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Review article

Graphene oxide nanoarchitectures in cancer biology: Nano-modulators of autophagy and apoptosis

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ABSTRACT

Nanotechnology is a growing field, with many potential biomedical applications of nanomedicine for the treatment of different diseases, particularly cancer, on the horizon. Graphene oxide (GO) nanoparticles can act as carbon-based nanocarriers with advantages such as a large surface area, good mechanical strength, and the capacity for surface modification. These nanostructures have been extensively used in cancer therapy for drug and gene delivery, photothermal therapy, overcoming chemotherapy resistance, and for imaging procedures. In the current review, we focus on the biological functions of GO nanoparticles as regulators of apoptosis and

Abbreviations: GO, graphene oxide; ROS, reactive oxygen species; TGF-β, transforming growth factor-beta; EMT, epithelial-to-mesenchymal transition; siRNA, small interfering RNA; IL, interleukin; TNF-β, tumor necrosis factor-α; IFN-α, interferon-gamma; CS, chitosan; PCD, programmed cell death; Cyt C, cytochrome C; MOMP, mitochondrial outer membrane permeabilization; TNFR1, TNF-receptor 1; TRAIL, TNF-related apoptosis-inducing ligand; TRADD, TNFR1 associated death domain; DISC, death-inducing signaling complex; GSK-3, glycogen synthase kinase-3; NF-κB, nuclear factor-kappaB; ncRNA, non-coding RNA; miRNAs, microRNAs; lncRNAs, long non-coding RNAs; ULK1, unc-51-like kinase 1; ATG, autophagy-related gene; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; PI3K, phosphoinositide 3-kinase; PI3P, phosphatidylinositol triphosphate; PKC, protein kinase C; GPX4, glutathione peroxidase 4; AMPK, AMP-activated protein kinase; HMSNs, hollow mesoporous silica nanoparticles; GO-AgNPs, GO-silver nanoparticles; PEG, polyethylene glycol; ICG, indocyanine green; MTH1, MutT homolog 1; GRP78, glucose-regulated protein 78; TLRs, toll-like receptors; MyD88, myeloid differentiation primary response gene 88; TRAF6, TNF receptor-associated factor 6; JNK, c-Jun N-terminal kinase; MMP, mitochondrial membrane potential; DOX, doxorubicin; AS, astrocytes; ERK1/2, extracellular regulated kinase 1/2; Nrf2, nuclear factor erythroid 2-related factor 2; HY, hypericin; P-l-Arg, poly-l-arginine; HDAC1, histone deacetylase kinase 1; HIF-1, hypoxia inducible factor-1; RPE, R-phycoerythrin; Akt, protein kinase-B; MDR, multidrug resistance..

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autophagy, the two major forms of programmed cell death. GO nanoparticles can either induce or inhibit autophagy in cancer cells, depending on the conditions. By stimulating autophagy, GO nanocarriers can promote the sensitivity of cancer cells to chemotherapy. However, by impairing autophagy flux, GO nanoparticles can reduce cell survival and enhance inflammation. Similarly, GO nanomaterials can increase ROS production and induce DNA damage, thereby sensitizing cancer cells to apoptosis. In vitro and in vivo experiments have investigated whether GO nanomaterials show any toxicity in major body organs, such as the brain, liver, spleen, and heart. Molecular pathways, such as ATG, MAPK, JNK, and Akt, can be regulated by GO nanomaterials, leading to effects on autophagy and apoptosis. These topics are discussed in this review to shed some lights towards the biomedical potential of GO nanoparticles and their biocompatibility, paving the way for their future application in clinical trials.

1. Introduction

1.1. Graphene oxide: Basics and synthesis

The application of carbon-based materials in science and technology has undergone significant advances, and different allotropes of pure carbon have been prepared with different combinations of sp1, sp2, and sp3 hybridization of their molecular orbitals [1–3]. Graphene oxide (GO) has a two-dimensional crystal structure with oxygen groups and a single atomic layer [4]. The large surface area and hydrophobic nature of GO emanate from an atomic layer that is comprised of sp^2 and sp^3 carbon atoms organized in a hexagonal grid as its basic skeleton [5–8]. Although Hummer's method is the most common strategy for synthesis of GO from graphite [9], the basic features of GO derived from various synthetic methods are similar, and there are changes in some characteristics, including lateral dimensions and number of oxygen groups. The water solubility of GO emanates from the presence of oxygen-containing groups, including hydroxyl groups, carbonyl groups, and epoxy groups [10]. The carboxyl group is the most important oxygen-containing group since it provides the basis for the development of functionalized GO. The aromatic lattice found in GO is essential for the modification and functionalization of GO via π-π stacking or hydrophobic interaction. The modification of GO with hydrophilic biomaterials can significantly increase the solubility and stability of GO nanostructures [11]. To date, a number of carbon-based nanomaterials have been prepared, which include sp2 hybridized carbon atoms in a hexagonal lattice typical of graphite [12]. However, a wide variety of carbon-based nanostructures with different structures, morphologies and characteristics can be synthesized. Four major categories of carbon-based nanomaterials have been defined, including zero-, one-, two-, and three-dimensional carbon nanostructures. In addition to nanodiamonds, three-dimensional carbon nanostructures include graphemic superstructures and nanotube-graphene hybrids, as well as one- and two-dimensional structures [13–16].

Graphene is composed of a single-atom thick layer of sp2 hybridized carbon atoms, with good electrochemical properties, such as high thermal conductivity, optical absorption, impermeability to gases, a large surface area, and good mechanical strength [17]. GO can be produced by Hummers and Offemann reactions involving oxidation of graphite in an acidic medium [18–21]. Fig. 1 demonstrates the synthesis of GO started via mixing graphite and H_2SO_4:HNO_3. Then, it is free-dried after mixing with deionized water. Next, H_2SO_4 and $KMnO_4$ are used to finally synthesize GO. These are considered as Hummers and Offeman methods for GO preparation [22,23].

GO has many unique physico-chemical properties that are important for its application in science and technology. The disruption of the conjugation between the sp2 hybridized bonds provides the electrical insulating property of GO. Moreover, the oxidation level is important since GO can also function as a semiconductor depending on the degree of oxidation [24]. The oxidation of graphene to produce GO generates numerous functional groups on the surface of GO, including hydroxyl and epoxy groups. Furthermore, the oxidation of graphene increases its hydrophilicity [19]. Its high surface area (890 m^2/g) and good mechanical strength are beneficial properties of GO [18,20]. Due to the presence of functional groups at the surface of GO, functionalization can be performed by attaching DNA, proteins, or antibodies to increase its

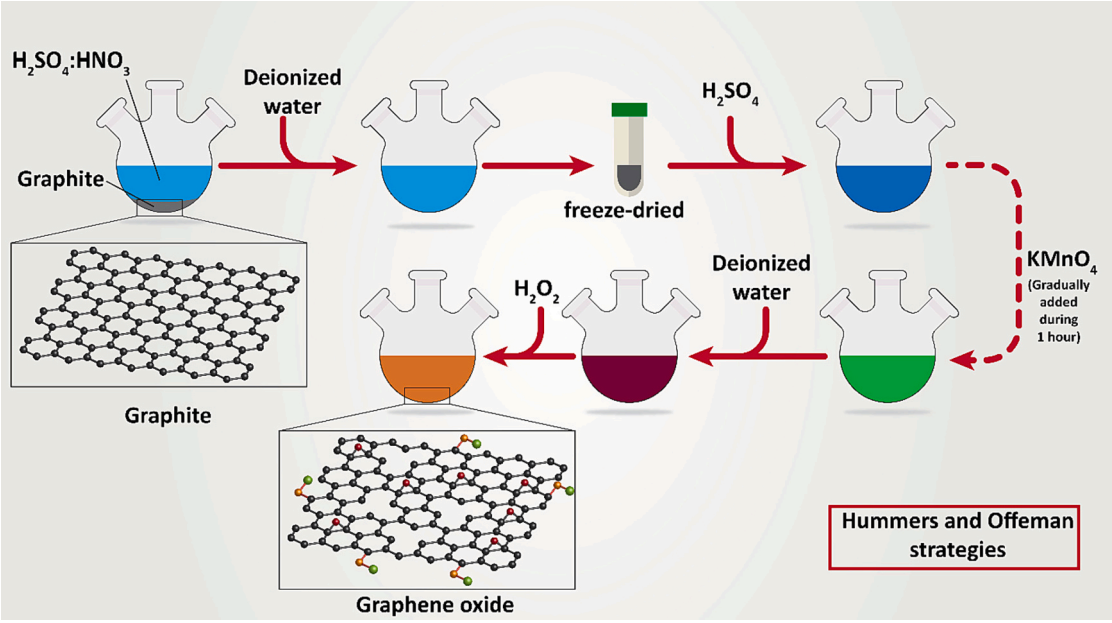

Fig. 1. A schematic representation of GO synthesis from graphite.

biomedical applications [25–27]. The high adsorption capacity for various molecules and the ability to undergo covalent conjugation with proteins are other benefits of GO [28].

1.2. Graphene oxide in cancer therapy: an overview

GO nanostructures have attracted much attention in biomedical science for drug and gene delivery. Because cancer remains a therapeutic challenge nowadays, many studies have focused on the use of GO nanoparticles in cancer therapy. Experiments have focused on developing smart GO structures, such as stimulus-responsive nanostructures, theranostics systems, and enabling photodynamic and photothermal therapy [29]. Cancer cells exposed to GO on their own can have their viability reduced by up to 52.7% [30]. The mechanism of GO-mediated reduction of cell viability has been attributed to increasing the production of reactive oxygen species (ROS). ROS can cause mitochondrial dysfunction and DNA damage, resulting in decreased cancer cell viability [31–36]. Several experiments have shown that GO nanostructures are generally biocompatible, with only limited toxicity towards normal cells [37]. Furthermore, GO nanostructures can suppress the migration and metastasis of cancer cells, and also inhibit prostaglandin-mediated inflammatory responses [38]. However, there is some controversy about the anti-metastatic activity of GO nanostructures. It was proposed that GO nanostructures could inhibit cancer cell invasion by disturbing the actin cytoskeleton [37]. However, another study suggested that GO could actually enhance the metastasis and migration of cancer cells. Low-dose GO has been shown to stimulate the production of transforming growth factor-beta (TGF-β) to induce the epithelial-to-mesenchymal transition (EMT), resulting in enhanced metastasis [39]. GO nanoparticles can interact with cancer cell membranes leading to internalization into the cytoplasm of cancer cells [40].

The important aspect related to GO platforms is their application for synergistic cancer therapy. For treatment of glioblastoma, bortezomib is used, and GO is able to elevate cytotoxicity of this drug against tumor cells [41]. A combination of nitrogen-doped GO and manganese oxide can be utilized for purpose of photodynamic therapy and imaging in cancer [42]. Moreover, by providing targeted drug delivery, they can exert a synergistic impact in tumor suppression [43,44].

GO has been widely investigated for drug delivery in cancer therapy. For instance, melittin (a bee venom peptide) was loaded onto GO-based magnetic nanocomposites to produce pores in cancer cell membranes and stimulate cell lysis. These carriers could provide targeted delivery and sustained release of melittin, increasing its cytotoxicity against cancer cells [45]. Another study evaluated the effectiveness of GO functionalized with folic acid and Pluronic F68 for the delivery of anticancer drugs. This dual-targeted nanosystem could enhance the blood circulation time of anti-tumor drugs, provide sustained release, and enhance tumor accumulation in order to suppress cancer progression [38]. Recently, our laboratory has synthesized aptamer-modified GO nanocarriers for cancer chemotherapy and theranostics applications. This platform provided simultaneous chemotherapy and optical imaging, and its functionalization with an aptamer enhanced its selectivity towards cancer cells [46].

Interestingly, GO has been shown to be useful for purposes of phototherapy in cancer. In a recent effort, manganese-oxide and palladium nanostructures were decorated with polypyrrole, and they found that by providing chemodynamic therapy and phototherapy, they significantly reduced the viability of tumor cells [47]. Moreover, both photothermal and photodynamic therapy can be provided by GO nanocomposites. For instance, methylene blue-loaded mesoporous silica-coated gold nanorods on GO can reduce breast tumor cell survival, induce cell death, and mediate photothermal and photodynamic therapy in cancer suppression [48]. Therefore, GO can be used alone or in combination with chemotherapy agents to provide phototherapy and/or chemotherapy [49]. More interestingly, GO platforms can provide phototherapy in enhancing potential of chemotherapy in cancer inhibition [50,51].

Moreover, GO can induce epigenetic alterations in order to inhibit cell growth. For instance, it has been shown that GO can induce transcriptional silencing of the EZH2 gene to inhibit glioblastoma growth [52,53]. Overall, GO nanostructures are promising candidates in cancer therapy owing to their ability to deliver drugs and theranostic imaging with molecularly targeted approaches [54–61]. In addition to drug delivery, GO can also be used for gene delivery in cancer therapy. In one recent study, the effectiveness of folate-functionalized GO nanostructures for the delivery of small interfering RNA (siRNA) against tumor-promoting genes was explored. The results demonstrated high cytotoxicity and growth inhibition induced by the siRNA-loaded GO nanostructures [62]. However, the toxicity of GO towards normal cells is a major issue that is discussed in the next section.

The anti-tumor activity of GO via regulating apoptosis and autophagy has been of importance in recent years to shed lights on the underlying mechanisms involved in tumor suppression. In ovarian cancer, the application of GO is important in changing some of the features of the tumor microenvironment. A combination of GO, cisplatin, and C6-ceramide promotes total exosome protein levels, increases acetylcholine esterase and neutral sphingomyelinase activity, and elevates the number of exosomes, which is correlated with an increase in apoptosis and oxidative stress [63]. Moreover, in the treatment of colorectal cancer, a combination of GO and doxorubicin stimulates 56% apoptosis, which is higher compared to GO (24%), and doxorubicin (31%), alone [64]. Hyaluronic acid-modified doxorubicin-loaded GO nanocomposites are able to mediate chemotherapy and phototherapy due to the conjugation of IR780 and loading of doxorubicin, which increase apoptosis and necrosis in tumor cells [65]. Therefore, GO nanocomposites are valuable structures for apoptosis induction in cancer therapy [66]. Moreover, autophagy, another cell death mechanism, can be regulated by GO in cancer therapy. Reduced GO is capable of stimulating apoptosis and autophagy in breast tumor cells, reducing their progression [67]. Furthermore, GO is able to increase ROS levels by regulating the AMPK/mTOR/ULK1 axis during apoptosis and autophagy induction in colorectal tumors [68]. These studies highlight the fact that apoptosis and autophagy are regulated by GO nanocomposites, which is the focus of the current manuscript. Table 1 provides a summary of some applications of GO in cancer therapy. (See Tables 2 and 3.)

1.3. Toxicological profile of graphene oxide

As the number of biomedical applications of GO nanomaterials continues to grow, more attention should be directed towards assessing the biocompatibility of GO nanosheets. A number of in vitro and in vivo experiments have demonstrated certain toxicity of GO composites toward normal cells [77]. GO nanomaterials can induce hemolysis due to the electrostatic interaction between the GO surface (negative charge) and the lipid bilayer of red blood cells [78,79]. It has been reported that uptake of GO nanomaterials by macrophages results in an inflammation response by promoting the secretion of pro-inflammatory cytokines, such as interleukin-6 (IL-6), IL-12, tumor necrosis factor-β (TNF-β), and interferon-gamma (IFN-β) [80]. Increasing evidence has demonstrated a proven dose-dependent cytotoxicity of GO nanomaterials [78,81–83]. It appears that the size of the nanoparticles is a major factor that determines the toxicity of GO nanoparticles against normal cells, so that it has been reported that GO nanoparticles with a size of 430–780 nm have lower toxicity towards fibroblast cells [78,83,84]. Recently published experiments have confirmed this finding [85,86]. The exposure of an experimental embryo to GO could cause defects. It has been reported that GO can increase teratogenicity in a concentration-dependent manner during embryogenesis [87]. Therefore, exposure of pregnant women to GO should be avoided. Exposure of fish larvae to GO was correlated with neurodevelopmental abnormalities and altered locomotor function [88]. GO appears to have poor hemocompatibility due to its effects on platelet aggregation and inducing erythrocyte hemolysis [89]. Furthermore, GO injection into rats reduced the size of red blood

Table 1
GO nanostructures in cancer therapy.

Nanostructure	Cancer type	Drug or gene delivery	Particle size (nm) Zeta potential (mV) Encapsulation efficiency (%)	Remarks	Refs
Carboxylated GO-trimethyl chitosan-hyaluronate	Colorectal Melanoma Breast	siRNA-HIF-1α Dinaciclib	94.56 nm 27.2 mV	High biocompatibility Enhanced cellular uptake Targeting of CD44-over-expressing cancer cells with hyaluronate modification Suppressed proliferation and metastasis Synergistic effect of combined gene and drug therapy	[69]
Chitosan hydrogel-containing GO	Glioblastoma	Irinotecan SLP2 shRNA	201–279 nm −11.7 to −19.3 mV	Sustained-release behavior Targeted delivery by binding to EGFR receptor Decreased invasion and proliferation	[70]
Glycyrrhetinic acid-modified GO	Liver	SiRNA-VEGF	20–30 mV	High stability Biocompatibility Partial hemolytic toxicity High transfection efficiency Enhanced cellular uptake Reduced gene expression	[71]
Nano-GO	Cervical	Doxorubicin siRNA-VEGF	100–250 nm 34.38 mV	Effective gene silencing Reduced tumor growth in vitro and in vivo Suppresed proliferation and metastasis	[72]
GO	Liver	Doxorubicin	252–260 nm	Increased cytotoxicity of DOX against cancer cells Reduced cell viability	[73]
GO	Breast	Doxorubicin Anti-miRNA-21	Less than 200 nm	Cell uptake by endocytosis Increased cell uptake Suppressed cancer progression	[74]
ZnO-dopamine GO	Breast	Doxorubicin	+17.9 mV	pH-responsive property Inhibited cancer growth	[75]
PEG-decorated GO	–	Curcumin	−13.9 mV	Sustained release of curcumin	[76]

Table 2
Autophagy regulation by GO nanostructures in cancer treatment.

Cancer type	In vitro/ In vivo	Cell line/Animal model	Particle size (nm) Zeta potential (mV)	Effect on autophagy	Remarks	Refs
Cervical, lung, prostate	In vitro	HeLa, A549, Tramp-C1 cells	–	Induction	Triggered early autophagy Enhanced nuclear trafficking Induced necrosis Increased chemosensitivity of cancer cells	[244]
Breast	In vitro	MCF-7, MDA-MB-231 cells	127 nm 9.2 mV	Inhibition	Loading miRNA for endosomal escape into cytoplasm High cell transfection Inhibited autophagy Triggered thermal stress Induced apoptosis	[272]
Osteosarcoma	In vitro	MG-63, K7M2 cells	–	Induction	Up-regulated ATG5 and ATG7 to stimulate autophagic flux and reduce viability Simultaneous induction of apoptosis via ROS generation	[254]
Cervical	In vitro	HeLa CCL2 cells	20–100 nm	Induction	A combination of cisplatin and GO-silver nanocomposites stimulated autophagy to reduce proliferation and sensitize apoptosis of cancer cells	[243]
Different cancers	In vitro In vivo	Tumor-bearing mice	–	Induction	Triggered autophagy and sensitized cell death Autophagy activation dependent on TLRs	[216]
Lung	In vitro	A549 cells	–	Inhibition	GO-chloroquine conjugate inhibited autophagy flux and enhanced autophagosome accumulation Induced necroptotic death in cancer cells	[256]
Colorectal	In vitro In vivo	CT26 cells, Mouse model	–	Induction	Autophagy stimulation and promoted nuclear import of LC3 Increased chemosensitivity	[257]

cells and enhanced the hemoglobin content within these cells [90]. In order to reduce the toxicity of GO nanosheets, some modifications have been performed. For instance, silanization of GO can increase the interlayer separation distance and cause certain disorders within the lattice, significantly improving the biocompatibility of GO [91]. Modification of GO with chitosan (CS), a naturally occurring polysaccharide, lessened the toxicity of GO towards normal cells [92]. Recently, we provided a comprehensive review of graphene modification by polysaccharides and evaluated the resulting biocompatibility [93].

2. Apoptosis machinery

2.1. Basics

Programmed cell death (PCD) is a conserved mechanism in all cell types with a major role in preserving homeostasis and also occurs to a large extent during embryonic development [94–98]. Apoptosis is a type of PCD that creates a balance between new cell formation and cell loss. Cells that are undergoing apoptosis show a number of particular morphological features, such as cell shrinkage, chromatin condensation, DNA fragmentation, and membrane disruption. The major factors

Table 3
GO nanoparticles to trigger apoptosis in cancer cells.

Nanocarrier	Cancer type	In vitro/ In vivo	Cell line/Animal model	Particle size (nm) Zeta potential (mV) Drug loading/ encapsulation efficiency	Remarks	Refs
GO decorated with Ru(II)-polyethylene glycol complex	Lung cancer	In vitro In vivo	A549 cells Nude mice	25 nm 128%	pH-dependent release Enhanced release after light irradiation Combined photothermal and photodynamic therapy Increased ROS levels, activated cathepsin-triggered apoptosis following irradiation Reduced cancer viability	[324]
GO-platinum nanoparticle nanocomposite	Prostate cancer	In vitro	LNCaP cells	–	Reduced expression of anti-apoptotic Bcl-2 and Bcl-xl Enhanced expression of pro-apoptotic Bax, Bak, caspase-9, p53, and p21 Induced apoptosis and DNA damage	[325]
Aminated GO	Colorectal cancer	In vitro	Colon26 cell line	560 nm 38.5 mV	Increased ROS levels Triggered DNA damage and apoptosis Reduced viability and proliferation of cancer cells	[326]
Metal nanoparticles-decorated reduced GO	Breast cancer	In vitro	MCF-7 cells	983 nm −27.5 mV	Reduced viability of cancer cells in a dose-dependent manner Induced cell death (up to 53%)	[327]
PEGylated GO nanoparticles	Breast cancer	In vitro	MCF-7 cells	161.5 nm −17.9 mV 0.376 mg/mg	Improved cancer chemotherapy Triggered apoptosis and necrosis	[328]
Magnetic GO nanocarrier	Glioma	In vivo	Tumor-bearing rat	68.4–91.5 nm −33 to −4 mV	Acted in a pH-responsive manner Good accumulation at brain tumor site Penetration through BBB Indued apoptosis via Bax/Bcl-2 up-regulation	[329]
GE11-modified GO nanoparticles	Oesophageal cancer	In vitro	KYSE-30, KYSE-150, EC9706, EC109 cells	196 nm −41 mV	Accumulated in lysosomes and reduced cancer cell viability Induced loss of mitochondrial membrane potential Apoptosis induction	[330]
Hydrogel-containing GO	Glioblastoma	In vitro In vivo	U87 cells Xenograft model	201–579 nm −11.7 mV	Suppressed tumor growth in vitro and in vivo Targeted delivery due to modification with cetuximab (anti-EGFR Mab) Combination drug and gene delivery Apoptosis induction	[70]
Pluronic-based GO-methylene blue nanocomposite	Cervical cancer	In vitro	SiHa cells	121.8 nm −16.7 mV	Combined photodynamic and photothermal therapy Increased ROS levels Caused oxidative stress Apoptosis induction	[331]
PAH-RGD functionalized GO nanoparticles	Breast cancer	In vitro	MCF-7 cells	115 nm	Provided photothermal activity Drug release in pH-responsive manner High biocompatibility Increased ROS levels to induce DNA damage and apoptosis	[332]

involved in apoptosis are caspases, which are enzymes that are originally produced in an inactive form (pro-caspase) and then undergo cleavage to be fully activated [99–103]. There are two types of caspases, including initiator caspases and executor caspases. Initiator caspases such as caspase-2, −8, −9, and − 10 are produced by auto-cleavage of pro-caspases, and then they activate executor caspases such as caspase-3, −6, and −7 to trigger apoptosis [104–108]. There are two pathways for apoptosis, classified as the classical and alternative pathways. In the classical pathway, there is a further sub-division into intrinsic and extrinsic pathways [109–112]. The alternative apoptosis pathway is mediated by activated T cells that express the granzyme-perforin pathway. In this pathway, perforin induces transmembrane pores to allow the entry of granzyme-loaded cytoplasmic granules released by T-cells, leading to target cell death [113,114].

2.2. Intrinsic apoptotic pathway

Mitochondria play a central role in triggering the intrinsic apoptotic pathway [115–118]. After mitochondrial outer membrane permeabilization (MOMP), following the loss of mitochondrial membrane potential, cytochrome C (Cyt C) is released to activate caspase-3 [119,120]. There is a no-return phase of apoptosis [121,122]. The release of cyt C from mitochondria is mediated by the upregulation of pro-apoptotic factors such as BCL-2, BCL-X, BCL-W, and MCL-1 inside the mitochondria and the downregulation of anti-apoptotic factors such as BCL-2, BCL-X, BCL-W, and MCL-1 outside the mitochondria [123–125]. Pores must be created in the mitochondrial membrane to enhance its permeability. For this purpose, BIM, BID, and PUMA function as activators to induce BAX and BAK1 [126–132]. Then, these pro-apoptotic proteins create conformational alterations and oligomerize among themselves to cause pore formation in the mitochondrial membrane [133]. In contrast, the anti-apoptotic proteins mentioned above create a binding groove to sequester the pro-apoptotic proteins, BAX and BAK1 [134]. The affinity of the interactions between each protein is different. For instance, it has been reported that PUMA, BID, and BIM selectively target and suppress MCL-1, while BAD selectively targets and negatively regulates BCL-2, BCL-W, and BCL-X [135].

2.3. Extrinsic apoptotic pathway

In the extrinsic apoptotic pathway, specific cell membrane receptors (known as death receptors) are involved. The death receptors include Fas, tumor necrosis factor (TNF) receptors 1 and 2 (TNFR1 and TNFR2), and TNF-related apoptosis-inducing ligand (TRAIL) receptors (DR4 and DR5) [136–150]. Pro-apoptotic death receptors enable a death-enabling protein-protein interaction and contain an evolutionarily conserved domain [151]. Upon stimulation of death receptors by a specific ligand, a conformational change (trimerization and aggregation) occurs to produce a death domain [152–155]. Then, activation of an adaptor protein known as FADD/tumor necrosis factor receptor type-1 associated death domain (TRADD) occurs, resulting in caspase-8 activation in cooperation with the death-inducing signaling complex (DISC), leading to caspase-3 and -7 activation, and finally to apoptotic cell death (Fig. 2) [156–158].

2.4. Apoptosis in cancer therapy: regulation and targeting

Apoptosis is the most important pathway that causes cancer cell death and is triggered by anti-tumor drugs and ionizing radiation treatment [159–162]. A wide variety of molecular pathways and specific agents or drugs have been shown to regulate apoptosis in cancer cells [163–165]. For instance, it has been shown that glycogen synthase kinase-3 (GSK-3) can stimulate nuclear factor-kappaB (NF-κB) signaling, which inhibits apoptosis in cancer cells [166]. In contrast, tumor-suppressor factors, such as LHPP, a protein histidine phosphatase, enhanced apoptosis in pancreatic cancer cells through caspase-3 up-regulation [167]. Olaparib and rosiglitazone are two anti-tumor drugs that can promote p53 phosphorylation to induce apoptosis in ovarian cancer cells, and prevent cell senescence [168]. Shikonin induces apoptosis in cancer cells independent of p53 by promoting p21 expression [169]. Non-coding RNAs (ncRNAs) are also potential regulators of apoptosis in cancer. MicroRNAs (miRNAs) and long non-coding RNAs (lncRNAs) can affect apoptosis in cancer cells via targeting the caspase cascade [168,170–175]. Taken together, apoptosis is tightly regulated by molecular pathways in cancer cells, and increasing apoptosis can suppress cancer progression and sensitize tumors to chemotherapy [176–180].

In particular, the ability of drugs and agents to regulate apoptosis in cancer therapy can be improved using nanocarriers. Loading silibinin into polymerosome nanoparticles significantly enhanced its ability to induce apoptosis in pancreatic cancer cells by enabling targeted delivery [181]. It has been reported that selenium nanoparticles can increase ROS levels to induce apoptosis in cancer cells [166]. Mesoporous silica nanoparticles (MSNs) encapsulating methotrexate plus fingolimod could induce apoptosis to suppress the malignant behavior of thyroid cancer cells [182]. Many apoptotic regulators can be loaded into nanostructures to improve their efficacy in cancer treatment [166,183–185].

3. Autophagy machinery

3.1. Basics

Autophagy is a “self-digestion” mechanism involved in the maintenance of homeostasis [186–193]. This catabolic degradation mechanism is evolutionarily conserved and delivers macromolecules, proteins, damaged organelles, or pathogens to lysosomes, where they are degraded by lysosomal hydrolysis, leading to the generation of metabolic substrates such as nucleotides, amino acids, sugars, and ATP that are finally recycled [194–202]. Because the products resulting from autophagy are vital for the normal functions of cells, it has been suggested that the autophagy mechanism evolved to ensure the survival of cells under stressful conditions such as starvation while also eliminating potentially toxic agents that are harmful for cells [203–207]. There are three different types of autophagy based on the morphological features of the organelles, including: (A) microautophagy; (B) chaperone-mediated autophagy; (C) macroautophagy [208,209]. The most well-known type of autophagy is macroautophagy, and going forward, we will use autophagy instead of macroautophagy.

The invaginations in the lysosomal or endosomal membranes surround the cytoplasmic cargo in microautophagy [210]. Chaperone-

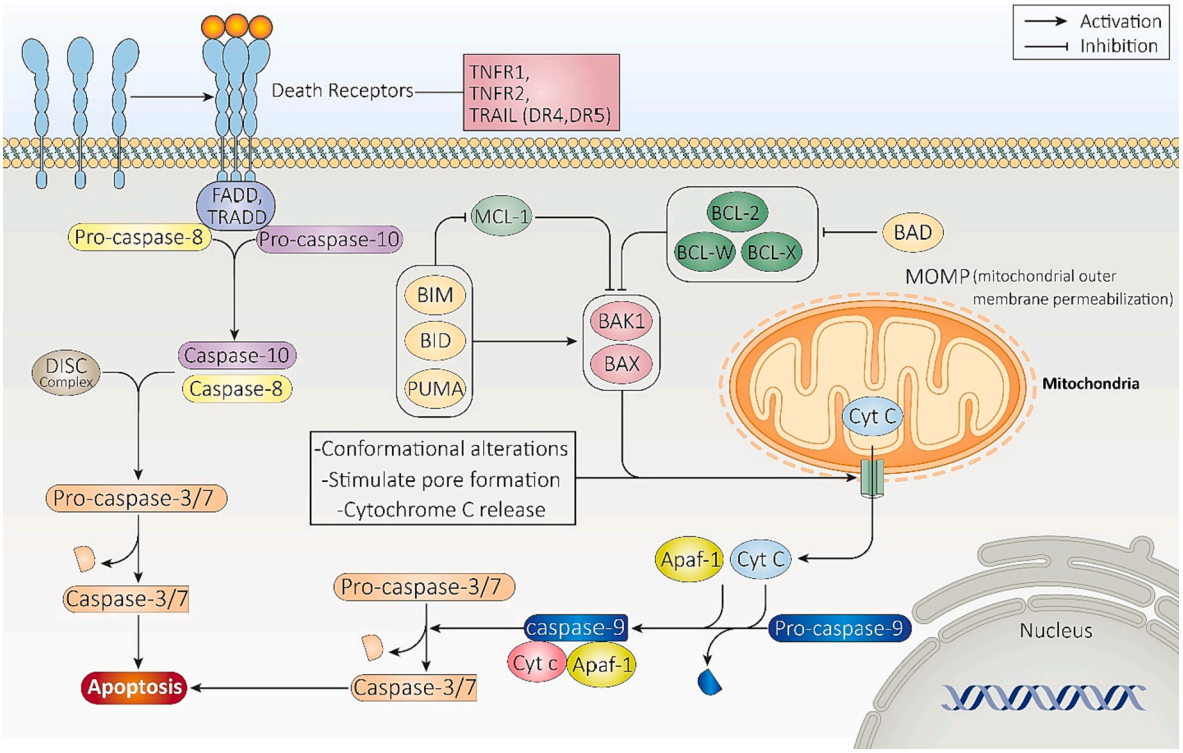

Fig. 2. Apoptosis machinery and related molecular pathways.

mediated autophagy is a selective type of autophagy where the endosomal cargo binds to a chaperone protein. If the cargo protein contains a KFERQ-like motif, the chaperone enables the transportation of the cargo across the lysosomal membrane via LAMP2a (lysosome-associated membrane glycoprotein 2a) [211]. In macroautophagy (aka autophagy), autophagosomes (double-membrane compartments) engulf the cargo to fuse with lysosomes, resulting in cargo degradation [212]. Each step of autophagy has its own characteristics and is regulated by specific molecular pathways, whose identification is important for the development of novel therapeutics to modulate autophagy in therapeutic applications [213–215].

The most well-known initiator of autophagy is unc-51-like kinase 1 (ULK1), a mammalian homologue of autophagy-related gene 1 (ATG1) kinase. As a serine/threonine protein kinase, ULK1 forms a complex with ATG13, ATG101, and ULK1 to induce autophagy [216,217]. The mechanisms of autophagy inhibition rely on the presence of sufficient amounts of nutrients. Given that activation of autophagy occurs under stress conditions or low energy conditions, the presence of excess amino acids inhibits autophagy via activation of the mammalian target of rapamycin (mTOR) complex 1 (mTORC1). mTORC1 triggers the phosphorylation of ULK1 and ATG13 to inhibit the activation of autophagy. However, in starvation or other stress conditions, dephosphorylation of ULK1 and ATG13 occurs by inactivation of mTORC1, located on the lysosomal interior surface, resulting in the induction of autophagy [218]. It appears that ULK1 is involved in regulating another complex, phosphoinositide 3-kinase (PI3K), which generates phosphatidylinositol triphosphate (PI3P) from phosphatidylinositol (PI). PI3P is considered a biomarker for autophagosome formation [219]. PI3K forms a complex with Beclin-1 and Vps15. It has been reported that ULK1 induces beclin-1 phosphorylation to activate the PI3K-beclin-1-Vps15 axis, leading to autophagy induction [220]. Furthermore, PI3K is capable of generating another complex with ATG14 to promote autophagy vesicle formation [221]. **Fig. 3** illustrates the molecular pathways involved in autophagy regulation.

3.2. Autophagy in cancer therapy

In addition to apoptosis, autophagy is also vital for effective cancer therapy. However, the scenario is a little distinct with regard to the role of autophagy in cancer. This is because autophagy can function either as a pro-survival or a pro-death mechanism. For instance, it has been reported that loss of protein kinase C (PKC) λ/ι significantly enhances liver cancer progression via autophagy induction [222]. This study revealed the role of autophagy as a pro-survival mechanism to promote cancer growth. Therefore, in this situation, autophagy inhibition should be considered to improve anti-tumor activity. Another study revealed that stimulation of autophagy under hypoxic conditions enhanced colorectal cancer proliferation and invasion [223]. On the other hand, autophagy can also act as a pro-death mechanism. In pancreatic cancer cells, mitochondrial DNA stress was associated with autophagy-dependent ferroptotic cell death via lipid peroxidation [222]. It seems that reducing the expression of glutathione peroxidase 4 (GPX4) using RNA interference inhibited the down-regulation of mTOR to induce autophagy-dependent ferroptosis, resulting in enhanced activity of the anti-tumor drug rapamycin [224]. Overall, the findings are in agreement that various signaling networks can regulate autophagy in cancer, and their understanding can pave the way to more effective cancer therapy [225–228].

Nanoparticles have been shown to evoke regulatory effects on autophagy in various forms of cancer. Nanostructures can down-regulate mTOR expression to stimulate autophagy [227]. Exposure of prostate cancer cells to silver nanoparticles stimulated the AMP-activated protein kinase (AMPK)/mTOR axis to inhibit autophagy [229]. Autophagy modulators can be loaded onto nanoparticles. Hydroxychloroquine-loaded hollow mesoporous silica nanoparticles (HMSN) were used to inhibit cell-protective autophagy, leading to colon cancer inhibition and increased sensitivity to radiotherapy [184]. Furthermore, silver nanoparticles triggered PI3K/Akt/mTOR signaling to induce both apoptosis and autophagy in cancer cells [230]. Therefore, nanocarriers are potential modulators of autophagy [231,232], and in the next sections, we will discuss the role of GO nanostructures in the regulation of autophagy and apoptosis from a mechanistic standpoint and summarize their anti-tumor activity and toxicological profile.

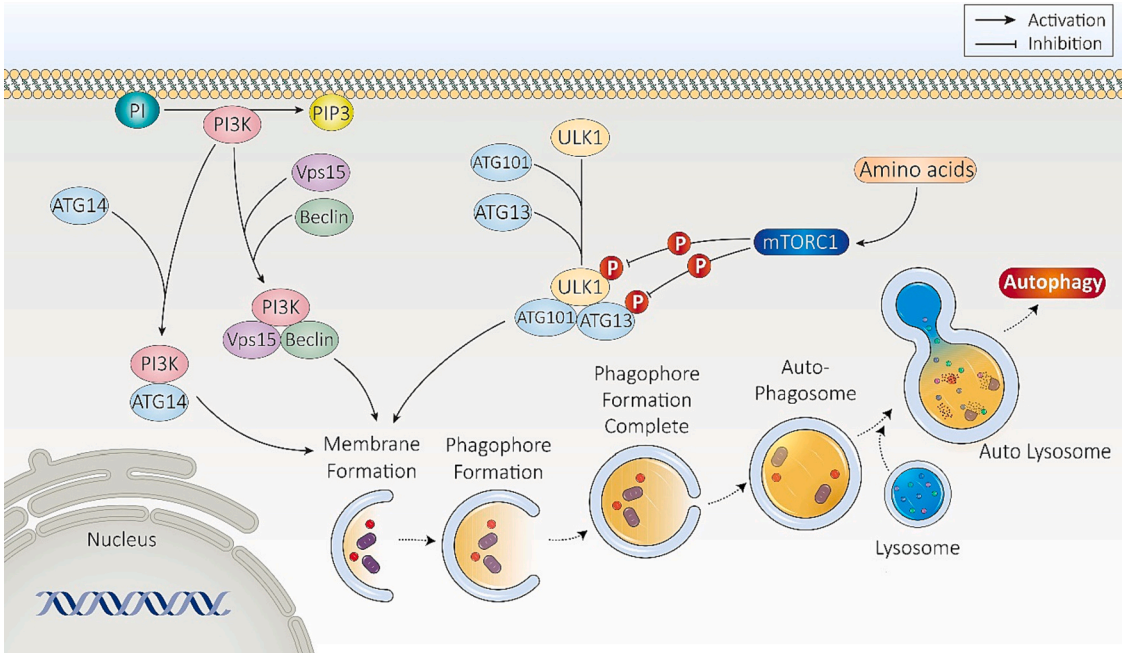

Fig. 3. A schematic representation of the molecular pathways participating in autophagy regulation.

4. Graphene oxide and autophagy

4.1. Cancer therapy

Chemotherapeutic drugs have long been used in cancer therapy. Nonetheless, different innovative natural, biological, and synthetic compounds are being examined in cancer chemotherapy to restrict the progression and metastasis of tumors [233,234]. Hormones, drugs, vitamins, vaccines, and minerals are all considered as chemopreventive agents [235,236]. However, the efficacy of chemotherapy depends on the response of cancer cells themselves. In fact, chemotherapy is beneficial for cancer suppression because it can induce cell death and inhibit cancer metastasis and invasion of distant cells and tissues. It has been reported that activation of tumor-promoting factors significantly increases the growth and metastasis of cancer cells, limiting the effectiveness of chemotherapeutic agents [237,238]. One of the molecular mechanisms that can help in anti-cancer chemotherapy is autophagy induction [239,240]. Combination chemotherapy with gemcitabine and rapamycin induces autophagy to inhibit the proliferation and metastasis of osteosarcoma cells [241]. Myricetin is an anti-tumor agent that stimulates autophagy via suppression of PI3K/Akt/mTOR signaling to inhibit colon cancer progression [242].

GO can affect autophagy to potentiate the efficacy of chemotherapeutic agents in cancer and can also lower drug resistance. One experiment evaluated the potential of GO-silver nanoparticle nanocomposites (GO-AgNPs) to enhance cisplatin-mediated cytotoxicity in cervical cancer cells. In this case, hybrid nanocomposites were first prepared using C-phycocyanin. The synthesized nanocarriers were spherical in shape with a particle size of 10 nm and were well dispersed and homogenous. Cell viability assay demonstrated decreased cancer cell viability caused by GO-AgNPs in a concentration-dependent manner. A combination of GO-AgNPs plus cisplatin reduced cell viability in a synergistic manner and induced autophagy via up-regulation of ATG3, ATG5, ATG6, and ATG7, which are responsible for autophagy stimulation. Then, the increased autophagy sensitized the cancer cells to apoptosis [243]. The role of autophagy was to promote cisplatin-mediated apoptosis. It appears that the GO nanocomposites can stimulate autophagy, and enhance the chemosensitivity of cancer cells. A recent experiment has shown how the induction of GO-mediated autophagy could enhance the sensitivity of different cancer cells to cisplatin chemotherapy. In cancer cells (colon, ovarian, cervical, and prostate cancer), GO nanoparticles promoted nuclear importation of cisplatin and induced autophagy via LC-3 up-regulation, leading to cell necrosis and increased chemosensitivity in cancer cells. However, it is uncertain whether the completion of the autophagy process is necessary for increasing chemosensitivity. It has been reported that early autophagy (just the initiation and progression) increases the effects of chemotherapy, and that is not necessary to complete the autophagy process (autophagosome and autolysosome formation). Nevertheless, the translocation of cisplatin to the nucleus is enhanced to allow its interaction with chromosomal DNA, causing cell death [244].

As previously discussed, autophagy can play two distinct roles in cancer, either pro-survival or pro-death. This means that the function of autophagy is context-dependent, and it can suppress/induce progression of tumor cells. Targeting autophagy in cancer therapy is complicated due to its oncogenic and oncosuppressor functions, and before its targeting, the exact function of this mechanism should be revealed. Autophagy has a close association with proliferation and invasion of tumor cells and can also determine the response to therapy in tumor cells. When autophagy has a pro-death function, it decreases the viability of tumor cells, while tumor-suppressor autophagy is capable of decreasing the progression of cancer cells [245–247]. A recent experiment employed GO conjugated to polyethylene glycol (PEG) plus folic acid (FA) as a surface modification that specifically targets folate receptor expressing cancer cells. Indocyanine green (ICG) was used as a photosensitizer to allow GO nanomaterials to be used for photodynamic therapy (PDT). Then, this GO nanocomposite was utilized for the delivery of MutT homolog 1 (MTH1), which induces oxidative stress. These nanocarriers allowed both chemotherapy and phototherapy to suppress the proliferation and invasion of osteosarcoma cells. Because of the delivery of MTH1, the GO nanoparticles enhanced the ROS generation caused by PDT to result in more cell death. ROS induce ER stress to promote apoptosis via the JNK/p53/p21 axis. Furthermore, these nanocarriers also stimulated autophagy in osteosarcoma cells. There was a protective function of autophagy against cell death, so that upon autophagy inhibition, osteosarcoma cells were sensitized to apoptosis (Fig. 4) [229].

The role of autophagy in mediating chemoresistance has been confirmed in several studies [248–250], and although GO nanomaterials are potential modulators of autophagy in cancer, we should consider the double-edged role of autophagy as either a pro-survival or a pro-death mechanism. Another aspect to be considered is the existence of a two-way traffic between apoptosis and autophagy. It seems that autophagy can either promote or decrease apoptosis in cells [251–253]. Since GO nanomaterials can induce both apoptosis and autophagy in cancer cells, we should consider how autophagy can affect the apoptotic pathway [254].

An association was highlighted between autophagy and apoptosis in a recently conducted study. GO nanoparticles were investigated in astroglioma cells, and in this way, these nanocarriers can induce both autophagy and apoptosis. GO nanoparticles enhance the expression levels of LC3II and p62 to trigger autophagy, while they decrease the expression levels of PI3K/Akt/mTOR. The accumulation of p62 as an inducer of autophagy promotes caspase-3 cleavage and induces apoptosis. Furthermore, the inhibition of autophagy can reduce apoptosis induction in cancer cells [255]. In this case, autophagy acts as an anti-tumor mechanism, and its induction increases apoptosis in cancer cells.

Because of the context-dependent role of autophagy, some agents capable of inhibiting this molecular mechanism have been developed. Moreover, GO can provide a platform for the delivery of drugs. Therefore, in order to maximize cancer cell death with GO nanomaterials, it is important to use an autophagy inhibitor, such as chloroquine. A GO-chloroquine nanoconjugate was prepared to suppress the proliferation of lung cancer cells (A549 cells). Exposing A549 cells to GO nanoparticles induced both autophagy and apoptosis. Autophagy inhibition by the addition of chloroquine potentiated the apoptotic cell death caused by GO nanocomposites [256]. In vivo experiments have also confirmed the role of GO nanomaterials in inducing autophagy and suppressing cancer growth. It was reported that GO nanoparticles stimulated autophagy upon intratumoral injection into mice bearing CT26 colon cancer tumors, leading to enhanced cisplatin sensitivity [257]. In addition to cisplatin, DOX has been widely utilized in cancer therapy. However, the development of resistance to DOX in cancer cells has resulted in the application of nanoparticles as new therapeutic strategies for boosting its anti-tumor activity and reversing chemoresistance [258–260]. Recently, GO nanocomposites have been used for DOX delivery in cancer therapy; surface modification of GO with gelatin and Arabic gum mediates pH-sensitive release of DOX and increases anti-tumor activity against lung cancer [261]. Moreover, co-delivery of curcumin and DOX with GO nanocomposites is vital for pH-sensitive cargo release and suppressing tumorigenesis [262]. DOX-loaded GO nanocomposites are able to modulate autophagy in cancer. DOX and cisplatin have stimulators of ER stress were loaded on GO, and it was shown that they can induce apoptosis and autophagy through ER stress while suppressing tumorigenesis in breast and lung cancers [263]. However, more experiments related to other chemotherapeutic agents such as 5-fluorouracil, docetaxel, paclitaxel, and oxaliplatin, among others, and their delivery with GO for regulation of autophagy in cancer should be performed.

Up until now, we have discussed the role of autophagy as a protective mechanism. Now, the question arises: are there other molecular

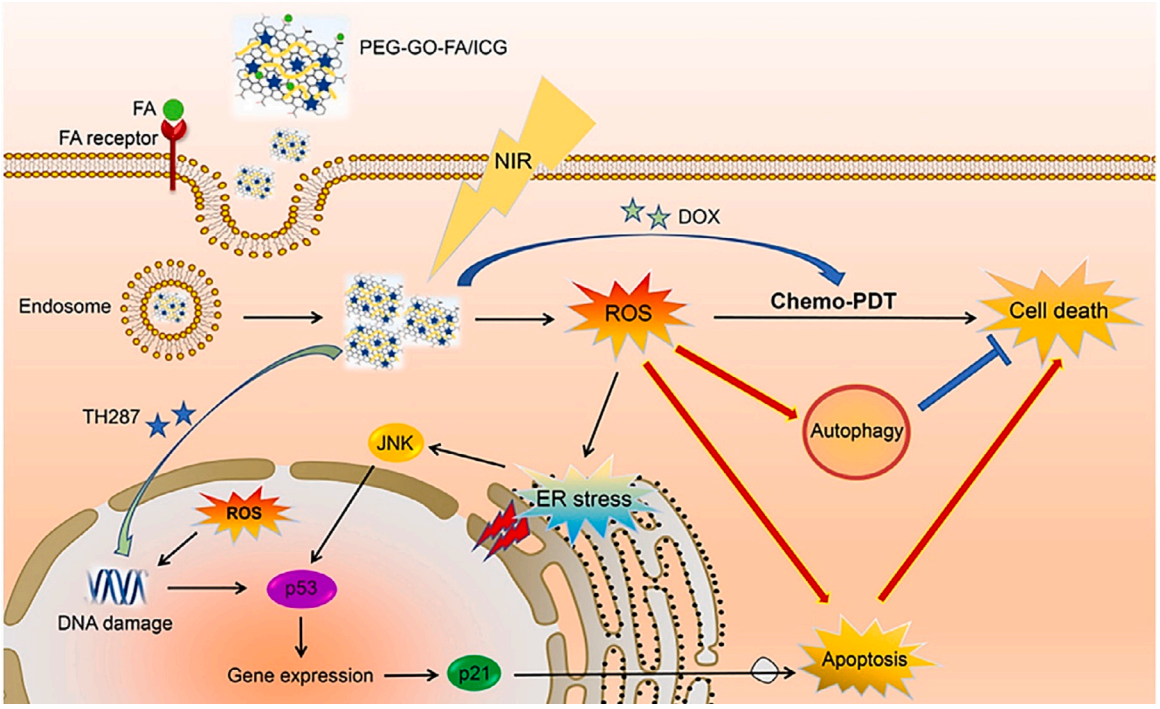

Fig. 4. The prepared GO nanostructures are able to enter cancer cells through binding to FA receptor and affecting molecular pathways in regulating autophagy. Reprinted with permission from Elsevier [229].

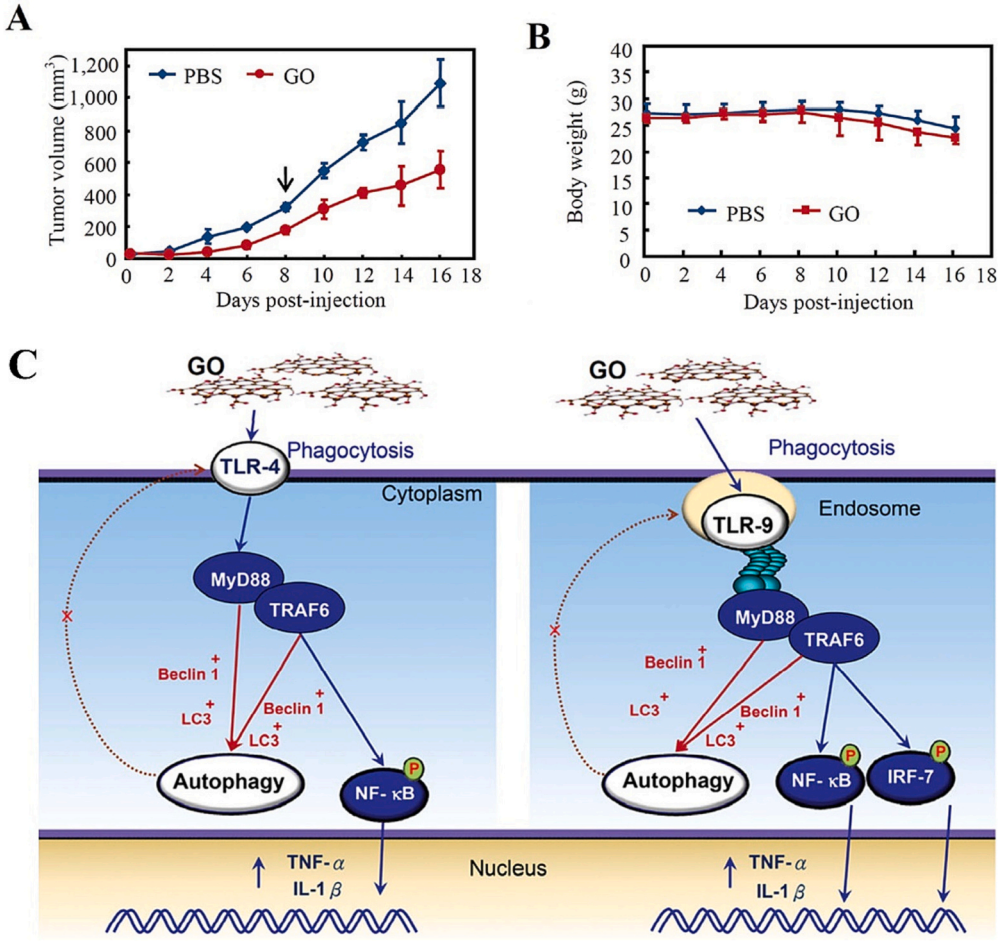

Fig. 5. A and B) Tumor volume and weight; C) Related molecular pathways in regulating autophagy. Reprinted with permission from Wiley [270].

mechanisms capable of reducing the anti-tumor activity of autophagy, and functioning as a pro-survival mechanism? The answer is that endoplasmic reticulum (ER) stress may play this role in tumor growth and survival [243,254,264]. On the other hand, it appears that ER stress can also function as an adaptive mechanism. In order to show how ER stress can affect GO-mediated autophagy in nasopharyngeal cells, one study exposed these cancer cells to GO nanomaterials. The results showed induction of apoptosis in a dose-dependent manner, accompanied by activation of both autophagy and ER stress. LC3 and autophagosome formation were up-regulated to stimulate autophagy. Furthermore, ER stress can activate autophagy via overexpression of glucose-regulated protein 78 (GRP78). Although GO nanoparticles can significantly decrease the viability of cancer cells, it seems that inhibiting ER stress (an adaptive molecular mechanism) can boost the anti-tumor activity of GO nanoparticles [265].

Toll-like receptors (TLRs) act as receptors for various danger-associated molecular patterns and are involved in regulating the immune response and cancer immunobiology [266]. Among the range of TLRs, TLR4 recognizes lipopolysaccharide, and TLR9 recognizes unmethylated DNA containing CpG motifs, both of which are derived from bacteria [267]. Both TLR4 and TLR9 can function as upstream regulators of autophagy [268,269]. GO nanoparticles can regulate the TLR/autophagy axis to affect the growth and progression of cancer. After phagocytosis of GO nanoparticles by colon cancer cells, these nanocarriers induced autophagy, and inhibited tumor growth. Investigation of molecular pathways showed that GO nanomaterials triggered autophagy by activating TLR4/9 signaling via the myeloid differentiation primary response protein 88 (MyD88) and TNF receptor-associated factor 6 (TRAF6) molecular pathways (Fig. 5) [270]. It should be mentioned that autophagy induction by GO nanoparticles does not always lead to cell death or cell cycle arrest in cancer [271].

Overall, the following conclusions can be drawn with regards to the effects of GO nanoparticles on autophagy in cancer therapy (Fig. 6).

1) Because autophagy can have both pro-survival and pro-death roles, the exact molecular mechanism should be determined in experiments exposing cancer cells to GO nanoparticles, and if autophagy helps cancer progression, autophagy inhibitors such as chloroquine can be used.
2) GO nanoparticles can stimulate both autophagy and apoptosis in cancer cells, and their interactions should be determined in each case.
3) By affecting autophagy-related processes, GO nanoparticles can enhance the efficacy of chemotherapy in various cancers.
4) Other molecular mechanism, such as ER stress, can play a protective role and could be inhibited to potentiate GO-mediated autophagy and tumor suppression.
5) Green synthesized GO nanoparticles have shown high cytotoxicity against cancer cells via induction of autophagy, and more studies are required to confirm their potential for cancer eradication.

4.2. Toxicological profile

In the previous section, we discussed the role of GO nanoparticles as potential regulators for autophagy in cancer, leading to tumor inhibition and enhanced responsiveness to therapy. It should be mentioned that autophagy also possesses normal physiological functions. Therefore, impairing autophagy with GO nanoparticles could lead to the development of pathological processes and toxicity. These side effects of GO nanocarriers would not only be important for scientists who work on basic science but also for those working on clinical application, since good biocompatibility and low toxicity of GO nanomaterials are vital in the treatment of cancer patients. Before discussing autophagy-mediated toxicity of GO nanoparticles, it is worth mentioning that these nanostructures can also induce autophagy in the treatment of conditions such as neurological diseases and others [273–275]. However, since the biocompatibility of GO nanoparticles is important, we use this section to discuss the toxicological profile of GO nanomaterials with a focus on autophagy mechanisms.

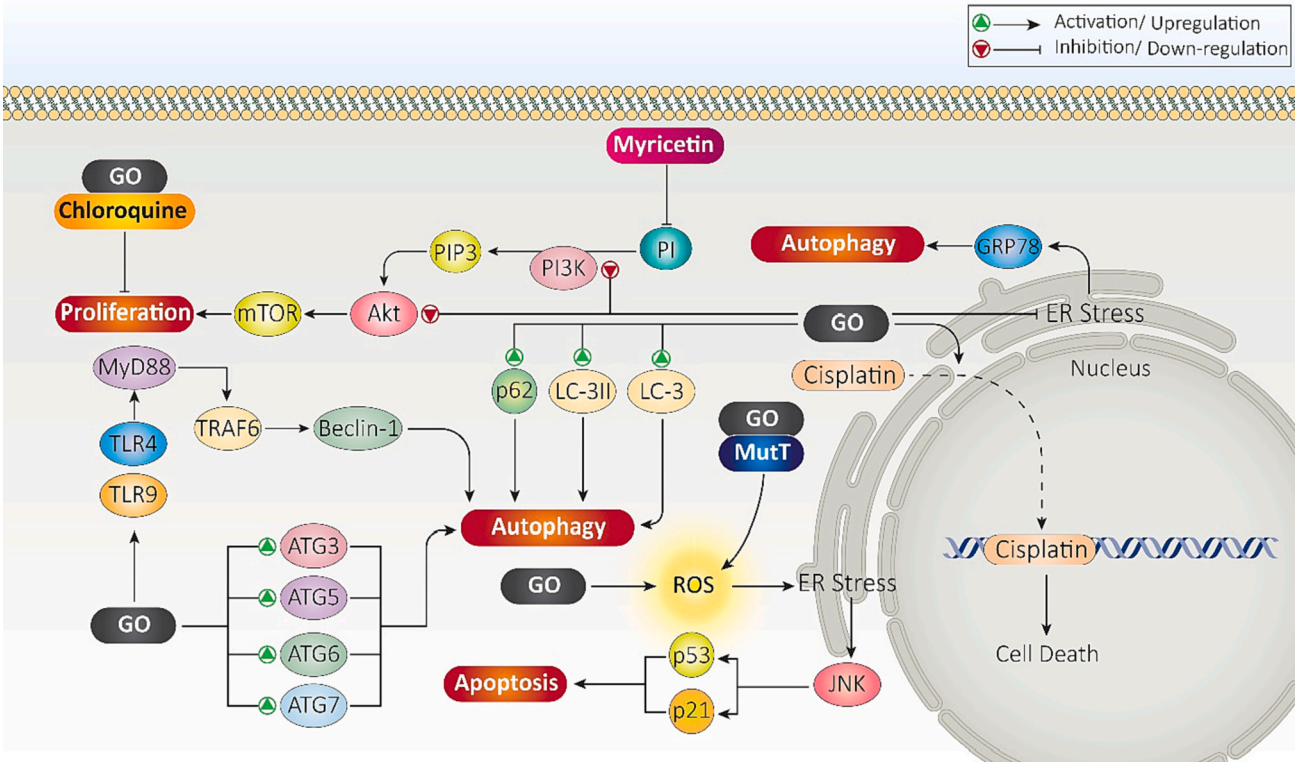

Fig. 6. Regulation of autophagy by GO nanomaterials and its application in cancer therapy.

A recent experiment has indicated that GO nanomaterials can evoke toxicity in mouse embryonic stem cells through regulation of autophagy. Exposure of embryonic cells to GO nanoparticles enhanced autophagosome formation and increased the expression levels of LC3 and p62. However, these findings are not in favor of autophagy induction, and it seems that GO nanoparticles could inhibit or prevent autophagy flux rather than induce it in these normal cells. Further investigation revealed that disruption of autophagy function was due to disturbing lysosomal homeostasis via lysosomal alkalization and lysosome membrane permeabilization [276]. It should be noted that the use of autophagy inhibition to improve the biocompatibility of GO nanoparticles could be self-defeating since they use autophagy to gain entrance into cancer cells [277]. In this case, we should attempt to prevent their entrance into normal cells, while still allowing them access to cancer cells.

Previously, it was mentioned that GO nanoparticles exert a neuroprotective effect via regulating autophagy. It has been reported that autophagy modulation by GO nanoparticles can also lead to neurotoxicity. This is due to the close relationship between autophagy and apoptosis. It seems that the neurotoxic effects of GO nanocarriers are both dose-dependent and time-dependent. Furthermore, by impairing lysosome degradation, GO nanoparticles can reduce autophagy flux in PC12 cells. This leads to intracellular accumulation of the autophagy substrate p62/SQSTM in the cells, followed by caspase-3-induced apoptosis [278]. In this case, the induction of autophagy could be used to ameliorate the apoptosis in PC12 cells and reduce the neurotoxicity of GO nanoparticles. One previous study showed apoptosis induction after inhibition of autophagy flux by GO nanomaterials, while another study demonstrated simultaneous induction of autophagy and apoptosis after exposure to these nanocarriers. GO nanoparticles induced phosphorylation of Bcl-2 by activating c-Jun N-terminal kinase (JNK), leading to dissociation of Beclin-1 from Bcl-2. This paved the way for autophagy induction and increased apoptotic cell death. Inhibiting autophagy reduced apoptosis in endothelial cells, showing how autophagy can affect apoptosis [279]. Another experiment showed that GO nanomaterials could induce autophagy, but this had no impact on neuron viability, although it could still affect the function of the neurons [280].

One issue with using GO nanomaterials in cancer therapy is their potential toxicity to normal cells. GO nanoparticles produce toxicity toward both normal and cancer cells in a time- and concentration-dependent manner. Furthermore, the size, charge, shape, and physicochemical properties of GO nanomaterials determine their toxicity [271,281–285]. Therefore, some efforts have been made to enhance the biocompatibility of GO nanocomposites, for instance, by modification with dextran [286]. Noteworthy, the green synthesis of GO nanoparticles can not only enhance their biocompatibility but can also increase their anti-tumor activity. One experiment used quercetin for the green synthesis of GO nanoparticles intended for neuroblastoma therapy. These green synthesized-GO nanoparticles showed high cytotoxicity against cancer cells even at low concentrations. Examination of the molecular mechanisms showed enhanced autophagosome formation and more autophagic vacuoles caused by the GO nanocarriers [287].

Increasing evidence has shown a close association between autophagy and inflammation. It was reported that the induction of autophagy using metformin improved mitochondrial homeostasis, resulting in a reduction in inflammation [288]. Furthermore, inhibition of caveolin-1 stimulated autophagy, which subsequently alleviated vascular inflammation and atherosclerosis [289]. These studies clearly show that stimulation of autophagy is favorable for decreasing inflammation. The association between autophagy and inflammation was investigated in a model of colitis. GO nanomaterials cause lysosomal dysfunction to suppress autophagy flux. Inhibition of autophagy led to a decrease in the levels of pro-inflammatory cytokines, including IL-6, IL-8, TLR4, and CXCL2, while it enhanced levels of IL-10, resulting in the amelioration of colitis [290]. It is worth mentioning that GO nanoparticles can also induce toxicity in normal cells via autophagy stimulation [291]. To date, a few studies have evaluated autophagy regulation by GO nanocarriers and investigated the toxicological profile [292]. Future studies should shed more light on this aspect.

5. Graphene oxide and apoptosis

5.1. Cancer therapy

Apoptosis is the most common avenue to kill cancer cells. GO nanomaterials can induce apoptosis in cancer cells by affecting both pro-apoptotic and anti-apoptotic proteins. It has been reported that exposing breast cancer cells (MCF-7 and MCF-10) to GO nanoparticles was correlated with an increase in the expression levels of p53, p21, and Bax (pro-apoptotic factors), while a decrease in Bcl-2 expression (an anti-apoptotic factor) occurred, leading to apoptosis induction in breast cancer cells [293]. GO nanoparticles function in a dose-dependent manner to reduce the viability and proliferation of cancer cells. Exposure of leukemia cells to GO nanomaterials resulted in the loss of mitochondrial membrane potential (MMP) and an increase in ROS levels, leading to cell death by apoptosis. GO nanomaterials can also stimulate apoptosis by triggering DNA damage in cancer cells [294]. Molecular pathways can be regulated by GO to stimulate apoptosis in cancer cells. Doxorubicin (DOX) was loaded onto GO nanoparticles functionalized with Pluronic F127 via non-covalent and π-π stacking interactions in a recent study. These nanocarriers increased caspase-3 expression to trigger intrinsic apoptosis pathway, with a loading capacity of 0.083 mg/mg and encapsulation efficiency of 83%. These nanocarriers also inhibited the expression of extracellular regulated kinase 1/2 (ERK1/2) to promote apoptotic cell death [242].

Many studies have shown that GO nanoparticles can decrease the viability and proliferation of cancer cells in a time- and dose-dependent manner. However, the molecular pathways that GO nanoparticles follow to induce apoptosis in cancer cells are important. Previously, it was shown that the overproduction of ROS is often involved in the stimulation of apoptosis in cancer cells because ROS can impair mitochondrial homeostasis. Therefore, biological pathways (anti-oxidants) that can reduce ROS levels could enhance cancer progression via suppressing apoptosis. Nuclear factor erythroid 2-related factor 2 (Nrf2) acts to regulate the redox state, and its primary role is to protect cells against oxidative stress-mediated cell death [295,296]. Recent experiments have shown the role of Nrf2 in cancer progression and chemotherapy resistance [297–299]. GO nanoparticles inhibited Nrf2 expression to promote ROS levels, resulting in apoptosis and cytotoxicity against osteosarcoma cancer cells [300].

Another approach to boosting the ability of GO nanoparticles to stimulate apoptotic cell death is conjugation with agents capable of selectively targeting mitochondria, which are the organelles involved in the intrinsic apoptosis pathway. Hypericin (HY) is a naturally occurring compound derived from *Hypericum perforatum* L, which is used as a photosensitizer for photodynamic therapy and localizes in mitochondria. DOX was loaded on HY-functionalized GO nanoparticles to provide simultaneous chemotherapy and phototherapy. Due to selective targeting of mitochondria, apoptosis was induced, leading to a significant decrease in viability and proliferation of cancer cells (Fig. 7) [301]. GO nanoparticles reduced the colony-formation capacity of cervical cancer cells by inducing apoptosis [37].

As mentioned previously, there is a dual relationship between autophagy and apoptosis. It is known that miRNAs can function as upstream mediators of apoptosis in cancer [252]. With respect to the role of GO nanoparticles as efficient gene delivery systems, they can be used for the delivery of specific miRNAs [74] to regulate apoptosis in cancer cells. Recently, cationic GO nanoparticles were developed for the delivery of miRNA-101 to induce apoptosis in cancer cells. The surface of the GO nanoparticles was modified by PEG and poly-l-arginine (P-l-Arg) to provide a cationic charge and enhance the near infrared absorption.

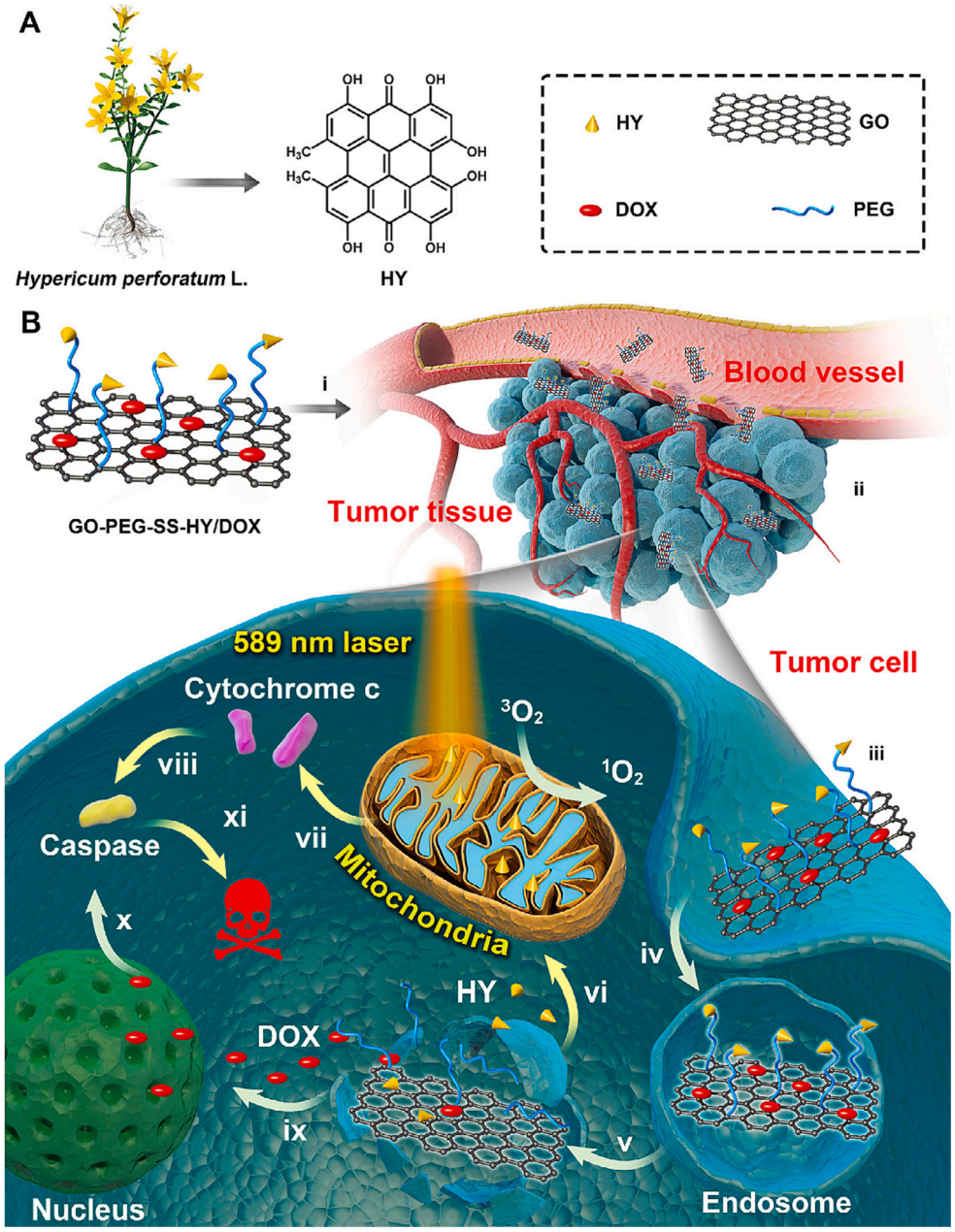

Fig. 7. A) Using HA for mediating mitochondrial targeted capacity of GO nanoparticles; B) Molecular pathways in affecting mitochondrion and triggering apoptosis. Reprinted with permission from Elsevier [301].

These nanostructures underwent endosomal escape to the cytoplasm and increased the internalization of miRNA-101 to increase apoptosis while at the same time suppressing autophagy [272]. However, the main pathway for apoptosis induction was increased ROS levels, while simultaneous stimulation of pro-death autophagy could reduce the viability and survival of cancer cells [254].

In addition to miRNA, GO nanoparticles can be used for the delivery of other kinds of nucleic acid-based therapeutic agents. Small interfering RNA (siRNA) is a newly discovered tool for suppressing tumor-promoting factors and inhibiting the progression of cancer cells [302–304]. GO nanoparticles have been used for targeted delivery of siRNA, protecting it against degradation, and enhancing its internalization [305–307]. Loading certain siRNAs onto GO nanoparticles can sensitize cancer cells to apoptosis. For instance, it has been reported that siRNA against histone deacetylase 1 (HDAC1) and siRNA against K-Ras can be loaded on PEGylated GO nanoparticles to provide simultaneous gene therapy and phototherapy, producing apoptosis in pancreatic cancer cells [308]. Hypoxia-inducible factor 1 (HIF-1) is another molecular pathway activated under hypoxic conditions to increase the proliferation and invasion of cancer cells [309–311]. SiRNA against HIF-1 plus dinaciclib (a selective small molecule inhibitor of CDK1, CDK2, CDK5, and CDK9) were co-loaded onto GO nanocarriers to increase the uptake into cancer cells, resulting in apoptotic cell death [69]. Several siRNAs can be designed and loaded onto GO nanoparticles to modulate the targeted molecular pathways and sensitize cancer cells to apoptosis. Furthermore, some anti-apoptotic factors, such as Bcl-2, can also be targeted by siRNA-loaded GO nanoparticles in cancer therapy. Another possibility that could be investigated is the loading of a CRISPR/Cas9 system, which is a genetic tool that could be used for targeting tumor-promoting factors and stimulating apoptosis in cancer cells. Based on the fact that GO nanoparticles have already been utilized for CRISPR/Cas9 delivery [312], future studies could evaluate how this combination

could result in apoptosis and cancer cell death.

Although we have emphasized that GO nanoparticles can induce apoptosis and can also be used for drug delivery in cancer, there are some reports showing contrasting results. In one study, DOX-loaded GO nanomaterials were tested to suppress the progression of multiple myeloma cells. This study revealed that although the DOX-loaded GO nanomaterials had low toxicity towards normal cells, they were not capable of inducing apoptosis in cancer cells. Furthermore, these nanocarriers did not change the anti-tumor activity of DOX [313]. However, most studies do not agree with these findings, and they support the potential of GO nanomaterials to induce apoptosis and inhibit the progression of cancer cells. One study evaluated the cytotoxicity of GO nanoparticles against glioma cells and showed they could stimulate apoptosis and decrease viability and proliferation at higher concentrations [314]. GO nanoparticles are generally considered promising candidates to trigger apoptosis in cancer cells [315]. Curcumin-loaded GO nanomaterials for breast cancer therapy are another example of GO nanoparticles used for drug delivery and apoptosis induction. These nanocarriers triggered apoptosis and morphological changes from an elongated to a rounded morphology [316].

Recently, much attention has been directed towards using various hybrid nanocomposites in cancer therapy, since they combine the beneficial properties of several different nanostructures that can exert additive or even synergistic effects in combination [317–320]. A GO-silver nanocomposite was prepared and studied for synergistic effects in ovarian cancer therapy. This hybrid nanocomposite was prepared using R-phycoerythrin (RPE), and exposure of ovarian cancer cells to the GO-silver nanocomposite significantly decreased the viability and proliferation of cancer cells. This nanocomposite increased ROS levels enough to disturb the mitochondrial membrane potential, leading to apoptosis in ovarian cancer cells. Furthermore, the GO-silver nanocomposite could promote the anti-tumor activity of salinomycin (a drug that causes lysosome membrane permeabilization and ferroptosis) [321]. Another study tested the GO-wrapped mesoporous silica nanoparticles for the co-delivery of DOX plus cinnamaldehyde in breast cancer therapy. These nanoparticles displayed high stability and enhanced intracellular accumulation of both DOX and cinnamaldehyde. In order to provide selectivity toward cancer cells, surface modification of GO-mesoporous silica nanoparticles with HA was carried out. In breast cancer cells, these pH-sensitive nanocarriers effectively stimulated the intrinsic apoptotic pathway [322]. A number of in vivo studies have also confirmed a role for GO nanocarriers in apoptosis induction and inhibition of cancer progression. Recently, ceramide-GO nanoparticles were shown to exert a synergistic effect in combination with sorafenib to suppress cancer cells through apoptosis induction. Furthermore, the combination of GO and sorafenib down-regulated protein kinase-B (Akt) as well as multidrug resistance (MDR) proteins, leading to increased apoptosis in hepatocellular carcinoma cells and decreased tumor growth in vivo [323].

Overall, the following conclusions can be drawn (Fig. 8).

1) GO nanoparticles can induce apoptosis in cancer cells and reduce their proliferation and viability.
2) A variety of tumor-promoting pathways can be affected by GO nanoparticles to trigger apoptosis.
3) Gene therapy using miRNA and siRNA loaded onto GO nanoparticles can be used for sensitizing cancer cells to apoptosis.
4) Anti-tumor and chemotherapeutic drugs can be loaded onto GO nanomaterials for stimulation of apoptosis.

5.2. Toxicological profile

The effects of GO nanomaterials on autophagy and apoptosis can result in toxicity to major body organs, as supported by findings from different studies. Exposing embryonic fibroblast cells to GO led to cell cycle arrest in the S phase, induced DNA damage, and induced apoptosis via TP53 up-regulation [333]. Hemoglobin and lymphocytes could be negatively affected by GO nanosheets, so that exposing cells to GO was associated with unfolding the quaternary structure of hemoglobin near tyrosine residues. Higher GO concentrations altered hemoglobin's α-helix structure. Furthermore, GO nanoparticles increased ROS and Bax levels while reducing Bcl-2 levels, leading to apoptosis [334]. The

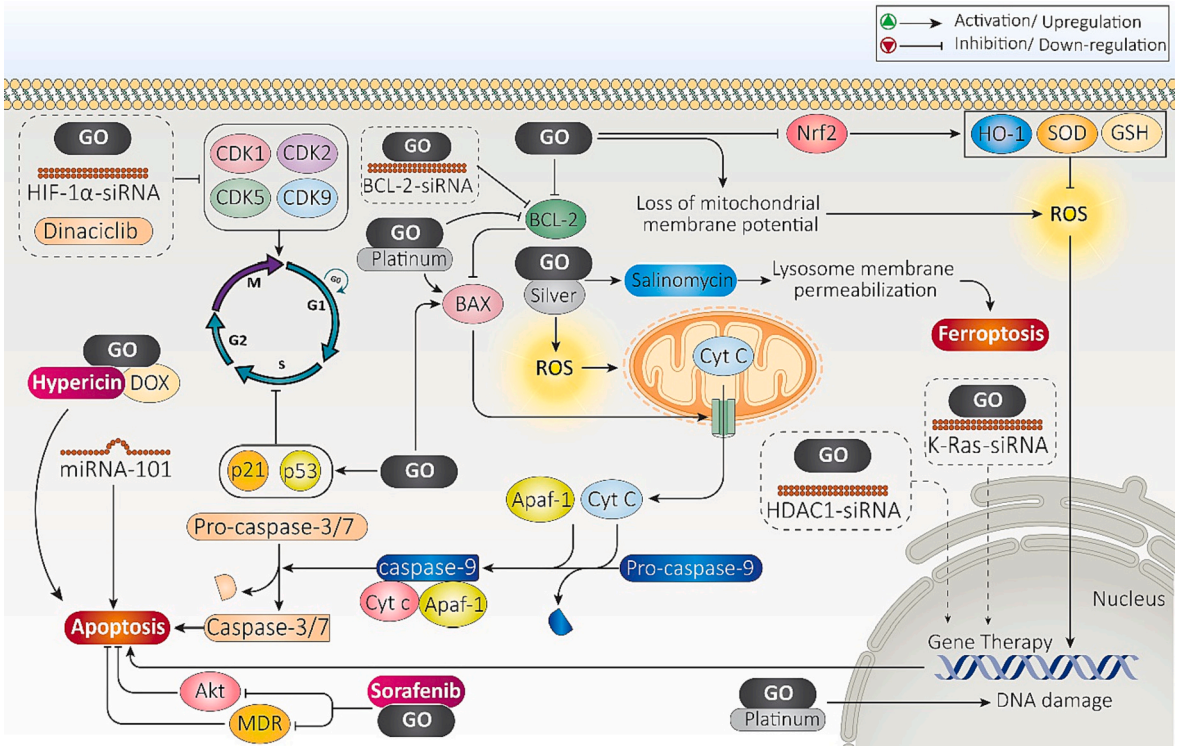

Fig. 8. Regulation of apoptosis by GO nanomaterials and its application in cancer therapy.

interactions of GO nanoparticles with biological molecules depend on their physico-chemical properties and also on their generation method. Surface modifications are also important in determining the biocompatibility of these materials [335,336]. In a size- and time-dependent manner, GO nanomaterials can cause cytotoxicity to cells and can damage whole organisms [337–339]. Besides, major body organs, such as liver, kidney, spleen, and heart, can be negatively affected by intravenous injection of GO nanoparticles [81,340]. Exposure of pre-osteoblast cells to GO nanosheets resulted in apoptosis and a decrease in cell proliferation [341].

An in vivo experiment using Swiss mice confirmed the toxicity of GO nanomaterials to normal and healthy cells. Oral administration of GO nanoparticles (10, 20, and 40 mg/kg) resulted in DNA damage and triggered apoptosis, necrosis, and inflammation [342]. These pre-clinical studies are necessary for the commercialization of GO nanoparticles and using them for treatment of diseases in clinical studies since high biocompatibility is considered to be a determining factor for introducing a new agent to the market.

In a dose-dependent manner, GO nanomaterials can increase ROS levels and induce loss of mitochondrial membrane potential, which are the primary steps for inducing apoptosis in normal cells [343]. Previously, it was mentioned that the toxicity of GO nanomaterials is size-dependent. The question now is whether larger sizes are more toxic than smaller sizes. The previous study in Swiss mice demonstrated that by increasing the dose of GO nanoparticles, their toxicity increased. However, the story is completely different with regard to size. A new study synthesized two different sizes of GO nanoparticles, 20 nm and 100 nm, and then examined their toxicity to Leydig and Sertoli cells. Both GO nanomaterials with 20 nm and 100 nm sizes induced ROS overproduction, DNA damage, and apoptosis in Leydig and Sertoli cells. Importantly, molecular pathways such as EGFR and Akt, which are responsible for cell survival and regulating apoptosis, were inhibited by these nanoparticles. Noteworthy, it was found that GO nanoparticles with a 20 nm size were more toxic to normal cells compared to GO nanocarriers with a 100 nm size [344].

Another pathway used by GO nanomaterials to induce apoptosis in normal cells is by adhering to the cell membrane, triggering cell membrane ruffles, methuosis, and apoptosis. Methuosis is a type of non-apoptotic cell death associated with the vacuolization of macropinosomes and endosomes. This was attributed to increased cell stress and enhanced ROS levels [345]. Taking everything together, these studies are in agreement with the fact that GO nanomaterials can show toxicity towards normal cells, which could be an impediment for their clinical application. In order to improve their biocompatibility, green synthesis procedures can be tested [93].

6. Conclusions

In this review, we summarized the mechanisms through which GO nanomaterials can regulate autophagy and apoptosis in both normal and cancer cells. Owing to ever-rising applications of GO nanomaterials in the field of biomedicine, especially cancer therapy, it is vital to understand how these nanostructures affect cells. In particular, at the time that these effects are not appropriate for their biomedical application, further improvements should be made in the synthesis of the GO nanoparticles, or combination treatments should be investigated.

In cancer cells, autophagy may function as either a pro-survival or a pro-death machinery. As discussed above, GO nanoparticles can either induce or inhibit autophagy. When autophagy plays a pro-survival role in cancer cells, autophagy inhibitors such as chloroquine can be used to enhance anti-tumor capacity. In this case, inhibition of autophagy would potentiate the efficacy of GO nanomaterials in triggering apoptosis in cancer cells. GO nanoparticles are promising candidates for drug and gene delivery and can be loaded with anti-tumor drugs such as DOX to promote apoptosis induction and cancer elimination. Furthermore, gene delivery by GO nanomaterials is possible in cancer treatment and can lead to increased apoptosis.

Although GO nanoparticles exhibit good promise in the regulation of autophagy and apoptosis in cancer treatment, it is likely that interference with these molecular pathways will negatively affect their biocompatibility. Autophagy is an adaptive mechanism, and its induction is of importance for cell survival and homeostasis. By impairing autophagy flux, GO nanomaterials can result in cell toxicity and development of pathological sequelae such as inflammation. Furthermore, GO nanoparticles can increase ROS generation and DNA damage to trigger apoptosis in normal cells as well as cancer cells.

These topics have been mechanistically discussed in the review to pave the way for future clinical applications of GO nanoparticles. In a first step, the green synthesis of GO nanoparticles is suggested to improve both biocompatibility and biomedical potential. Then, because GO nanoparticles are distributed in the major organs of the body and mainly function by increasing ROS levels, it is suggested that antioxidant agents could be co-administered to reduce toxicity to normal cells. Further experiments are required to shed some lights towards the genuine potential of GO nanoparticles and whether their application in cancer therapy can be achieved with a manageable toxicological profile. One of the important drawbacks of current experiments is that they have mainly focused on the material part, while molecular pathways have been partially ignored. Therefore, it is recommended that future studies focus more on related molecular pathways of apoptosis and autophagy and the regulation of signaling networks by GO nanocomposites in cancer therapy.

One of the reasons of using nanoparticles in delivery approaches in increasing accumulation and internalization in tumor cells. The problem related to composites and metal platforms is that their rigidity may reduce internalization and therefore, green modification has been suggested to improve internalization ability [346]. Since green modification and coating composites have been effective in improving tumor internalization, similar strategy can be employed in GO architectures in cancer therapy field. Notably, graphene and its derivatives have been synthesized using green products and in an eco-friendly method for different applications [347–350]. In order to synthesis nanoparticles in a green way, bacteria, actinomycetes, yeasts, fungi, algae and plants can be utilized. Notably, two approaches can be used for synthesis of nanoparticles including bottom-up and top-down strategies that green synthesis is included in bottom-up category [351].Another important aspect is that in addition to increasing tumor internalization, green modification is beneficial in improving biocompatibility and decreasing adverse impacts of nanoplatforms. Therefore, a special attention should be directed to this case. However, this is still the beginning point of using GO platforms in cancer therapy via regulating apoptosis and autophagy, and more experiments in future should focus on green-synthesized and -coated GO architectures in cancer therapy and regulating cell death pathways.

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Declaration of Competing Interest

MRH declares the following potential conflicts of interest. Scientific Advisory Boards: Transdermal Cap Inc., Cleveland, OH; Hologenix Inc. Santa Monica, CA; Vielight, Toronto, Canada; JOOVV Inc., Minneapolis-St. Paul MN. Consulting; USHIO Corp, Japan; Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany.

The other authors declare no conflict of interest.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2023.01.028.

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