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Review Article
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AUTOPHAGY: THE MECHANISM AND A POTENTIAL CANCER THERAPY

Omkar Kulkarni*[1] and Harsh Priya[1]

1Department of Pharmaceutical Sciences and technology,

Institute of Chemical Technology, Mumbai, India

***Corresponding Author Email:** omkarkulkarni2101@gmail.com

ABSTRACT

Autophagy is a regulatory mechanism essential to maintain homeostasis and is a kind of Programmed cell death (type II). Role of autophagy in tumor genesis is a complex one. In early stages of tumorigenesis, a number of activated oncoproteins suppress autophagy, indicating that suppression of autophagy in early stages helps the tumor cells to grow and proliferate. Whereas in the metastasis stage of cancer, autophagy induced due to chemotherapy, helps the tumor cells to survive the chemotherapeutic attack as it eliminates the drug molecules from the cells. This suggests a dual and paradoxical role of autophagy in early and late stages of tumor genesis. Designing drug delivery systems, which can modulate autophagy in these cells can be a ray of hope for cancer patients. A keen choice of therapeutic agents and a well formulated drug delivery system can give us a promising answer to cancer.

KEY WORDS

Autophagy, cancer, tumorigenesis, oncogene, Nano technology, chemotherapy, phytoconstituents.

1. INTRODUCTION

Cellular homeostasis is a principal factor in the development and functioning of a cell, which is basically controlled by balancing protein synthesis and degradation[1]. For maintaining this balance, a large number of regulatory mechanisms are present which are invoked after sensing specific kinds of environmental signals and giving appropriate responses for the same. Programmed Cell Death (PCD) is one of the most important regulatory mechanisms. Failure of its regulation can lead to development of resistance to death in malignant cells or affect the efficiency of neurons in case of neurodegenerative disorders.

Although apoptosis is the best known PCD and categorized as type I PCD, it can occur in more than one way. Autophagy being one of them is classified as type II PCD. Unlike apoptosis, autophagy plays a role in both survival and death depending on the context. Apoptosis involves processes which stimulate catabolic pathways, which eventually lead to nuclear chromatin condensation, nuclear fragmentation along with formation of different apoptotic bodies[2]. On the other hand, autophagy comprises of membrane trafficking pathway and is responsible for the degradation of cytosolic proteins and organelles by lysosomes.

1.1 Autophagy:

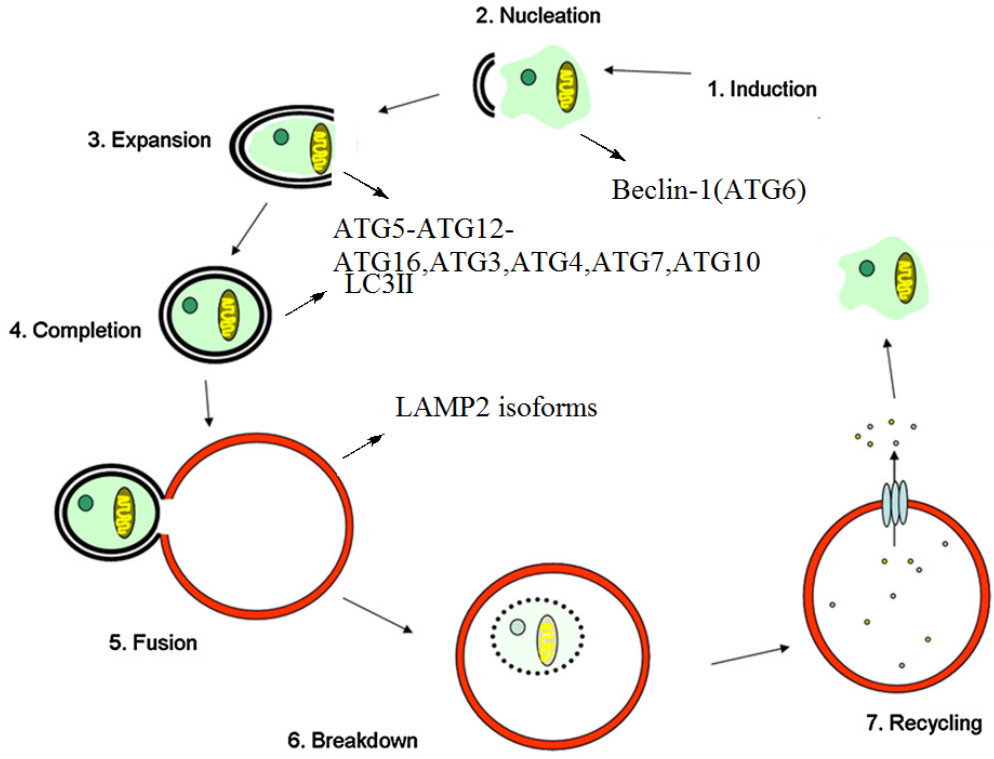
Excessive and damaged organelles and aggregated proteins in a cell are removed by the process known as autophagy rendering it a vital mechanism for cytoplasmic quality control. Autophagy is induced under starvation condition and also in response to certain hormones in case of mammalian cells. Under normal physiological

circumstances, autophagy is able to maintain homeostasis by elimination of cellular cargo. But overstimulation can lead to dire consequences like cell death instead of homeostasis.

Autophagy is a collective term for different selective and nonselective processes encompassing micro autophagy, chaperone-mediated autophagy and macro autophagy. In case of micro autophagy, formation of lysosomal invagination takes place which later results into direct and nonspecific sequestration and breakdown of cytosolic components[3]. In chaperone-mediated autophagy, unfolded proteins are specifically recognized and bound by a chaperone complex, which is then translocated into lysosome lumen[4]. But in most of the cases and also in the following review, autophagy refers to the process of macro autophagy.

1.2 Mechanism of autophagy:

Fig 1[5]: Dissection of the macro autophagy pathway. A schematic dissection of macro autophagy is presented, together with the core proteins involved in each step .At nitrogen limitation conditions (1) macro autophagy is induced leading to (2) nucleation, (3) expansion and (4) completion of membrane formation for sequestration of cytoplasmic components. (5) Subsequent fusion step and uptake in the vacuole. (6) Degradation by vacuolar hydrolases and (7) building blocks (e.g. amino acids) recycle to the cytosol.



Macro autophagy, or simply autophagy, is a complex multistep process which is coordinated by key proteins encoded by autophagy-related genes, i.e., Atg genes. In short, a part of the cytoplasm is sequestered in typical double-membrane vesicles which is referred to as auto phagosomes that fuse with lysosomes to form autolysosomes, a process termed 'autophagy flux'. Next, the delivered lysosomal enzymes break down the inner membrane and cargo of

the auto lysosome[6]. The recruitment and working of a phosphatidylinositol 3-kinase Class III (PI3KCIII) complex is essential for the nucleation of phagophore which is the first step of autophagy. Beclin-1 (Atg6) acts as a platform for other proteins required for auto phagosome formation rendering it an important component of this multi protein complex[7]. After phagophore elongation and simultaneous sequestration of cytoplasm, the vesicle closes to form the typical double membrane auto phagosome. These auto phagosomes are used as markers in autophagy research. The characteristic degradative power of the phenomenon is related to the formation of auto phago lysosomes which is a consequence of the fusion of an auto phagosome with a lysosome. Finally, the content of the auto phagosome and inner membrane is digested with the enzymes present in auto lysosome[8]. Permeases are the enzymes which now come into the picture and play an important role of transporting the digested material to the cytoplasm where they can be utilized for de novo synthesis of cellular components or energy generation purposes[9].

36 identified proteins are involved in the macroautophagy pathway[10]. The core machinery contains 16 essential proteins. The inactivation of mammalian target of rapamycin (mTOR) brings about the initiation of autophagy. ULK1, which is Atg1 human homologue in yeast, activates the Beclin 1 complex. As mentioned above, Beclin 1 recruits other proteins and elongates the phagophore. ULK1 is maintained in phosphorylated form by mTOR. Certain stimuli which induce autophagy releases ULK1 from mTOR repression[11].

For elongation of the phagophore, LC3 (1A/1B Light chain 3) protein is converted to the LC3-II form. Ubiquitin like conjugation systems bring about this conversion. Two ubiquitin systems are utilized for membrane elongation. In one system, Atg7 and Atg10 are used to form a covalent linkage (irreversible) between Atg5 and Atg12. Atg16L1 then associates to form the Atg5-12-16L1 complex, which causes membrane elongation. The other ubiquitin-like conjugating system incorporates LC3-II into the double membrane of the autophagosome. The terminal amino acid(s) from LC3 is cleaved by Atg4, forming LC3-I. Phosphatidylethanolamine is added to LC3-I, resulting in formation of LC3-II and its subsequent incorporation into the autophagosome, by the activities of Atg3 and Atg7 proteins (with or without involving the Atg5–12–16L1 complex). After elongation of autophagosome, Atg5 – Atg12 – Atg16L1 dissociates from the autophagosomal membrane. LC3-II is recycled and converted to LC3-I again by Atg4[12].

Fusion of lysosome with autophagosome is mediated by LAMP2 isoforms which are expressed on the lysosomal surface. Inner autophagosomal membrane as well as its protein/ organelle cargo is degraded by reactions catalyzed by Lytic lysosomal enzymes[13]. To degrade specific target substrates, the autophagy system is dependent on certain adapter proteins. p62/SQSTM-1 is one of the most suitable adapter proteins. P62 has both LC3 and ubiquitin binding domains. Therefore, it can bind to ubiquitinated substrates and target them to the autophagy pathway[14].

1.3 Autophagy and Cancer:

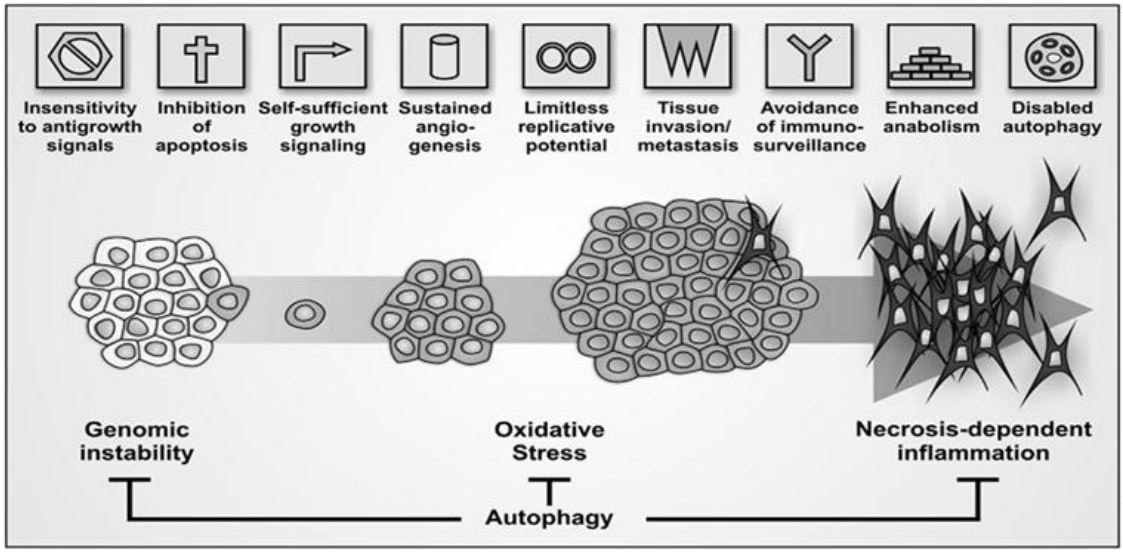
The role of autophagy in cancer and tumorigenesis is highly complex.Autophagy is regulated by multiple cancer associated pathways. These multiple signaling pathways do not function independently. Constant crosstalk takes place among different pathways. Also, certain signals can dominate over others during different stages of tumorigenesis. For example, p53 system can induce autophagy through sestrin expression despite the presence of oncogenes. Therefore, the ultimate induction or inhibition of autophagy seen in cancer cells is the result of integration of signals coming from oncogenes as well as the tumor suppressors.

During cancer, autophagy is activated by signals such as nutrient starvation, the unfolded protein response and hypoxia. Autophagy is also observed during the treatment of cancer with broad spectrum cytotoxic agents.

The tumor-promoting role of autophagy is well established but certain tumor-suppressing effects of autophagy have been discovered recently and it has intrigued scientists causing an extensive research on using autophagy to fight against cancer.

The exact mechanism by which suppression of autophagy promotes tumorigenesis is not yet known. Three hypotheses have been put forward to explain the effects of reduced autophagy on tumorigenesis (Fig 2). First, inhibition of autophagy leads to cell death by necrosis within the tumor. This leads to aggravated local inflammation which might promote tumor growth[15]. Second,old and damaged organelles might accumulate due to compromised autophagy. This may promote tumorigenesis as the damaged organelles can be sources of intrinsic genotoxic or tumorigenic chemical species such as ROS from uncoupled mitochondria[16]. Third, oncogene activation might occur due to chromosomal instability, particularly in cells which have been subjected to metabolic stress. This chromosomal instability occurs due to compromised autophagy[17].However, the exact mechanism by which autophagy preserves genome integrity is not yet known.

Fig 2: Effects of the suppression of autophagy[18]:



2.1. Early phase of Tumorigenesis:

2.1.1 Beclin 1:

Genetic studies of Beclin 1 first revealed that autophagy may have a possible role as a tumor suppressor. Beclin 1 is the mammalian ortholog of the *Saccharomyces cerevisiae* atg6/VPS30 gene. It is an essential protein required for autophagy.

Beclin 1 engages with class III PI 3-kinase complex. This complex converts PtdIns to PtdIns(3)P. PtdIns(3)P is a lipid which is needed for phagophore formation[19]. Class III PI 3-kinase complex contains the catalytic subunit Vps34/PIK3C3 in addition to the regulatory subunit Vps15/PIK3R4 as well as accessory proteins such as Beclin 1 , UVRAG, ATG14L and Rubicon. ATG14L and Rubiconmutually bind to Beclin 1 and act as regulators of PIK3C3 and autophagy. ATG14L increases PIK3C3 activity and autophagy while on other hand, Rubicon inhibits both[20].This indicates that at least two different complexes with different roles are formed by Beclin 1 in autophagy[21].

It was observed that in a significant percentage of breasts, ovarian and prostate cancers, Beclin 1 was monoallelically deleted. Also, decreased Beclin 1 expression was seen in human breast carcinomas when compared to normal breast tissue. On the other hand, ectopic expression of Beclin 1 reduced proliferation of cancer cells *in vitro* and also decreased the tumorigenic potential *in vivo*. For example, autophagy potential was fully restored in tetraploid MCF-7 cells and reduced cell proliferation in xenograft tumors (*in vivo*) and *in vitro*[22]. These observations clearly indicate that Beclin 1 may act as a tumor suppressor. This hypothesis was further confirmed by knockout mouse technology. Beclin 1 heterozygous mice almost certainly develop lung and liver cancers on aging, in addition to hyperproliferative and preneoplastic mammary lesions. The second Beclin 1 allele was found to be retained in all the tumors which developed in Beclin 1 +/- mice. Also, it was neither mutated nor silenced[23]. The above findings further emphasis that beclin 1 is a haploinsufficient tumor suppressor. In addition to this, immortalized baby mouse and immortalized mouse mammary epithelial cells (iMMECs)[24], which under metabolic stress normally exhibit compromised autophagy, showed increased tumorigenesis in nude mouse allografts[25].

2.1.2: UVRAG:

UVRAG is a protein which that shows resistance against UV sensitivity in cells of xerodermapigmentosum, hence it is named as UV irradiation resistance-associated gene protein (UVRAG)[26].UVRAG associates with Beclin 1 and PI3KIII to facilitate formation of autophagosome as well as autophagy activation and inhibition of proliferation and tumorigenicity of human colon cancer cells[27]. In addition, UVRAG also interacts with C-Vps complex and enhances maturation of autophagosome as well as degradation of autophagic cargo. C-Vps is an important component of the endosomal fusion machinery. It regulates autophagy, independent of Beclin 1[28]. The above recently established the role of UVRAG as a positive regulator of Beclin 1.

The UVRAG gene is present on chromosome band region 11q13. This region is usually found altered in malignancies such as breast and colon cancer. Apolyadenine tract (A10 in exon 8) in the UVRAG gene, acts as a target for frameshift mutations, which decreases the autophagy potential with microsatellite instability (MSI) in gastric and colon cancers[29].

2.1.3: mTOR:

Mammalian target of rapamycin (mTOR) is the best known regulator of autophagy. It forms two complexes viz. mTORC1 and mTORC2 (not sensitive to rapamycin). mTORC1 plays an important role in determining the nutrient

availability as well as energy status and detecting the mitogenic signals[30]. mTORC1 activity is inversely related to autophagy induction.

A number of pathways that control mTORC1 activation converge on the TSC (Tiberius sclerosis complex) and Rheb (Ras homolog enriched in brain). Rheb is a small GTPase and activates mTOR complex 1 in its GTP-bound form[31]. TSC is composed of TSC1 (hamartin) and TSC2 (tuberin)[32]. TSC has a GTPase activating protein (GAP) function. GAP hydrolyzes GTP and negatively regulates Rheband inhibits mTORC1 activity[33].

In majority of human cancers, mTORC1 is found to be deregulated as most of the mTORC1 regulating signaling molecules are oncogenes and tumor suppressors. Rheb is found to be overexpressed and this promotes tumorigenesis. High Rheb activity inhibits autophagy and causes cell death[34].

Furthermore, activation of PI3K and its downstream components such as AKT kinase activate mTORC1. Increased PI3K activity is often observed in cancers due to kinase mutations or gene amplification. A deregulated PI3K/AKT/mTOR axis suppresses autophagy as well as induces translation of proteins and proliferation of cells, which acts as a major driving force in tumor growth. Conversely, inhibition of AKT kinase inactivates mTORC1 and leads to increased autophagy[35]. In addition, overexpression of PTEN (Phosphate and tensin homolog), a tumor suppressor and negative regulator of PI3K/AKT pathway also causes increased autophagy[36].

2.1.4. Dual role of p53:

p53 is an important tumor suppressor protein. p53 is encoded by TP53(tumor protein 53) gene in humans. It is a transcriptional factor that regulates apoptosis and cell cycle arrest. In cancer cells, it causes irreparable genomic alterations and induces apoptosis and/or senescence of cancer cells[37]. p53translocates between the cell nucleus and cytoplasm. In the nucleus, it brings about target gene expression which results in arrest of cell cycle and apoptosis. p53 is mainly known as a nuclear transcription factor. However, recently, it has been discovered that even cytoplasmic p53 induces permeabilization of outer membrane of mitochondria as well as apoptosis.

The role of p53 system in regulation of autophagy is dependent on its location in the cell. Nuclear p53 controls transcription of regulators of mTOR pathway and thereby induces autophagy. AMPK (5'adenosine monophosphate-activated protein kinase) (beta), TSC2 & PTEN (Phosphatase and tensin homolog), which are negative regulators of mTORC1, are target genes of p53[38]. In addition, sestrin1 and sestrin2 are two additional p53 target genes which provide the link between mTORC1 activity and activation of p53[39]. Sestrin stimulates TSC activation (mediated by AMPK) and consequently inhibits mTORC1 activity. More importantly, expression of sestrin overrides mTOR activation and cell growth mediated by oncogenes. Also, sestrin2 is needed for induction of autophagy in response to stress and nutrient starvation[40]. DRAM (damage regulated autophagy modulator) is another target of p53. DRAM is a positive regulator of autophagy[41]. DRAM is required for p53-mediated induction of autophagy and apoptosis. However, the exact mechanism by which DRAM induces autophagy is not yet known[42].

On the contrary, cytoplasmic p53 inhibits autophagy in response to autophagy-inducing agents, independently of its transcriptional function in the nucleus. Active p53 accumulates in the cytoplasm, activates Bax(Bcl2 associated protein) and/or inhibits anti-apoptotic members of Bcl2 protein family and consequently induces mitochondrial cell death[43]. Furthermore,

cytoplasmic p53 exerts a tonic inhibition of the autophagic flow. Indeed, human, mouse and nematode cells when subjected to pharmacological inhibition of p53 showed several signs of enhanced autophagy, both *in vitro* and *in vivo*. In addition, inhibition of p53 also induces autophagy in enucleated cells further suggesting that cytoplasmic p53 functions independently of nuclear p53[44]. The above clearly shows that p53 regulates autophagy differentially dependent on its location.

In almost 50% of cancers, p53 is found to carry mutations[45]. Also, numerous studies (as described above) point out that autophagy is found to be suppressed in early phase of tumorigenesis. It can be speculated that the cancer associated mutations hit p53 activity in multiple ways. Such mutations may inactivate the mitochondrial (pro-apoptotic) and nuclear (pro-apoptotic and pro-autophagic) functions of p53 and may not affect the suppression of autophagy mediated by cytoplasmic p53[46].

2.1.5: ROS and autophagy:

ROS (Reactive oxygen species) are a group of molecules that includes superoxide anion, hydroxyl radical and hydrogen peroxide. These ROS molecules are produced in the cells through oxygen metabolism. They can be important signaling molecules at low levels. However, ROS can lead to oxidative stress due to elevated intracellular levels, which can cause damage to proteins, lipids and DNA. In a normal cell, majority of ROS is formed in the mitochondria through the electrons leaked from the electron transport chain during oxidative phosphorylation.

The induction of autophagy due to stress and nutrient starvation requires some amount of H_2O_2 production. H_2O_2 oxidizes ATG4. ATG4 is involved in maturation of ATG8 protein. ATG8 maturation leads to LC3 associated phagosome formation[47]. ROS can also regulate autophagy indirectly through activation of AMPK. Activated AMPK inhibits mTORC1 which leads to induction of autophagy[48].

In early phase of tumorigenesis, suppression of autophagy leads to increased ROS production due to accumulation of damaged mitochondria. Increased ROS generation leads to activation of pro-inflammatory factors like NLRP3 (NOD-like receptor family containing pyrin domain), inflammasome (a multiprotein complex that causes maturation and secretion of pro-inflammatory cytokines). Increased inflammation causes release of certain molecules like the cytokines, chemokines, etc. from macrophages and mast cells. This leads to recruitment and infiltration of leukocytes at the site of inflammation. Inflammation is considered an important risk of cancer initiation in liver, lungs, etc[49].

2.1.6. Bif-1:

Bif-1(BAX interacting factor-1) has recently been identified as a tumor suppressor. Bif-1 increases the activity of class III PI 3-kinase by interacting with Beclin 1 through UVRAG. Conversely, formation of autophagosome is suppressed by loss of Bif-1. Also, development of spontaneous tumors was enhanced on knocking out Bif-1 protein. The exact way by which Bif-1 acts as a tumor suppressor has still not been established, it can be speculated that Bif-1 is involved in autophagy and through this involvement, it suppresses tumors. However, it should also be noted that cell survival is prolonged under starvation conditions by deletion of Bif-1. This may occur due to increased genome instability or due to down regulation of apoptosis. Therefore, it can be assumed that Bif-1 may primarily act as a regulator of apoptosis along with autophagy[50].

2.1.7. Atg4C:

It has recently been found that depletion of Atg4C also leads to reduced autophagy in cells. Atg4C is a protease that mediates formation of autophagosome by processing of LC3/ATG8. Furthermore, ATG4C -/- mice shows reduced starvation-induced autophagy. It also displays increased susceptibility to develop fibrosarcomas induced by chemical carcinogens[51].

2.1.8. Summary of the early phase of tumorigenesis:

Table 1: Role of different proteins and regulators of autophagy:

Proteins	Role in normal cell	Role in cancer cell
Beclin1	Beclin 1 engages with class III PI 3-kinase complex. This complex converts PtdIns to PtdIns(3)P. PtdIns(3)P is a is needed for phagophore formation. Beclin 1 recruits other proteins and elongates the phagophore.	Beclin 1 is found to be monoallelically deleted in a significant percentage of breasts, ovarian, prostate cancers and other cancers.
UVRAG	UVRAG associates with Beclin 1 and PI3KIII to facilitate formation of autophagosome. It also enhances maturation of autophagosome as well as degradation of autophagic cargo.	UVRAG gene is subjected to frameshift mutation which decreases the autophagy potential.
Bif 1	Bif-1 increases the activity of class III PI 3-kinase by interacting with Beclin 1 through UVRAG	Bif 1 is found to be eliminated.
Atg4c	It is a protease that mediates formation of autophagosome by processing of LC3/ATG8.	Not specified
mTOR	Negative regulator of autophagy.	In majority of human cancers, mTOR is found to be deregulated which leads to increased mTORC1 activity and consequently suppression of autophagy.
Nuclear p53	Nuclear p53 controls transcription of regulators of mTOR pathway and thereby induces autophagy.	It is found to be mutated in almost 50% of cancers.
Cytoplasmic p53	Cytoplasmic p53 inhibits autophagy in response to autophagy-inducing agents	p53 mutations do not affect the activity of cytoplasmic p53 (speculated).

ROS	Low level of H_2O_2 oxidizes ATG4 which is involved in maturation of ATG8 protein. ATG8 maturation leads to LC3 associated phagosome formation. Also activates AMPK.	Suppression of autophagy leads to increased ROS production which leads to activation of pro-inflammatory factors.

2.2. Later stages of Tumorigenesis:

Metastasis, angiogenesis and epithelial-mesenchymal transition (EMT) characterize tumor progression and aggressiveness. Metastasis is a process which causes cancer cells to spread to distant organ sites. The first step of metastasis is EMT which leads to loss of epithelial properties and acquirement of mesenchymal properties. This leads to increased mobility of tumor cells. Increased autophagy during later stages of tumorigenesis is shown to be associated with metastasis. Autophagy activates EMT and promotes metastasis in hepatocellular carcinoma (HCC) and mammary epithelial cells (MEC). Autophagy also promotes resistance to anoikis (detachment-induced cell death). Anoikis is necessary for metastasis of tumor cells. Autophagy also promotes tumor cell survival under metabolic stress conditions[52].

Increased autophagy activity (seen by increase in the level of LC3-II-FITC punctate, a biomarker of autophagy) seen in tumor cells in response to cancer treatment represents an adaptive response by the tumor cells so as to survive the therapeutic attack. In addition, accumulation of autophagosomes is also observed in tumor cells after exposure to chemotherapeutics such as the DNA alkylating agent temozolomide, etc. It can be hypothesized that inhibition of autophagy will synergize with other chemotherapies and facilitate in effectively eliminating cancer cells. Recent studies support this hypothesis in multiple tumor types and in response to wide range of chemotherapeutic agents. Inhibition of autophagy is normally brought about by 3-methyladenine (3-MA), bafilomycinA1 (BafA), and chloroquine (CQ). 3-MA inhibits autophagy at the sequestration step (early stage). BafA, a vacuolar H+-ATPase inhibitor, prevents lysosomal function and blocks auto phagosome degradation, a late stage of the process. CQ, a weak base blocks cargo degradation. It gets trapped in acidic vesicles and increases intra lysosomal pH. Although these compounds can effectively suppress autophagy and are widely regarded as autophagy inhibitors, none are specific for autophagy alone. These drugs also have multiple side effects and affect other cellular activities, such as endocytosis, intracellular trafficking and lysosomal biogenesis and function[53].

The effect of inhibiting autophagy in combination with anti-cancer therapies has been studied in multiple tumor models, including glioma, multiple myeloma, breast, colon and prostate cancer as shown in Table 2.

Table 2[54]: Autophagy inhibition during cancer therapy

Cancer type	Primary treatment	Target	Method(s) of autophagy inhibition
Breast	Trastuzumab	Her 2	3-MA, BafA, RNAi (LC3)
	CPT	DNA Topoisomerase	3-MA, BafA, RNAi(BECN1,ATG7)
CML	Imatinib	BCR/ABL	CQ, BafA, RNAi(ATG5,ATG7)
	INNO-406	BCR/ABL	CQ
	SAHA	HDAC	CQ,3-MA
Colon	Vorinostat	HDAC	CQ,RNAi(ATG7)
	Radiation	DNA damage	RNAi(BECN1,ATG3,ATG4B,ATG5)
Glioma	AKTi-1/2	AKT	CQ
	PI-103	PI3k,mTOR	CQ
	Imatinib	Tyrosine kinase	BafA
	Radiation	DNA damage	3-MA,BafA RNAi(BECN1,ATG5)
	Radiation	DNA damage	3-MA,BafA
Malignant rhabdoid tumor	FK228	HDAC	CQ
Multiple myeloma	8-aminoadenosine	DNA synthesis	CQ
Prostate	ADI-PEG 20	Arginine in blood	CQ,3-MA,RNAi(BECN1)

Abbreviations used:BafA, bafilomycin A1; CPT, camptothecin; CQ, chloroquine; HDAC, histone deacetylases; MNNG, N-methyl-N'- nitro-N-nitrosoguanidine; mTOR, mammaliantarget of rapamycin; PI3K, phosphoinositide 3-kinase; RNAi, RNA interference (ATG target is in parentheses).

3. Modulation of autophagy promotes chemotherapeutic efficacy:

Manipulations of autophagy can potentially influence treatment outcomes in cancer[55]. Treatment by chemotherapy and radiation often result in increased autophagy activity in tumor cells. A lot of studies have shown that tumor cells get sensitized to respond to a range of cancer therapies by inhibition of autophagy[56]. On the other hand, certain nanomaterials and plant phytoconstituents induce autophagy and promote cell death by overstimulation of autophagy[57]. It can be said that treatment of cancer by modulation of autophagy is highly dependent on the stage of cancer. Therefore, proper diagnosis and judgment greatly influence the treatment of cancer by modulation of autophagy.

3.1. Nanoparticles:

Development of nano drug delivery systems for established drugs has recently generated a lot of interest. A broad range of composite materials such as metals, polymers or semiconductors having different geometrical shapes such as sphere, rod or prism have been developed so as to be utilized in a variety of cancer diagnostic procedures and treatment[58]. Nanoparticles (NPs) are recognized as a novel class of materials that can induce autophagy in cells. Studies indicate that nanomaterials like the Quantum Dots (QD),

nanowires, etc. are capable of inducing autophagy in mesenchymal stem cells, HeLa cells as well as other cell types[59]. Studies have also shown that NPs are also capable of selectively over stimulating autophagy in cancer cells causing cell death without having a significant effect on the level of autophagy in normal cells. Thus, NPs are capable of exhibiting an intrinsic toxicity towards cancer cells.

On the other hand, co administration of autophagy inhibitors like 3-methyladenine (3-MA) and Chloroquine (CQ) has been shown to significantly increase the therapeutic effects of certain selected nanoparticle formulations. Thus, nanoparticles can bring about modulation of autophagy. Also, after endocytosis of nanoparticles, autophagy significantly affects their therapeutic effects as a drug carrier. Thereforee, it is essential to study the effect of NPs on autophagy both from a nano toxicological as well from as a therapeutic viewpoint[60].

3.1.1. Combination of autophagy inhibitors and drug-loaded biodegradable polymer nanoparticles[61]:

Targeted drug delivery can be achieved by using nanoparticles. The most extensively used polymer for the synthesis of NPs is Poly (lactide-co-glycolide) (PLGA). PLGA is a polymer which is biodegradable and is FDA approved[62]. The PLGA-based NPs are applicable for both hydrophilic or hydrophobic drugs and biological therapeutic macromolecules such as peptide and protein drugs[63]. The interaction between tumor cells and the nanoparticles can be significantly improved by modification of the surface of PLGA-based NPs, thus resulting in longer half-life, higher cellular uptake and better targeting effects. The moiety that is used most often for surface modification is polyethylene glycol (PEG). Modification of PLGA NPs with PEG and TPGS (d-alpha tocopheryl polyethylene glycol 1000 succinate) improves biocompatibility of the NPs, providing a hydrophilic layer on the surface and enabling them to evade the reticulo-endothelial system (RES)[64].

Coumarin-6-loaded or docetaxel (DTX)-loaded nanoparticles of PLGA or PLGA/PEG or PLGA/TPGS with or without cholic acid conjugation denoted by CA-PLGA, PLGA-b-PEG, CA-PLGA-b-PEG, PLGA-b-TPGS, and CA-PLGA-b-TPGS NPs respectively, were prepared by a modified nano precipitation technique. Drug release, cellular uptake and bio distribution of NPs largely depends on theirparticle size and surface properties[65].Thereforee, size, size distribution and other characteristics were measured using dynamic light scattering (DLS). These properties are shown in Table 3.

Table 3:Characterization of DTX-loaded NPs

Polymer	Size(nm)	PDI	ZP(mV)	LC(%)	EE(%)
PLGA	136.23±5.1	0.146	-23.6± 3.4	8.34	78.36
CA-PLGA	118.6±3.7	0.141	-25.2±2.7	9.17	84.31
PLGA-b-PEG	125.3±4.1	0.120	-15.8±2.1	8.49	86.05
PLGA-b-TPGS	123.7±4.5	0.137	-18.2±2.7	8.75	85.91
CA-PLGA-b-PEG	101.9±3.2	0.133	-11.9±1.9	9.86	95.27
CA-PLGA-b-TPGS	98.6 ±3.7	0.125	-13.9±3.3	10.08	97.85

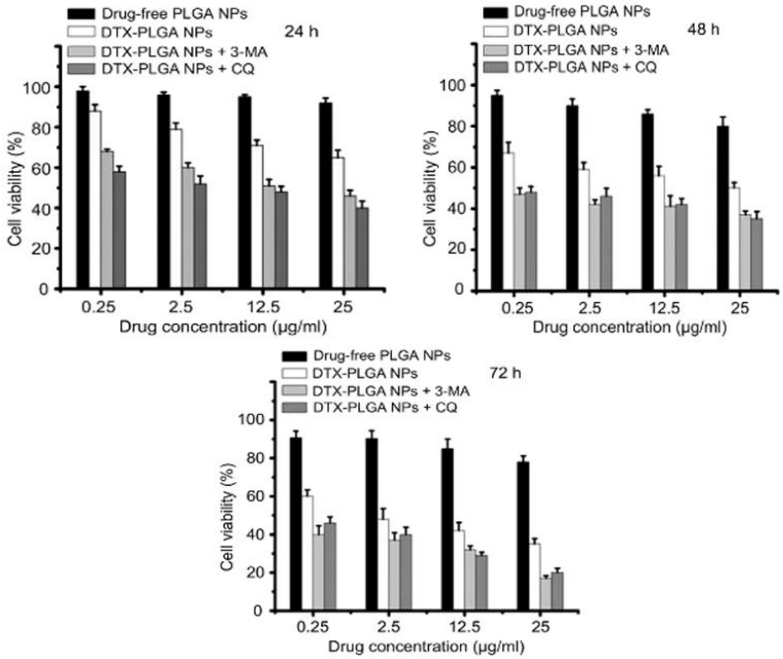
PDI =polydispersity index, ZP = zeta potential, LC = loading content,
EE =encapsulation efficiency, n =3.

As mentioned earlier, autophagy inhibitors when used in combination with chemotherapeutic drugs, enhance the intracellular drug delivery by nanoparticle formulation. Therefore, autophagy

inhibitor substances such as 3-MA and CQ were used in combination with DTX-loaded PLGA-NPs. Apparent reduction in proliferation of MCF-7 cells was observed in cells treated with DTX-loaded PLGA in time and dose dependent manner.

The survival rate of MCF-7 cells apparently decreased. The IC 50 values of MCF-7 cells after 24, 48, 72 h incubation with the DTX-PLGA NPs, DTX-PLGA NPs þ 3-MA and DTX-PLGA NPs þ CQ are shown in Fig 3

Fig 3: Graphs of cell viability (%) vs drug concentration (mcg/ml) at time intervals of 24hrs, 48hrs and 72hrs



In addition to this, the curative effect of chemotherapeutic drug-loaded NPs in combination with autophagy inhibitors was investigated using the xenograft SCID mice model with the MCF-7 cell line. Mice received intraperitoneal injections of DTX-loaded PLGA NPs and Taxotere every 4 days for five consecutive cycles. CQ was used as autophagy inhibitor. Therefore, mice also received intraperitoneal injections of CQ every 2 days for five consecutive cycles. The tumor size of the mice was recorded every 4 days until the 20th day. Control mice were injected with physiological saline solution. The tumor growth observed in the physiological saline group was similar to the growth seen in drug-free PLGA-based NPs. Also, injection of CQ alone did not have significant inhibition effect on the tumor growth. When the DTX-loaded PLGA NPs were combined with CQ, then only the tumor growth was significantly reduced. The weight and volume of the tumor observed in mice treated with the PLGA NP formulation (with CQ) for 20 days was half of that seen in mice treated with PLGA NP based formulation (without CQ).

In conclusion, it can be said that cholic acid conjugated docetaxel loaded PLGA nanoparticles combined with autophagy inhibitors such as 3-MA and CQ can greatly enhance the therapeutic effects both in vitro and in vivo.

3.1.2. Induction of autophagy in lung epithelial cancer cells by iron oxide nanoparticles[66]:

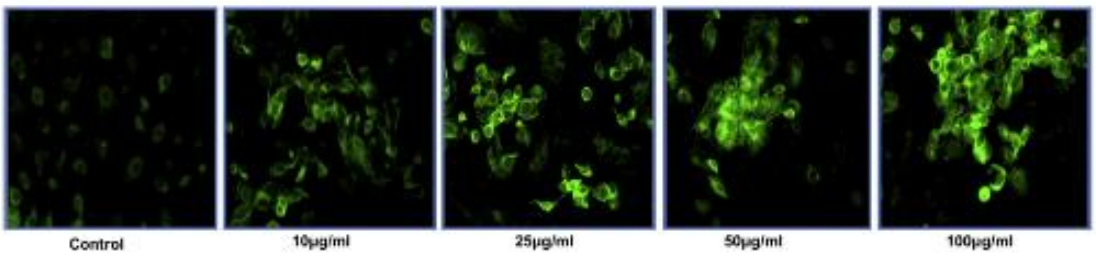
Iron oxide nanoparticles can selectively induce autophagy in lung epithelial cancer cells leading

to overstimulation of autophagy and ultimately cell death by necrosis[67]. Iron oxide NPs were synthesized to assess their autophagy potential and cytotoxic effect (cell death) on both A549 and IMR-90 cells (lung epithelial cells), which were used as test models for cancer cells and normal cells, respectively. A549 cells are observed in early stage lung cancer. Iron oxide NPs were synthesized by sol-gel method. Synthesized iron oxide NPs was analyzed by Transmission electron microscopy (TEM) to assess their particle size. Particle size range was observed to be between 30 and 65 nm with an average size of 51.34±14.71 nm. Similarly, X ray diffraction (XRD) patterns of Iron oxide NPs revealed that the two major XRD peaks are obtained at 2θ= 32.34° and 34.84°. Also, from the data it was observed that the nanoparticles were in cubic form with primitive lattice type.

Cells were exposed to increasing concentrations of Iron oxide NPs (10-100 mg/ml) and cell death was analyzed by Annexin/PI staining. Significant increase in cell death was observed at exposure concentrations >25 mg/ml when compared to control. In addition to this, a certain percentage of cells stained positive for propium iodide (PI). This indicates a loss of membrane structure which clearly suggests cell death by necrosis. Also, experiments related to important events which occur during cell death by necrosis such as loss of mitochondrial membrane potential (MMP), depletion of ATP, etc. were carried out. It was seen that significant loss of MMP and ATP occurs in A549 cells on exposure to increased concentration of Iron oxide NPs (Fig 4a and 4b). Again, this suggests that Iron oxide NPs induce cell death in tumor cells by necrosis and not by apoptosis. To further verify the claim that Iron oxide NPs selectively induce necrotic cell death in cancer cells and not normal cells, normal human lung fibroblast cells (IMR-90 cells) were treated with Iron oxide NPs and insignificant cell death was observed in comparison with control.

In addition, experiments were performed to find modulation of ROS in the experimental system as ROS is a known inducer of autophagy. 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) was loaded on to A549 cells. H2DCFDA is a ROS measuring probe. Iron oxide NPs induced ROS generation was deduced by observing the increase in fluorescence intensity in time and concentration dependent manner (Fig 4). Exposure to 100 mg/ml generated the highest amount of ROS after 24 hrs, indicating about 10 fold increases over untreated A549 control cells.

Fig 4: Fluorescence intensity in time and concentration dependent manner indicating ROS generated by iron oxide NPs



Further studies to properly evaluate the toxicity of Iron oxide NPs have so far proved to be inconclusive. Some studies indicate low or no toxicity while others indicate significant toxicity of Iron oxide NPs. Tests performed by Karlsson and his coworkers on human lung epithelial cell line (A549 cells) have shown that up to concentrations of 20-80 mg/ml, bare iron oxide NPs have very low toxicity. However, many other tests contradict this result. Tests performed by

Mohd Imran Khan and his coworkers' revealed significant cytotoxicity of bare Iron oxide NPs to A549 cells but not to normal human lung fibroblast cells (IMR-90). In conclusion, Iron oxide NPs induced autophagy can be used for cancer therapy. However, more studies are needed to carry out to ascertain the cytotoxicity of this nanomaterial.

3.2.Phytoconstituents:

3.2.1. 6-Shogaol[68]:

6-Shogaol is an active constituent of Ginger, a well-known herbaceous perennial plant, which is widely distributed in the tropical and subtropical regions. A number of studies have pointed out the anti-inflammatory and anti-helicobacter pylori effects of 6-Shogaol. 6-Shogaol has also been shown to induce apoptosis in human colorectal carcinoma cells.

Jen-Yu Hung et al. investigated the cell growth inhibition activity of 6-Shogaol on human non-small cell lung cancer cell line, A549.The results indicated that 6-Shogaol adversely affected the cell proliferation and clonogenic survival of A549 cells. Clonogenicity reduced in a concentration dependent manner after treatment with 6-Shogaol. Furthermore, 6-Shogaol had no significant effect on IMR-90 cells. IMR-90 cells are the normal lung cell line (Fig. 5). The results also showed that 6-Shogaol induces only a small amount of apoptotic cell death in A549 cells after 24 and 48 hours of treatment. This was determined by using a pan-caspase inhibitor to block caspase activity in A549 cells (Fig. 6). The transmission electron microscopy (TEM) results also showed that the nuclei maintained their integrity and displayed dispersed chromatin in most of the cells. This is not consistent with apoptosis.

Fig. 5: A - Cell proliferation inhibition effect of 6-shogaol in A549 cells. B- The effect of 6-shogaol on the cell proliferation of IMR-90 cells.

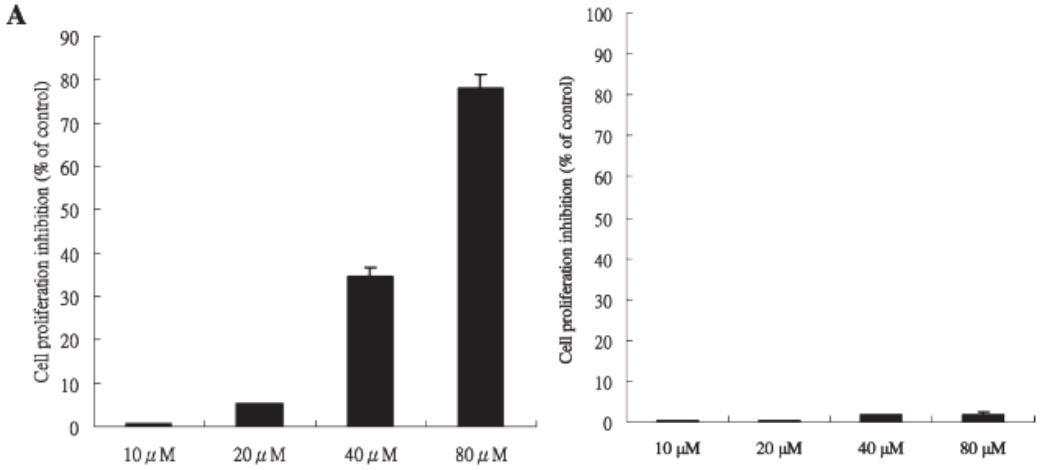
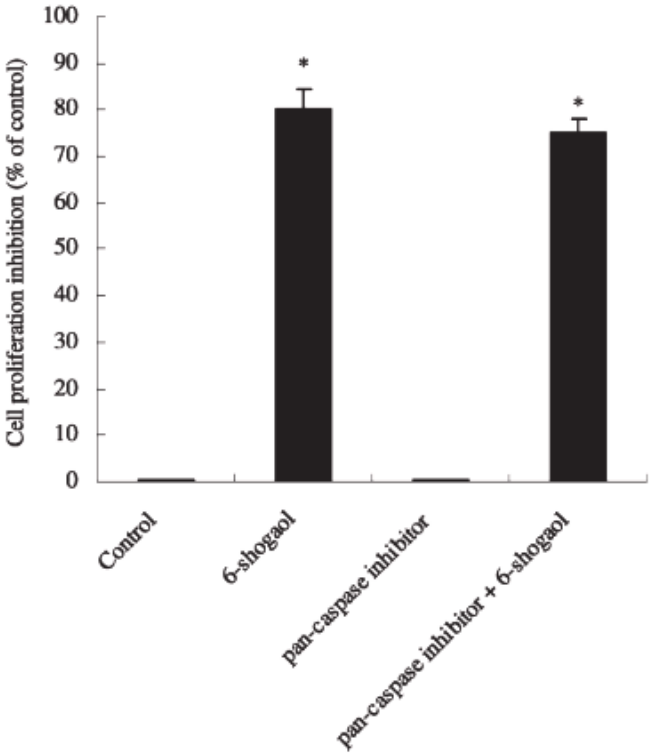
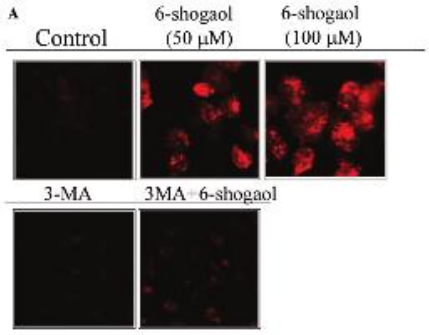


Fig. 6:The effect of pan-caspase inhibitor on 6-shogaol mediated proliferation inhibition.



The results further indicated the accumulation of the autophagic vacuoles after 24 hours of treatment with 6-Shogaol. This was again determined by increased fluorescence expression ofmonodansylcadaverine(MDC). MDC was used to label autophagic vacuoles in A549 cells. In addition, use of 3-methyladenine (3-MA), an autophagy inhibitor decreased the red fluorescence in both control and 6-Shogaol treated cells. This confirmed that 6-Shogaol induces autophagy in A549 cells.

Fig. 7:MDC staining using a fluorescence microscopy for different combinations of treatment

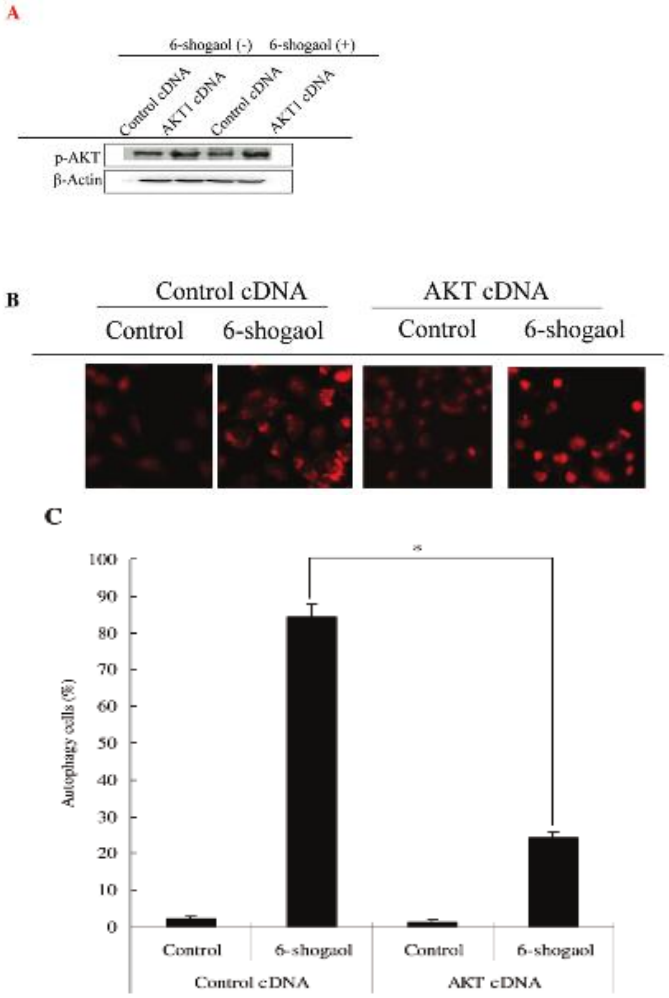


Shogaol treatment also led to a significant time-dependent decrease in the phosphorylation of AKT proteins in A549 cells but did not cause any change in the protein levels of total AKT. Diminished level of phosphorylated form of mTOR was also seen. mTOR is a downstream target of AKT. This indicates that 6-Shogaol inhibits the AKT/mTOR signaling pathway.

To further confirm that AKT/mTOR signaling pathway is targeted in 6-Shogaol induced

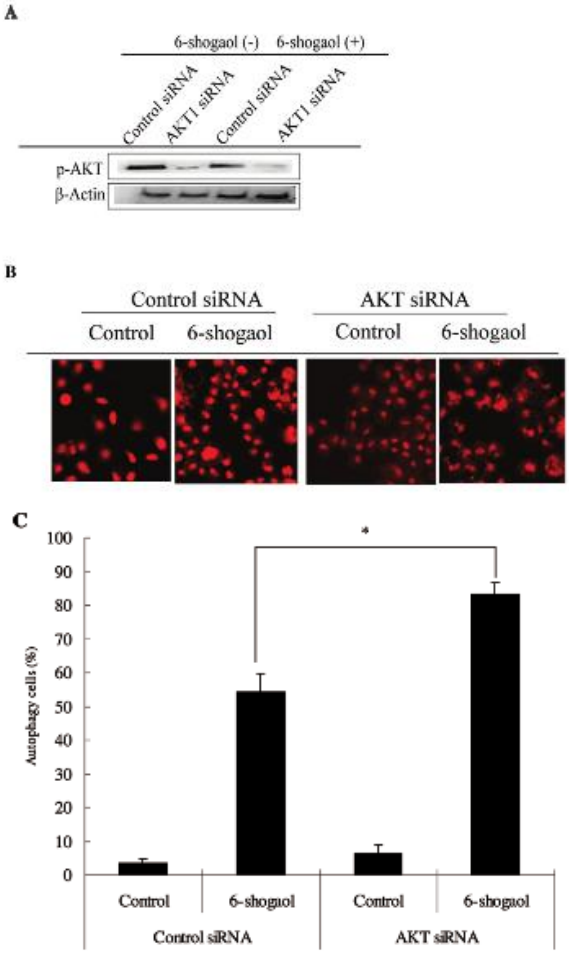
autophagy, A549 cells were transaffected by a constitutively active form of AKT cDNA. The transaffected cells over expressed AKT proteins. The effect of 6-Shogaol on these transaffected cells was observed. The transaffected cells were clearly more resistant to 6-Shogaol induced autophagy.Acridine orange and MDC stain were used to determine the degree of cell death by autophagy.

Fig. 8:A-Up regulation of AKT phosphorylation by active AKT cDNA transfection. B- The induction of autophagy of 6-shogaol in AKT cDNA transfected cells. C- The quantification of MDC staining using a fluorescence microscopy.



On the other hand,A549 cells were transfected with control oligonucleotide or AKT siRNA, then treated with 6-shogoal (100 µM) for the indicated times (30 min for phospho-AKT assay and 24 h for autophagy assay). Again, acridine orange and MDC stain were used to determine the degree of cell death by autophagy.. Exposure to 6-Shogaol led to a significant increase in the number of autophagy cells in AKT siRNA transaffected cells than in control siRNA trans affected cells (Fig. 9).

Fig. 9:A- Genetic suppression of AKT phosphorylation by AKT siRNA transfection. B- 6-Shogaol induced autophagy in control and AKT siRNA transfected cells. C- The quantification of MDC staining using a fluorescence microscopy.



The above results clearly demonstrate that 6-Shogaol induces autophagy in A549 cells by inhibiting the AKT mTOR pathway, which leads to autophagic cell death by overstimulation of autophagy. Therefore, the use of 6-Shogaol for lung cancer therapy can definitely be explored.

CONCLUSION

As it has been thoroughly emphasized throughout the article, autophagy itself and role of autophagy in cancer are highly complex phenomena. Current evidence directs that autophagy suppresses tumorigenesis, especially at early stages of tumor formation. On the other hand, in a tumor which is already established autophagy will offer it a survival pathway in response to stresses like chemotherapy and will enable it to progress. Hence we can clearly surmise here that the selection of an approach of autophagy modulation depends on crucial parameters like the type of tissue, the stage of cancer, ongoing chemotherapy and availability of autophagy modulating agents. So the success of the therapy will lie in accurate recognition of functional status of autophagy in that particular tumor and the choice of autophagy modulating agents.

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*Corresponding Author:
omkarkulkarni2101@gmail.com