

Autophagic Effects of Bisbenzylisoquinoline Alkaloid of Neferine: A Mechanistic View

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Received: September 04, 2024

Revised: September 10, 2024

Accepted: September 20, 2024

ePublished : December 10, 2024

Abstract

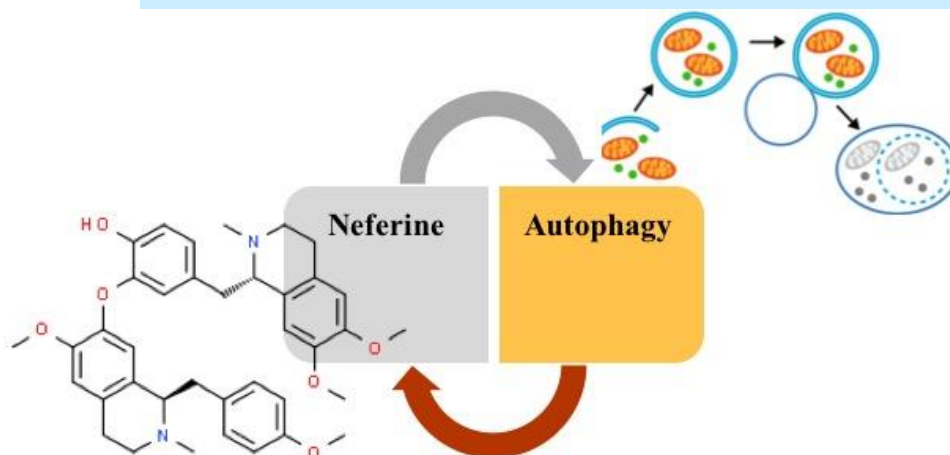
Background: Neferine is known as one of the major bioactive alkaloids. It is present in the seed embryo of *Nelumbo Nucifera*, which belongs to the family of bisbenzylisoquinoline alkaloids. This review article aimed to discuss the influences and benefits of Neferine administration through the autophagy process in order to provide a comprehensive overview of the autophagy benefits of Neferine on different medical conditions.

Materials and Methods: This review was performed on *in vivo* and *in vitro* research focused on autophagic features of Neferine on a variety of medical conditions, disorders, and malignancies.

Results: Due to its widespread therapeutic and bioactive features, Neferine could be regarded as a promising and applicable therapeutic choice. Neferine supplementation alleviates and attenuates the undesired and adverse effects of anticancer drugs against normal healthy cells, while potentially enhancing their therapeutic effects against cancerous cells. Additionally, Neferine augments the activity of detoxifying enzymes and may diminish the detrimental effects of toxic substances. Cytotoxic and cytoprotective effects of Neferine depend on incubation time, dose, and type of cells.

Conclusion: Neferine exerts context-dependent bidirectional effects, with its protective/toxic features being mediated through activation or inhibition of the autophagy process in a cell- and situation-dependent manner.

Keywords: Autophagy, Bisbenzylisoquinoline alkaloid, Cytoprotective, Cytotoxic, Neferine



Graphical Abstract



Introduction

Autophagy explicitly refers to a self-eating procedure at the subcellular level, which has been recognized for many years and has attracted increasing attention in recent years. Autophagy is known as an evolutionarily conserved catabolic process through which cells decompose cytosolic aggregated proteins and damaged organelles. Cells turn over their own required and essential constituents, such as amino acids, lipids, carbohydrates, and other essential components, through autophagy induction (1-4).

Autophagy is a cellular mechanism traditionally regarded as a degradative process; however, it is more accurately described as a recycling pathway because of its crucial role in maintaining cellular homeostasis. Through autophagy, essential cellular components are recycled and reused as sources of energy and building blocks for the synthesis and renewal of macromolecules, including proteins, lipids, and carbohydrates (5). Indeed, the autophagy process can be defined as a type II programmed cell death, which is an innovative, intelligent, and tunable cell death process in such a way that is fundamentally distinct from the apoptosis process (6).

The autophagy process is characterized by the formation of double-membrane structures, known as autophagosomes, within which the process of protein breakdown, degradation of cytosolic components, and damaged cellular organelles take place (7). Establishment of autophagosome/autolysosome and ATG8/LC3 vacuole transposition are regarded as the prominent morphological hallmarks of autophagy induction (8). Autophagy, by degrading aggregated proteins and eliminating damaged molecules, plays a strategic role and facilitates control of cellular renovation, and also provokes energy yield by enhancing ATP production (9,10).

Autophagy occurs, at least at basic levels, in virtually all cell types from prokaryotes to eukaryotes and is regarded as a vital regulator of cellular homeostasis. For example, whenever cells are exposed to a series of stressful situations, like infectious conditions, oxidative stress, starvation, growth factor deprivation, and even at the time of structural remodeling, autophagy generally

upregulates (11). Cumulatively, there are three main distinct, well-defined kinds of autophagy processes, namely macro-autophagy, micro-autophagy, and chaperone-mediated autophagy.

All of these autophagy methods can stimulate lysosomal sequestration and decomposition of protein constituents and damaged cytosolic organelles in cells. Macro-autophagy is a process in which cytoplasmic consignments are transferred to the lysosome via a double-membrane structure, known as autophagosome degradation by lysosomal enzymes. Macro-autophagy can be more precisely classified into two main types of autophagy, namely selective and non-selective autophagy. Selective autophagy consists of ribophagy, pexophagy, mitophagy, and xenophagy. Mitophagy is also known as the process in which the selective removal of damaged mitochondria is performed (12,13). In contrast to macro-autophagy, micro-autophagy is another type of autophagy in which cellular constituents are directly picked up by the lysosomes through their membrane introversion. Huge constructions can be engulfed through macro and micro-autophagy by either selective and non-selective apparatuses (14).

During chaperone-mediated autophagy, selected cytosolic proteins are recognized by heat-shock cognate protein (HSC-70) and delivered to the lysosomal membrane, where they interact with lysosome-associated membrane protein 2A (LAMP-2A). This interaction initiates the breakdown and sequestration of proteins, followed by their incorporation into the cells (9,15).

The autophagy process comprises three distinct and major stages; the first one is the establishment of the autophagosome and transfection of cytosolic components and proteins into the vacuole structures. This procedure contains three fundamental steps: 1) initiation, which consists of phagophore establishment; 2) nucleation, known as the stage of engulfment of cytosolic components and proteins by the phagophore; 3) elongation/enclosure, the process in which the membrane layer is expanded and fused to develop the autophagosome.

The second stage comprises creation of the autolysosome compartment followed by combination and conjunction of the external membrane of the autophagosome with the lysosome.

The third stage involves sequestration and breakdown of cytosolic components, protein inclusions, and damaged cellular organelles (6,16). To date, more than approximately 30 autophagy-related genes (ATGs) have been identified. These genes are evolutionarily well-preserved in eukaryotes, from yeasts to mammals, which primarily lead to the development and establishment of applicative complexes acting as determinants and key compartments in modulating autophagy molecular signaling pathways (13).

Establishment of the phagophore, the autophagosomal originator, is initiated by the stimulation of class III phosphoinositide-3 kinase, which includes Beclin-1, Atg14, and vps15, all of which are prominent regulators of the autophagy process. A series of proteins, including Atg5, Atg12, Atg16, and focal adhesion kinase (FAK), play a key role in the primary steps of autophagy induction in combination with Atg1 and Atg13. One of the vital and determinant factors in the development of the autophagosome is the expansion of the membrane layer, which contains two reactions similar to the ubiquitination reaction.

Moreover, Atg7 mediates the intermixing of Atg12 with Atg5, which is identical to the E1-ubiquitin-stimulating enzyme. Likewise, Atg10 is known to be similar to an E2-ubiquitin-stimulating enzyme (17). Another ubiquitin-like organization that participates in autophagosome establishment is the LC3B protein (microtubule-associated protein light chain 3), encoded by the mammalian homologue of Atg8. The LC3B presents as a cytosolic protein in almost all kinds of cells, which is proteolytically broken down by Atg4 (cysteine protease) in order to produce LC3B-I. The carboxyterminal glycine is already cleaved by Atg4, then stimulated in an ATP-dependent way through E1-like Atg7. Subsequently, stimulated LC3B-I is delivered to the Atg3 protein (E2-like carrier protein) before conjugation with phosphatidylethanolamine and finally creates the complete LC3B-II protein.

The LC3B-II is present on both the external and internal sides of the autophagosome membrane, where it determines marked consignment for

decomposition and stimulates the membrane hemifusion process. Status levels of LC3 protein are regarded as the prominent hallmark of cell autophagy induction. The LC3B-II protein presents as a 'receptor' on the external and internal surface of the phagophore structure, which connects to 'adaptor' molecules on the surface of marked molecules (protein aggregates and organelles) to stimulate their selective transfer and decomposition. The p62/SQSTM1 protein is regarded as the most well-known adaptor protein that stimulates retrieval of poly-ubiquitinated protein aggregates (9,18). Fusion and combination between the external layer of the autophagosome and the lysosome practically require a series of proteins, such as SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor), VAMP8 (vesicle associated membrane protein 8), and VTI1B (vesicle transport through interaction with T-SNARE 1B) (Fig. 1) (17).

Generally, medicinal herbs possess a variety of bioactive ingredients, including flavonoids, glycosides, steroids, alkaloids, terpenoids, and some other bioactive constituents. Some of these chemical compounds are potentially beneficial for the treatment of diseases and are usually prescribed in order to preserve human health¹⁹. Lotus and its family species (sacred blue lotus) were prevalent on the beachside of the Nile. Lotus was gradually transferred from Egypt to several countries, and then expansively harvested all over India, China, and Persia.

Nelumbo Nucifera (scientific name) is an aquatic plant, which nowadays has become a popular decorative herb in most countries all over the world. *Nelumbo Nucifera* has turned into an economically important plant that is extensively harvested in China and India(20). It is regarded as a perennial, large aquatic root-like plant, which possesses a long and creeping stem with nodal roots. The color of its flowers varies from white to ruddy with a pleasant and balmy aroma. The average width of the flowers is approximately 10–25 cm with a bald and oval-shaped structure. Fruits of this plant are gloomy with oval-shaped seeds (19).

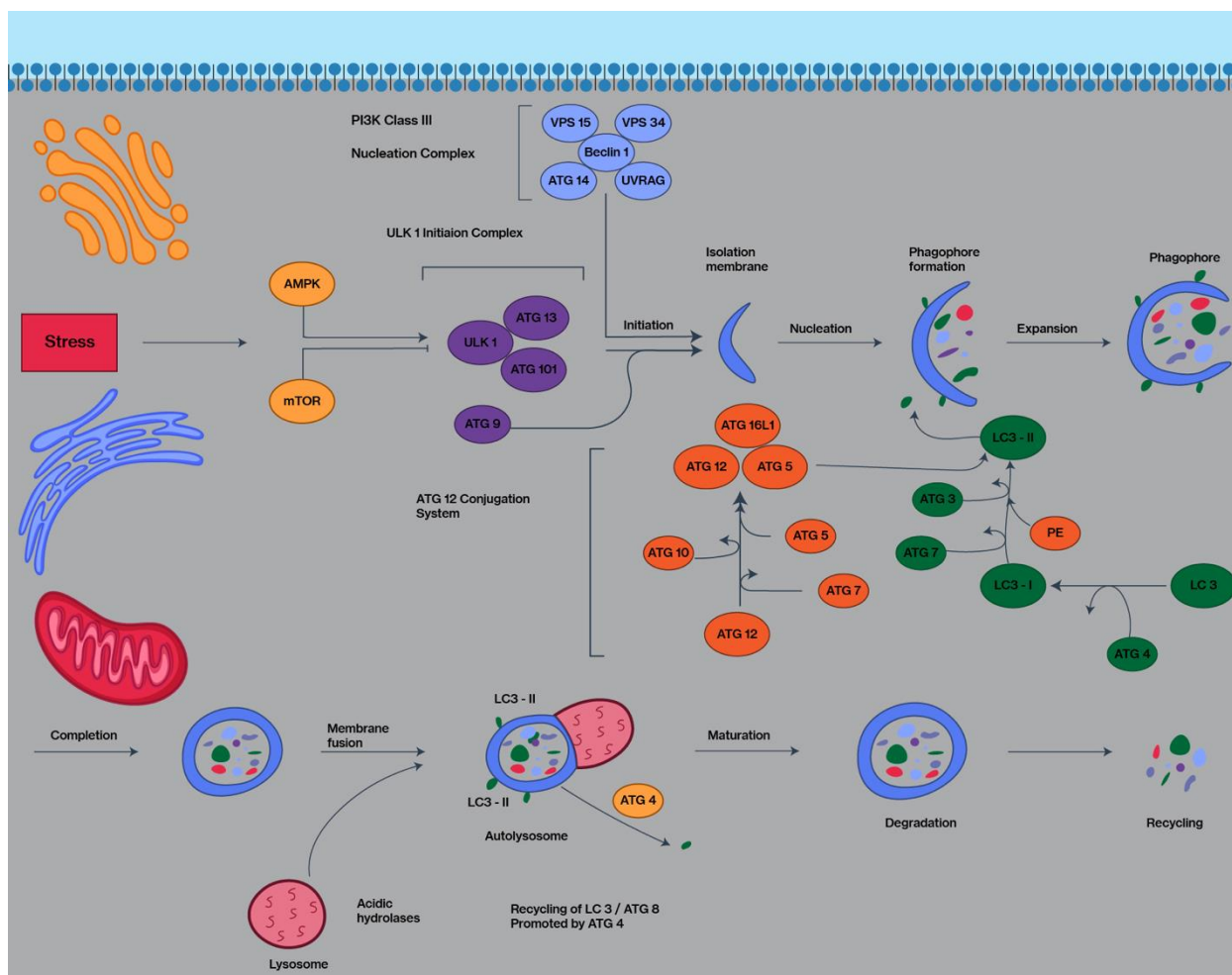


Fig. 1. Autophagy process.

Nelumbo Nucifera belongs to the family Nelumbonaceae and is found in abundance in Australia, Asia, and Russia (21). Conventionally, it has been known as a valuable, nutritious, and magical plant in traditional herbal medication. In this regard, all parts of *Nelumbo Nucifera*, such as seeds, fruits, leaves, embryos, stems, and flags, possess innumerable health benefits. They can be applied as a therapeutic agent toward an extensive range of medical disorders, such as skin disease, heart disease, hypertension, obesity, liver disease, AIDS, pectoralgia, insomnia, diabetes, and malignancies (21).

Nelumbo Nucifera contains high levels of reduced glutathione in footstalks and cotyledons. Content of reduced glutathione is regarded as a determinant factor in the incidence of the budding process (20). Its seeds contain high levels of essential nutrients, such as carbohydrate, fat, minerals, asparagine, fiber, starch, proteins, tannin, unsaturated fatty acids, and amino acids. It also serves as a rich source of

essential minerals, like potassium, calcium, magnesium, sodium, manganese, iron, zinc, copper, and chromium (22). Recently, lotus seed alkaloids have attracted special attention due to their outstanding pharmacological features (23-25).

Among lotus alkaloids, there are three major bioactive alkaloids, including neferine, liensinine, and isoliensinine, which possess outstanding medicinal properties (21). Different parts of lotus have their own unique chemical profiles and pharmacological features. Anti-inflammatory and anti-diabetic properties are attributed to the presence of steroid triterpenoids in the root, whereas the leaves have ameliorating features on nose-bleeding, hematuria, and hemoptosis. Furthermore, bioactive compounds present in the flowers represent anti-cholera, anti-diarrhea, anti-fever, and anti-hyperdipsia features, while the seeds possess anti-inflammatory, anti-cancer, and antidotal properties. The lotus embryo, known as Lian Zi Xin, has various outstanding pharmacological benefits, such

as neuroprotective and cardioprotective features, which in this regard are routinely applied in traditional Chinese medicine. Seeds of *Nelumbo Nucifera* have various alkaloids, which represent antioxidant, anti-tumor, anti-inflammatory, and sedative features(19,26). Nowadays, lotus seeds are very popular among Asian researchers due to their outstanding bioactive ingredients, such as flavonoids, terpenoids, and alkaloids (27,28). Embryo of *Nelumbo Nucifera* possesses a range of essential nutrients, including proteins, amino acids, phospholipids, sugars, and vitamins (29). Generally, until now, approximately 249 different chemical compounds and essential nutritional ingredients have been detected in lotus, which belong to the families of flavonoids, steroids, alkaloids, terpenoids, fatty acids, carbohydrates, proteins, essential oils, tannin, vitamins, and minerals (30).

One of the major alkaloids isolated and purified from the embryo seed of *Nelumbo Nucifera* is Neferine, which belongs to the family of bisbenzylisoquinoline alkaloids. Due to its outstanding and widespread therapeutic features, it serves as a promising therapeutic choice for the treatment of cardiovascular diseases, pulmonary diseases, cancer, neurodegenerative diseases, and inflammatory conditions (31).

This manuscript aimed to review a series of studies in which Neferine has succeeded in improving and attenuating a series of medical conditions, diseases, and malignancies through the stimulation or inhibition of autophagy signaling pathways, which will be discussed in detail below.

Autophagy Properties of Bisbenzylisoquinoline Alkaloid of Neferine

Autophagy in lung cancer

Lung carcinoma has a short survival period, and cigarette smoking is known as a major risk factor for lung cancer, while another prominent risk factor is aging. The increase in age, along with enhanced DNA damage, causes a rise in the susceptibility of lung cancer incidence (32). Lung cancer is recognized as one of the lethal types of cancer and is mainly categorized into two primary subgroups, including small cell lung carcinoma and non-small cell lung carcinoma (33).

In a study conducted by Poornima et al., the therapeutic effects of Neferine on lung adenocarcinoma (A549 cell) were evaluated. They found that Neferine can cause cell death followed by induction of the autophagy process through different molecular mechanisms. Neferine noticeably enhanced the conversion rate of LC3-I to LC3-II, substantially triggered accumulation of acidic vesicle compartments, and also remarkably stimulated downregulation of the PI3K/AKT/mTOR pathway in a dose-dependent manner without affecting the expression levels of cellular PI3KC3 and Beclin-1. This event confirmed that Neferine stimulated autophagy, which was not reliant on the canonical PI3K and Beclin-1 molecular pathway. In other words, Neferine triggered autophagy by blockage of mTOR signaling pathway. Additionally, Neferine exposure meaningfully augmented the production of reactive oxygen species (ROS) in a dose-dependent manner, and in consequence triggered autophagy through ROS hyper-generation (34).

Sivalingam et al. evaluated the protective effects of Neferine supplementation on Diethylnitrosamine (DEN)-induced lung cancer. They claimed that DEN exposure enhanced the expression of PI3K/AKT/mTOR, while Neferine supplementation substantially reversed the expression of PI3K/AKT/mTOR signaling. According to their study, Neferine supplementation consequently caused cancer cell death through induction of the autophagy process via downregulation of the PI3K/AKT/mTOR signaling pathway in the DEN-induced lung carcinoma model (35).

Selvi et al. assessed the cytotoxic mechanisms of Neferine on lung adenocarcinoma. They stated that exposure to Neferine and Cisplatin enhanced the intracellular ROS production and, in consequence, triggered the autophagy process through ROS hyper-generation in A549 cells. Neferine exposure noticeably augmented the acidic vesicular compartment and enhanced the expression of LC3B mRNA and conversion rate of LC3-I to LC3-II. Moreover, Neferine exposure diminished the expression of PI3K and Beclin-1 mRNA and protein, which affirms that Neferine stimulated autophagy that is not reliant on the canonical autophagy pathway. Furthermore, Neferine

exposure substantially reduced the expression levels of PI3K/AKT/mTOR mRNA and protein, which consequently stimulated the autophagy process through the non-canonical autophagy pathway (36).

2.2. Autophagy in prostate cancer cells

Prostate cancer is statistically categorized as the second most prevalent type of cancer, and age is regarded as a prominent risk factor in its incidence. Environmental and genetic factors affect the susceptibility of prostate cancer. In this regard, the incidence rate of prostate cancer is greater in the African-American population compared to the white race³⁷. The PI3K/AKT signaling pathway is involved in the development of different malignancies. Both apoptosis and autophagy processes are triggered through modulation of the PI3K/AKT signaling pathway. The PI3K/AKT signaling pathway is involved in both cell survival and cell death mechanisms and plays a pivotal role in the regulation of the autophagy process (7,8,16).

Erdogan et al. investigated the anti-proliferative effects of Neferine on human prostate cancer cells (PC3, LNCaP, and CD44⁺). They stated that Neferine exposure remarkably enhanced the expression levels of p-JNK in PC3 and CD44⁺, and also upregulated the expression levels of p-38 MAPK in PC3 and CD44⁺ CSCs. Neferine exposure markedly declined the expression levels of PI3K protein in CD44⁺ CSCs, while noticeably augmenting the expression levels of PI3K protein in PC3 cells. Neferine did not exert any remarkable change in AKT protein levels in PC3 and CD44⁺ CSC cells. They noted that Neferine substantially augmented the levels of LC3-II protein, which subsequently provoked the autophagy process in prostate cancer cells. They concluded that the anti-cancer features of Neferine on prostate cancer probably proceed through stimulation of autophagy via modulation of autophagy signaling pathways (38).

Nazim et al. evaluated the signaling pathways by which Neferine treated prostate cancer cells (DU145). They claimed that Neferine exposure substantially enhanced the expression levels of LC3-II protein, reduced the expression levels of P62 protein, and remarkably augmented the autophagic vacuoles formation, which confirmed that Neferine

exposure noticeably provoked the autophagy process in DU145 cells. However, Neferine exposure markedly elevated the expression levels of p-JNK, which provoked TRAIL (tumor necrosis factor (TNF)-related apoptosis-inducing ligand)-triggered DU145 cell death. Accordingly, it seems that Neferine treatment caused DU145 cell death via TRAIL-triggered apoptosis through autophagy flux and JNK signaling (39).

Liu et al. investigated the anti-androgenic effects of Neferine on prostate cancer cells (PC3, LNCaP, and DU145). They noted that Neferine exposure markedly elevated the expression levels of LC3-II protein in LNCaP cells, and noticeably upregulated the PI3K/AKT signaling pathway by stimulating the autophagy process through modulation of PI3K/AKT/mTOR signaling and imposed cytotoxic effects on prostate cancer cells (40).

Autophagy in hypoxic muscle cells

Oxygen plays a pivotal role in cellular homeostasis and vital physiological functions. Oxygen shortage can cause impairment in biological activities and metabolic stress. Hypoxic inducible factor, a transcription factor, suppresses hypoxic situations (41).

Baskaran et al. investigated the autophagic modulatory features of Neferine supplementation on hypoxic muscle cells (Rhabdomyosarcoma cells). They realized that hypoxia, through the inhibition of PI3K/AKT/mTOR signaling, substantially triggered the autophagy process. Neferine pretreatment hindered autophagy induction through activation of AKT/mTOR signaling (as a negative regulator of autophagy) and down-regulation of Beclin-1, PI3KC3, and LC3B-II in cells that were subjected to hypoxic conditions. Additionally, Neferine exposure blocked hypoxia through activation and up-regulation of Nrf-2 protein signaling. The Nrf-2 protein is known as the positive mediator of antioxidant enzymes, and Neferine pretreatment markedly enhanced the activity of antioxidant enzymes through upregulation of Nrf-2 protein in hypoxic muscle cells. It seems that Neferine, by upregulating the PI3K/AKT/mTOR signaling pathway, exerted a negative impact on autophagy induction and, in consequence, down-regulated the autophagy process in hypoxic muscle cells (42).

Autophagy in hepatocellular carcinoma

Liver cancer is known as the seventh most frequent cancer among cancer cases, and also the second most lethal type of cancer. Hepatocellular carcinoma is regarded as the major type of liver cancer, which is responsible for three-quarters of the total liver cancer cases⁴³. Cirrhosis and chronic liver disease are regarded as two well-known risk factors in the incidence of liver cancer (44).

Yoon et al. evaluated the anti-cancer mechanism of Neferine on hepatocellular carcinoma (Hep 3B and SK-Hep1) and stated that Neferine exposure substantially enhanced the expression levels of LC3-II protein, and consequently, augmented the establishment of autophagosome compartments. Furthermore, the proportion of cytoplasm membrane-bound LC3-B progressively augmented following Neferine treatment in a time- and dose-dependent pattern. Incidence of these autophagy-related events appropriately implicates the triggering induction of the autophagy process in HeP3B cancer cells (45).

Autophagy in Neuroblastoma Cancer

Neuroblastoma is known as the most common type of cancer that occurs in newborns and is responsible for about 12-15% of mortalities in cancer cases (46). The FAK and 70-kDa ribosomal S6 kinase 1 (S6K1) proteins are regarded as prominent and determinant targets for anti-cancer purposes in several malignancies due to their vital role in cell integrity, viability, proliferation, and migration (47).

Pham et al. evaluated the therapeutic benefits of Neferine exposure on human neuroblastoma cancer (IMR32 cells) and found that Neferine substantially triggered IMR32 cell death in a dose-dependent manner. They also reported that Neferine treatment triggered induction of apoptosis and autophagy in IMR32 cells via reduction in the expression levels of FAK and S6K1 proteins. Additionally, Neferine exposure markedly upregulated the expression of Beclin-1 protein and LC3-II accumulation in a dose-dependent pattern. This signified that Neferine, by downregulation of the expression of FAK and S6K1 proteins and triggering up-regulation of Beclin-1 and LC3-II protein, stimulated cell death via autophagy

process activation in IMR32 neuroblastoma cells (48).

Autophagy in Graves Orbitopathy fibroblasts

Graves is known as an inflammatory autoimmune disorder and has various symptoms, such as extra adipogenesis, proliferation triggering, and excess generation of extracellular matrix (49). The Nrf-2 is regarded as a critical transcriptional factor that triggers up-regulation of the P62 gene in oxidative stress situations and protects cells against oxidative stress-induced injury (50).

Li et al. evaluated the therapeutic effects of Neferine supplementation on Graves orbitopathy condition. They suggested that IL-13 noticeably triggered autophagy induction through decreasing the levels of P62 protein and elevating the levels of Atg-7, Beclin-1, and LC3-II protein. Furthermore, IL-13 remarkably enhanced the levels of ROS generation, IL-1 β , IL-6, and TNF- α , which represents an impressive relationship between inflammation and autophagy induced in Graves orbitopathy (GO) orbital fibroblasts. Moreover, the levels of collagen-1, collagen-3, bFGF (basic fibroblast growth factor), and α -smooth muscle actin (α -SMA) were remarkably elevated by IL-13 administration. Neferine exposure dramatically reversed the upregulation of TNF- α , IL-1 β , IL-6, MCP-1, and ROS generation, and incompletely reversed the upregulation of collagen-1, collagen-3, bFGF, and α -SMA that was stimulated by IL-13.

In addition, Neferine exposure markedly triggered the AKT/mTOR signaling pathway and upregulated the levels of Nrf-2 protein. Therefore, it acts as a negative regulator of the autophagy process. Additionally, Neferine substantially downregulated the levels of Beclin-1, Atg-7, and LC3-II proteins, and markedly upregulated the levels of Nrf-2 and P62 protein, which counteracted the IL-13-induced autophagic effects, and consequently, attenuated autophagy in GO orbital fibroblasts, and substantially restricted inflammation and adipocyte development in GO fibroblasts (51).

Autophagy in Alveolar epithelial type-2 cells

Alveolar type-2 cells (AEC-II) are known as the major building blocks of distal lung epithelium. Due to having a high number of mitochondria, they play

a key role in renovating alveolar epithelium which mediates secretion of surfactant proteins and immune mechanisms. The malfunction of AEC-II cells causes lung disease, like chronic obstructive pulmonary disease (COPD) (52). The COPD is a respiratory syndrome with symptoms related to airflow limitation and inflammatory reactions. Environmental and genetic factors are recognized as major risk factors in the occurrence and progression of respiratory disease. In this regard, cigarette smoking is categorized as a major environmental risk factor in the incidence of respiratory disease (53).

Wang et al. evaluated the protective effects of Neferine on cigarette smoking extract (CSE)-induced apoptosis in AEC-II cells. They claimed that CSE substantially enhanced the status levels of Atg-5 and LC3-II proteins and decreased the levels of P62 protein in a time- and dose-dependent manner. Besides, Neferine exposure remarkably enhanced the expression levels of LC3-II and Atg-5 proteins and reduced the P62 protein level, which affirms that Neferine potentially induced autophagy induction in AEC-II cells. Therefore, it can be concluded that Neferine facilitated anti-apoptotic protection via autophagy activation in AEC-II cells. Neferine remarkably elevated the formation of autophagosome compartments and autolysosomes in AEC-II cells. Moreover, as a potent stimulator of autophagy, it substantially triggered the autophagy process by inhibiting the AMP-activated protein kinase (AMPK)/mTOR signaling pathway, and therefore could act as a potent cytoprotective agent in AEC-II cells against CSE-triggered cell death and apoptosis. Neferine can be considered a novel and promising tactic toward alleviating CSE-induced apoptosis and cytotoxicity and accordingly be regarded as a suitable alternative treatment choice for COPD (54).

Autophagy in NRK-52E cells

Acute kidney injury (AKI) is a critical medical disorder (55). Drugs that have potent renal toxicity may lead to an increased rate of AKI occurrence. Platinum-based drugs, such as cisplatin, are recognized as nephrotoxic agents. Cisplatin nephrotoxicity comprises clumping of cisplatin in proximal and distal tubules, which can cause

mitochondrial dysfunction and impairment in the function of membrane transporters (56).

Li et al. investigated the mechanism by which Neferine alleviates cisplatin-induced nephrotoxicity in NRK-52E cells. They claimed that cisplatin exposure substantially decreased the expression levels of LC3-II, decreased the conversion rate of LC3-I to LC3-II, and markedly enhanced the levels of P62 protein in NRK-52E cells in a dose- and time-dependent manner. Furthermore, cisplatin simultaneously triggered maximum apoptosis activity. Meanwhile, Neferine exposure remarkably enhanced the formation of autophagosome compartments and the levels of LC3-II protein and diminished the levels of p62 protein in NRK-52E cells in a dose- and time-dependent pattern. This shows that Neferine appropriately protected cells against cisplatin-triggered apoptosis. Neferine exposure substantially enhanced the phosphorylation levels of p-AMPK involved in autophagy induction. Accordingly, it can be concluded that Neferine notably triggered autophagy through upregulation of the AMPK/mTOR signaling pathway and therefore effectively protected NRK-52E cells against cisplatin-triggered apoptosis by autophagy induction in epithelial tubular NRK-52E cells (57).

Autophagy in cervical cancer cells

Cervical cancer is the fourth most prevalent cancer and is regarded as a challenging health problem all over the world. The major cause of its incidence is human papillomavirus (HPV) infection, which is known as the most prevalent sexually transmitted disease and recognized as a major risk factor in %99.7 of cervical cancer cases (58).

Dasari et al. investigated the anti-cancer features of Neferine against cervical cancer. They noted that the viability of cells that were exposed to Neferine markedly declined. They also reported that HeLa cells were more susceptible to Neferine cytotoxicity, compared to SiHa cells. The cytotoxic effects of Neferine remarkably diminished in the presence of an autophagy inhibitor, such as 3-methyladenine (3-MA), which indicates that the autophagy process plays a pivotal role in the anti-cancer mechanism of Neferine through oxidative stress.

In their study, Neferine exposure meaningfully augmented the conversion rate of LC3-I to LC3-II

and enhanced the levels of LC3-II, Beclin-1, Atg4, Atg5, Atg12, and P62 protein in SiHa and HeLa cells. Moreover, Neferine remarkably provoked autophagy through the formation of a lysophagosome and the induction of the accumulation of autophagic vesicles. By enhancing the Neferine dosage, the levels of Atg4 protein increased, which signified that ROS hyper-generation is involved in Neferine-induced autophagy in SiHa and HeLa cells. According to the results, it seems that the anti-cancer benefits of Neferine against cervical cancer (SiHa and HeLa) are reliant on the activation of autophagic signaling pathways (59).

Autophagy in high grade serous ovarian carcinoma

Ovarian carcinoma is categorized as the most problematic issue in the public health system. It is known as the most fatal type of gynecological malignancies and the 7th most prevalent cancer. Alteration in the epithelial cells is responsible for the incidence of 90% of ovarian carcinoma, and the rest of the cases are derived from sex-cord stromal tissue and germ cells (60).

Zhang et al. evaluated the anti-angiogenesis mechanism of Neferine on high-grade serous ovarian carcinoma and stated that Neferine exposure augmented the formation of autophagosome and autolysosome compartments and remarkably decreased the phosphorylated levels of P70S6K1 (ribosomal S6 kinase-1) and p-mTOR (mammalian target of rapamycin), while it did not impose any substantial alteration in the levels of phosphorylated 4EBP1 (Eukaryotic translation initiation factor 4E binding protein-1). Furthermore, Neferine treatment promoted the expression levels of LC3-II, Beclin-1, Atg7, and Atg5 proteins, while substantially decreasing the phosphorylated levels of unc-51 like kinase 1 (ULK1) in ovarian cancer cells. Accordingly, it seems that Neferine treatment, by downregulating p-mTOR, upregulating autophagy-related markers, and subsequently upregulating the autophagy process, markedly suppressed ovarian carcinoma (61).

Xu et al. assessed the therapeutic effects of Neferine against human ovarian cancer cells. They reported that Neferine exposure remarkably declined cell

viability of A2780 and FTE187 cells in a dose-dependent pattern. Neferine exposure notably elevated the formation of autophagosome compartments, augmented the levels of LC3-II and Atg7 proteins, and markedly declined the levels of p-70S6K and 4EBP1 in a dose- and time-dependent pattern, while not imposing any remarkable alteration in the expression levels of P62 and Beclin-1 proteins. This indicates that the autophagy process triggered in ovarian cancer proceeds through an mTOR-dependent signaling pathway. They further noted that the phosphorylation levels of P38 and JNK substantially augmented in a dose- and time-dependent pattern. This signaling had a positive regulatory impact on the expression of LC3-II and subsequently, the stimulation of autophagy induction in ovarian cancer (62).

Autophagy in head and neck squamous carcinoma

Head and neck squamous cell carcinoma (HNSCC) stems from the mucosal epithelium within the oral cavity, such as the pharynx, hypopharynx, larynx, and nasopharynx. The HNSCC is categorized as the 6th most frequent malignancy, and the incidence of HNSCC can be related to tobacco consumption and drinking of alcohol. Tumors originating from the oropharynx are related to previous infection by some oncogenic strains of HPV, such as HPV-16 and HPV-18 (63).

In a study conducted by Zhu et al. on assessing the inhibitory effects of Neferine on HNSCC, it was reported that the cell viability of HN6, HN30, and CAL27 treated with Neferine dramatically decreased in a dose- and time-dependent manner.

According to their findings, Neferine exposure notably augmented the conversion rate of LC3-I to LC3-II and the levels of Beclin-1 and P62 proteins. Moreover, it notably provoked the formation of autophagosome structure, prevented autophagosome degradation, and hindered autophagic flux in the ultimate steps of degradation in HN30 and CAL27 cells in a dose- and time-dependent pattern. Neferine treatment remarkably enhanced the ROS production levels in HN30 and CAL27 cells. The ROS plays the role of an initiator in the induction of Beclin-1 expression via activation of ASK1/JNK signaling (64,65); therefore, Neferine triggered the autophagy process through ROS hyper-generation.

Accordingly, it seems that by the inhibition of autophagic flux, Neferine potentially provoked induction of apoptosis (66).

Autophagy effects on inhibition of epidermal growth factor

The epidermal growth factor receptor comprises four associated receptors, including ErbB1 (HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4), which have similar chemical structures and play vital roles in modulating cell proliferation and migration of cancer cells. Incidence of several malignancies, such as breast, ovarian, non-small cell lung, prostate, colorectal, laryngeal, and esophageal cancers, is related to alterations in the structure of ErbB family receptors (67).

Ozal et al. conducted a study on the anti-proliferative effects of Neferine on retinal pigment epithelial cells. They clearly claimed that cell growth triggered by EGF remarkably declined after Neferine treatment. Neferine treatment dramatically decreased the phosphorylated levels of PI3K and AKT proteins, which were already triggered by EGF. In addition, Neferine exposure alongside EGF remarkably decreased the phosphorylated levels of P38 MAPK. This event signified that Neferine induced the autophagy process through inhibition of the PI3K/AKT/mTOR signaling pathway, which is responsible for the anti-proliferative effects of Neferine (68).

Autophagy in neurodegenerative disease

Neurodegenerative diseases comprise a variety of neurological disorders. The most prominent hallmark of neurodegenerative disorders is misfolded protein aggregation into insoluble inclusions in the CNS. Different protein inclusions are accumulated depending on the type of disease, such as amyloid- β and tau protein in Alzheimer disease, α -synuclein in Parkinson disease, TAR DNA-binding protein 43 and superoxide dismutase 1 in amyotrophic lateral sclerosis, and mutated huntingtin (HTT) in Huntington disease (69,70).

Tang et al. investigated the protective effects of Neferine and Neferine exosomes on mutant neurodegenerative disease proteins (Alzheimer disease) in PC12 cells. They realized that Neferine exposure substantially declined the expression levels

of HTT 74, P301L tau, and A53T α -syn mutant proteins. Neferine treatment notably augmented the expression levels of LC3-II protein and remarkably diminished the expression levels of P62 protein in PC12 cells. This confirmed the therapeutic efficiency of Neferine and Neferine exosomes in inducing the autophagy process. Moreover, Neferine and Neferine exosomes, by upregulating the autophagy process, exerted their attenuating and alleviating effects on mutant neurodegenerative disease proteins, which subsequently caused protection against neurodegenerative disease (Alzheimer's disease) (71).

Wong et al. performed a study on the autophagic effects of Neferine on protein levels of mutant huntingtin in PC12 cells. They found that Neferine promoted the establishment of GFP-LC3, enhanced the production rate of LC3-II, elevated the creation of autophagosomes, markedly elevated the phosphorylated levels of AMPK protein, and simultaneously reduced phosphorylation levels of ribosomal protein S6K1 in a dose-dependent manner. Neferine markedly elevated the clearance of EGFP-tagged mutant huntingtin in cells that possessed wild-type Atg7 but not in the Atg7-deficient MEF cells, which were resistant to autophagy induction. In consequence, Neferine positively regulated autophagy through the AMPK/mTOR signaling pathway in PC12 cells in a dose-dependent pattern (72).

Autophagy effects on Ryanodine receptors

Ryanodine receptors (RyRs), known as intracellular calcium channels, can regulate the release of calcium from the endoplasmic/sarcoplasmic reticulum stores. Ryanodine receptors are present in all types of mammalian cells and have vital functions, such as signal transmission in synapses, cardiac and skeletal muscle contraction, vascular muscle tone, and pancreatic beta cell secretion. There are three different types of ryanodine receptors, including RyR1, RyR2, and RyR3. The RyR1 is regarded as the main type in skeletal muscle, while RyR2 is known as the major type in cardiac muscle. RyR3 mainly presents in the brain (73).

Law et al. investigated the autophagy mechanism in apoptosis-resistant cancer cells via ryanodine

receptor (HeLa, MCF-7, PC3, HepG2, Hep3B, H1299, A549, and LLC1). They claimed that Neferine, through stimulating Ryanodine receptors and subsequently enhancing calcium release, triggered the autophagy process through the ULK/CaMKK2-AMPK (ULK-Ca²⁺/calmodulin-dependent protein kinase kinase 2 activated protein kinase (AMPK)-mTOR)-dependent pathway and augmented the formation of autophagosomes. Neferine exposure, by upregulation of a series of genes, such as P62 (SQSTM1), protein kinase R-like endoplasmic reticulum kinase (PERK), ULK1, P-PERK, and Cxcr4 (chemokine receptor type 4), triggered autophagy in apoptosis-resistant tumor cells.

According to their research, Neferine exposure noticeably elevated the levels of phosphorylated-AMPK and simultaneously declined the expression levels of phosphorylated P70S6K. They stated that Neferine triggered the autophagy process via the CaMKK-2-AMPK-mTOR signaling pathway. Moreover, Neferine exposure markedly blocked SERCA (Sarcoendoplasmic Reticulum Calcium ATPase) and subsequently stimulated autophagy induction. Neferine mobilized cytosolic calcium through RyR (Ryanodine receptor) stimulation, and exerted cytotoxic effects by triggering the expression of RyR1 in Hep3B, HepG2, HeLa, and H1299 cells except MCF-7. They concluded that the anti-cancer effects of Neferine against apoptosis-resistant cancer cells proceed through the autophagy pathway that depends on calcium release regulated via ryanodine receptors (74).

Autophagy effects on doxorubicin-treated H9c2 cardiomyocyte

Doxorubicin (DOX) imposes cytotoxic impacts by triggering several molecular signaling pathways, such as DNA intercalation, free radical production, blockage of the mitochondrial respiratory cycle, membrane lipid oxidation, disruption of the helicase enzyme function, and apoptosis stimulation via inhibition of topoisomerase II(75).

Priya et al. evaluated the protective effects of Neferine on DOX-treated H9c2 cardiomyocytes, and they realized that Neferine pretreatment remarkably reversed the downregulation of IGF-1R and PI3K/AKT/mTOR signaling in H9c2 cells

rather than DOX administration. Neferine also reduced the expression levels of ULK1, Beclin-1, ATG7, and LC3-II in cells exposed to Doxorubicin. Furthermore, Neferine pretreatment notably diminished the number of autophagosomes formed in H9c2 cells exposed to Doxorubicin. Accordingly, it seems that Neferine, by upregulation of PI3K/AKT/mTOR signaling, hindered the autophagy process, and it can be concluded that the therapeutic effects of Neferine on Doxorubicin-treated H9c2 cardiomyocytes are reliant on autophagic inhibitory effects of Neferine on H9c2 cells (76).

Autophagy in Ischemic stroke

Stroke is the fifth leading cause of death in the USA, that lead to the death of approximately 130,000 Americans each year. It is the most prevalent type of stroke and happens when blood vessels in the neck or brain occlude (77).

Wu et al. assessed the mitochondrial protective mechanism of Neferine through the Nrf2 signaling pathway. Neferine exposure demonstratively elevated the expression levels of Nrf2 in the nucleus, and substantially elevated the translocation of Nrf2 from the cytoplasm to the nucleus. According to the positive and determinant role of Nrf2 in autophagy induction, this event appropriately affirmed the mitochondrial protective mechanism of Neferine through autophagy induction. Moreover, Neferine treatment notably augmented the expression levels of LC3-II and declined the expression levels of P62 protein (78).

Sengking et al. investigated the protective effects of Neferine against brain injury in permanent cerebral ischemia. They stated that Neferine administration substantially decreased infarct size and ameliorated the neurological damage. Furthermore, Neferine substantially downregulated the expression levels of LC3-II, Beclin-1, P62 protein, and autophagosome establishment. In addition, Neferine treatment notably improved cerebral ischemia by modulating the autophagy process through Ca²⁺ dependent AMPK/mTOR signaling pathway. They concluded that Neferine supplementation ameliorated the cerebral ischemia through downregulation of the autophagy process (79).

Autophagy in lysosome malfunction

Xu et al. evaluated the mechanism by which Neferine induces macroautophagic flux and clearly stated that the levels of LC3-II, the proportion of LC3-II/LC3-I, and the expression levels of p62/SQSTM1 were remarkably elevated with the increase of Neferine dosage in ARPE-19 cells, and a series of cell lines (hTERT-RPE1, HEK-293, HeLa, and Neuro-2a), which indicates that Neferine attenuated the autophagic flux. They found that the elevated levels of LC3-II protein followed by Neferine administration cannot be ascribed to the stimulation of autophagic flux, but can be appropriately attributed to the disturbed

decomposition process which occurred within autophagolysosomes.

According to their study, Neferine did not notably augment the co-localization of LAMP2 and LC3, which represented that the fusion process took place, while subsequent decomposition process of LC3 was remarkably blocked. Neferine remarkably attenuated the decomposition of rhodopsin. In other words, Neferine potentially attenuated the lysosomal operation and consequently inhibited the decomposition of the autophagosome (80).

Table 1 is the summary of the mentioned contents, and Fig. 2 illustrates some of the pathways related to Neferine.

Table 1. Summary of the Neferine effects on signaling pathways and expressed proteins relating to autophagy.

Neferine dosage	Experiment duration	Cell lines or animals	Disorders	Signaling pathway or expressed proteins	Ref.
20 μ M	48 h	A549 cells	Lung carcinoma	• PI3K/AKT/mTOR $\downarrow$	[34]
50 μ M	48 h	PC3, LNCap, CD44 ⁺ CSC cells	Prostate cancer	• PI3K/AKT/mTOR $\downarrow$	[38]
0.5 μ M	36 h	Rhabdomyosarcoma cells (RD cells)	Hypoxic muscle	• AKT/mTOR $\uparrow$ • Beclin-1 $\downarrow$	[42]
5, 10, 15, 20, 25, 30 μ M	24 h	Hep3B, SK-Hep1, THLE-3 cells	Hepatocellular carcinoma	• LC3-II $\uparrow$	[45]
30 μ M	24 h	IMR32 cells	Neuroblastoma cancer	• LC3-II and Beclin-1 $\uparrow$	[47]
0.5 μ M	7 days	Fibroblast cells	Graves orbitopathy	• LC3-II, Beclin1, Nrf2, and Atg7 $\uparrow$ • P62 $\downarrow$	[50]
2, 4, 8 μ M	24 h	Alveolar epithelial type2 cells	Chronic obstructive pulmonary disease	• AMPK/mTOR $\uparrow$ • LC3-II and Atg5 $\uparrow$ • P62 $\downarrow$	[53]
2, 4, 8 μ M	24 h	NRK52E cells	Nephrotoxicity	• AMPK/mTOR $\uparrow$ • LC3-II $\uparrow$ • P62 $\downarrow$	[55]
10, 25, 50 μ M	48 h	SiHa and HeLa cells	Cervical cancer	• LC3-II, Atg4, Atg5, Atg12, Beclin1 $\uparrow$ • ROS generation $\uparrow$	[57]
8 μ M	24 h	HUVEC and mouse macrophage cell line (Raw264.7)	High-grade serous ovarian carcinoma	• PI3K/AKT/mTOR and S6K1 $\downarrow$ • LC3-II, Beclin1, Atg5, Atg7 $\uparrow$	[59]
5, 10, 15, 20 μ M	24, 48 h	HN6, HN30, CAL27 cells	Head and neck squamous carcinoma	• LC3-II, Beclin1 and P62 protein, and ROS generation $\uparrow$	[62]

Neferine dosage	Experiment duration	Cell lines or animals	Disorders	Signaling pathway or expressed proteins	Ref.
5, 10 μ M	24, 48 h	Retinal pigment epithelial (RPE) cells	Proliferative vitreoretinopathy	PI3K/AKT/mTOR $\uparrow$	[64]
2.5, 5, 7.5 μ M	24 h	PC-12, MEF cells	Huntington disease	AMPK/mTOR, S6K1, LC3-II and p-AMPK $\uparrow$	[68]
2.5, 5, 10, 15, 20 μ M	24 h	HeLa, MCF-7, PC3, HepG2, Hep3B, H1299, A549, LLC1 cells	Apoptosis-resistant cancers	- ULK/CaMMK2-AMPK-mTOR, LC3-II, P62, PERK, ULK1, CxCr4, RyR1 protein $\uparrow$ - SERCA $\downarrow$	[70]
10, 15, 20 mg/kg	20 days	Albino Wistar rat	Lung carcinoma	PI3K/AKT/mTOR $\downarrow$	[35]
10 μ M	24 h	H9c2 cells	Cardiomyoblast	IGF-1R, PI3K/AKT/mTOR, LC3-II, Beclin1, Atg7, ULK1 $\uparrow$	[72]
1, 5, 10 μ M	24 h	PC-12 cells	Ischemic stroke	- Nrf2 expression and translocation, LC3-II $\uparrow$ - P62 $\downarrow$	[74]
0.5, 5, 50 μ g/ml	24 h	Neuron-2a, PC-12 cells	Neurodegenerative disease (Alzheimer's disease)	- LC3-II $\uparrow$ - P62 $\downarrow$	[67]
10 μ M	24, 48 h	ARPE-19, hTERT-RPE-1, HeLa, Neuro-2a cells	Lysosome malfunction	- LC3-II $\uparrow$ - P62 $\downarrow$	[76]
0, 5, 10, 20 μ M	18 h	DU145, LNCaP cells	Prostate cancer	- Phosphorylation of JNK and LC3-II $\uparrow$ - P62 $\downarrow$	[39]
10, 20 μ M	12, 24, 48, 72 h	A549 cells	Lung carcinoma	- Expression of mRNA LC3B $\uparrow$ - mRNA and protein levels of Beclin1 and PI3KC3 $\uparrow$ - ROS generation $\uparrow$ - PI3K/Akt/mTOR $\downarrow$	[36]
5, 10, 15 μ M	24, 48, 72 h	HO8910, SKOV3, A2780, FTE-187 cells	Human ovarian carcinoma	- LC3-II, Atg7, mTOR $\uparrow$ - Phosphorylation levels of p38 and JNK $\uparrow$ - Expression levels of p-70S6K and 4EBP1 $\downarrow$	[60]
1, 10, 100 μ M	24, 48 h	PC3, LNCaP, DU145 cells	Prostate cancer	- LC3-II $\uparrow$ - PI3K/AKT signaling $\uparrow$	[40]

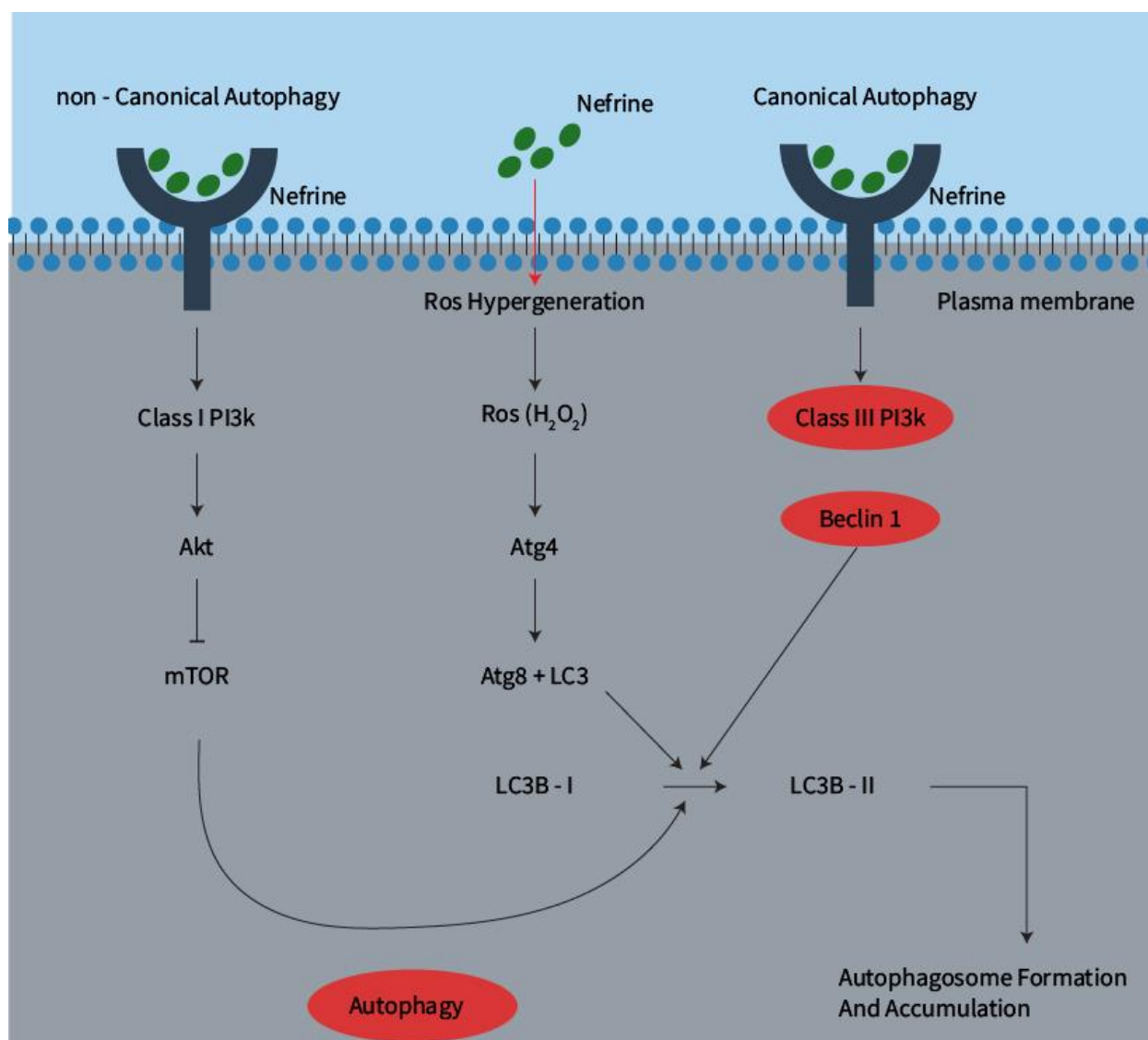


Fig. 2. Autophagy effects of Neferine.

Conclusion

In conclusion, according to the many studies that have been conducted on the pharmacological and toxicological features of Neferine until now, and also based on the observations, pieces of evidence, and obtained results from the above-mentioned studies about the beneficial effects of Neferine, it can be said that it possesses special therapeutic potential in the treatment of many malignancies. It also has the potential to be introduced as a potent and beneficial therapeutic agent with minimal adverse and undesirable effects on normal healthy cells and organs.

Considering its advantageous effects on the treatment of a variety of medical disorders which were mentioned above, this alkaloid composition

works more effectively and ideally with more desirable anti-cancer effects and fewer undesirable effects, compared to the common medications available in the pharmaceutical market. It is expected that Neferine will be applied as a beneficial medicinal agent in the pharmaceutical market in the near future; however, further detailed and accurate studies are needed to reach a precise understanding of its therapeutic mechanisms.

Acknowledgement

There is no financial support.

Author contributions

Khashayar Sanemar: Draft preparation

Mojdeh Mohammadi, Katayoun Derakhshandeh, Mohammad Abdollahi: Supervision, Reviewing and Editing

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

The authors declare that they have no conflicts of interest. All the authors approved the manuscript.

Funding

No funding.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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