinase; mTORC1, mammalian target of rapamycin complex 1; mLST8, mammalian target of rapamycin associated protein; Wnt, Wingless/integrase 1; PKC, protein kinase C; TFEB, transcription factor EB; PI3K, phosphatidylinositol 3 kinase; Akt, protein kinase B; p70S6K, p70 ribosomal S6 kinase; HIF-1α, hypoxia-inducible factor-1α; BNIP3, Bcl2/adenovirus E1B 19kD-interacting protein3; AMPK, AMP-activated protein kinase; Ca^{2+}, calcium; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; CaMKKβ, Ca^{2+}/calmodulin dependent protein kinase kinase β; ER, endoplasmic reticulum; UPR, unfolded protein response; PINK1, phosphatase and tensin homolog-induced kinase 1; TOM20, translocase of the outer membrane 20; Nur77, nuclear receptor-77; LXRα, liver X receptor α.

disrupted (84). Prospective studies are required to clarify the presence of causality between the regulation of autophagy and the ubiquitin-proteasome system mediated by celastrol, as well as the possible molecular machinery involved. A common feature of these investigations is the focus on the regulatory activities on macroautophagy, while the impact on the other two forms of autophagy has remained largely elusive. Questions still remain as to whether microphagy or chaperone-mediated autophagy directly have a functional role in response to celastrol.

In conclusion, celastrol possesses potent autophagic-regulatory effects with a broad potential pharmacological utility. In addition, celastrol may serve as a promising agent for studying autophagy. Further advances in unveiling the detailed molecular mechanisms and the specific functions of celastrol-mediated autophagy may provide effective strategies for drug development, as well as valuable therapeutic approaches for the treatment of certain autophagy-related diseases.

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Authors' contributions

YW conceived and supervised the present study. CZ prepared the first draft of the manuscript. WW, CD, HL, KZ, ZL, YC and SL reviewed the manuscript for important intellectual content. All authors have read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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