THE EMERGING ROLE OF PARAPTOSIS IN TUMOR CELL BIOLOGY: PERSPECTIVES FOR CANCER PREVENTION AND THERAPY WITH NATURAL COMPOUNDS

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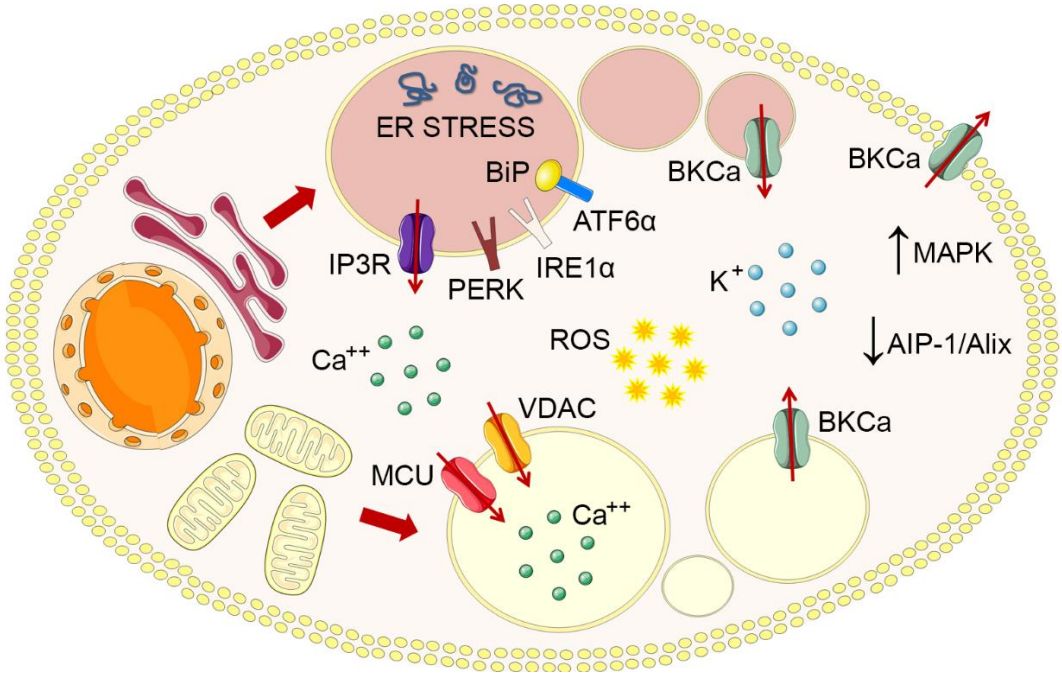
ABSTRACT

Standard anti-cancer therapies promote tumor growth suppression mainly via induction of apoptosis. However, in most cases cancer cells acquire the ability to escape apoptotic cell death, thus becoming resistant to current treatments. In this setting, the interest in alternative cell death modes has recently increased. Paraptosis is a new form of programmed cell death displaying endoplasmic reticulum (ER) and/or mitochondria dilation, generally due to proteostasis disruption or redox and ion homeostasis alteration. Recent studies have highlighted that several natural compounds can trigger paraptosis in different tumor cell lines. Here, we review the molecular mechanisms underlying paraptotic cell death, as well as the natural products inducing this kind of cell death program. A better understanding of paraptosis should facilitate the development of new therapeutic strategies for cancer prevention and treatment.

Keywords: Paraptosis; Apoptosis; Programmed cell death, Natural compounds; Novel therapeutic strategies

GRAPHICAL ABSTRACT



1. INTRODUCTION

In a multicellular organism, cells are arranged into highly organized tissues and organs. The number of cells in these structures is effectively controlled, not simply by regulating cell division, but also cell death. When overabundant or damaged, cells activate an intracellular death program, thus committing suicide: this process is called apoptosis [1–3]. Standard anti-cancer therapies activate different pro-death modes, including pro-apoptotic mechanisms, to eliminate highly proliferative tumor cells. In particular, apoptosis is often induced upon chemotherapeutic treatment. In this case, the downregulation of pro-apoptotic pathways allows cancer cells to escape these treatments, resulting in tumor survival and chemoresistance [4–6]. For this reason, the identification/characterization of novel cell death pathways could help defining complementary or alternative strategies to those based on the activation of apoptosis [7].

Paraptosis is a programmed cell death form involving endoplasmic reticulum (ER) and/or mitochondria dilation [8]. Although its molecular mechanisms are complex, it differs substantially from apoptosis. In particular, paraptosis appears to be implicated in neurodegeneration [9] and is also frequently observed in tumor cells treated with different natural compounds *in vitro* and *in vivo* [10]. This article provides a comprehensive review of paraptotic cell death, its features and how it can be induced by natural products in cancer cells to overcome the phenotype responsible for tumor drug-resistance.

2. APOPTOSIS

The term "apoptosis", from the Greek "dropping off", refers to a highly selective process in which a cell commits suicide after receiving certain stimuli. It was first reported by Kerr *et al.* in 1972, and it was observed to play a crucial role in both physiological and pathological conditions [11].

2.1. Morphology of Apoptosis

The main morphological changes associated with apoptotic cell death have been highlighted by light and electron microscopy [12]. In the early stages of apoptosis, cells undergo shrinkage and pyknosis, appearing smaller in size, with tightly packed organelles in the cytoplasm and condensed chromatin within the nucleus. In particular, cells stained with hematoxylin and eosin are round- or oval-shaped and show a dark acidophilic cytoplasm and violet chromatin fragments aggregating peripherally under the nuclear membrane. Subsequently, extensive plasma membrane blebbing is observed, accompanied by karyorrhexis, the nuclear membrane rupture and the release and degradation of its content, and budding, a process where the cell is fragmented into membrane-bound vesicles called “apoptotic bodies”. These bodies are then engulfed by macrophages and other phagocytic cells before they can release their content into the surrounding interstitial tissue. In particular, dying cells are removed by phagocytes in an orderly way, without evoking any inflammatory response. It should also be noted that apoptosis generally involves individual cells or small cell clusters.

2.2. Apoptotic pathways

The two best-characterized apoptotic pathways are the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway [13,14].

2.2.1. Apoptotic extrinsic pathway

The extrinsic pathway is mediated by cell surface death receptors belonging to the tumor necrosis factor receptor superfamily (TNFRS). They possess an extracellular cysteine-rich domain, a transmembrane domain and a cytoplasmic domain called “death domain”, responsible for the transmission of the death stimulus from the cell membrane to the intracellular molecular signaling. So far, the best-known ligand/death receptor systems are TNF-α/TNFR1, FasL/FasR, Apo2L/DR4, Apo2L/DR5 and Apo3L/DR3.

The extrinsic pathway is activated by the binding of a specific death receptor with its extracellular ligand. The obtained composite then recruits death domain-containing protein (FADD) and pro-caspase-8, forming

the death-inducing signaling complex (DISC). This results in the activation of pro-caspase-8, which leads to the cleavage of pro-caspase-3, the main enzyme responsible for apoptosis execution [15,16].

2.2.2. Apoptotic intrinsic pathway

The intrinsic pathway is regulated by mitochondrial enzymes, particularly by those belonging to the Bcl-2 family [17]. This group is divided into two different sub-categories of proteins, the pro-apoptotic proteins (*e.g.* Bax, Bak, Bad, Bcl-Xs, Bim, Bid, Bik and Hrk) and the anti-apoptotic proteins (*e.g.* Bcl-2, Bcl-X_L, Bcl-W, Bfl-1 and Mcl-1). The anti-apoptotic proteins block cell death by preventing the cytochrome *c* mitochondrial release, which is on the contrary stimulated by the pro-apoptotic proteins, so the balance between these inhibitors/inducers determines whether the apoptotic cascade would be activated or not. In particular, when the pro-/anti-apoptotic protein ratio is increased, outer mitochondrial membranes become permeable to internal cytochrome *c*, which is released into the cytosol. Cytochrome *c* then recruits apoptotic protease activating factor-1 (Apaf-1) and pro-caspase-9 to form the so-called "apoptosome", which triggers apoptosis via caspase-9/3 cascade induction [18–20].

2.2.3. The caspase cascade

Caspases are a family of protease enzymes implicated in apoptosis and inflammation. They are synthesized as inactive zymogens called "pro-caspases", which are activated by post-translational modification only after proper stimulation, allowing a rapid and tight regulation of the enzyme. In particular, the caspase activation mechanism involves dimerization/oligomerization of the pro-enzyme and its subsequent cleavage into a small and a large subunit. The two peptides then associate with each other to generate an active heterodimeric complex.

Caspases have been broadly classified by their roles in apoptosis (caspase-2, -3, -6, -7, -8, -9 and -10 in mammals) and in inflammation (caspase-1, -4, -5, -12 in humans and caspase-1, -11, and -12 in mice). Apoptotic caspases have been subdivided on the basis of their mechanism of action into initiator caspases (caspase-8 and -9) or executioner caspases (caspase-3, -6, and -7). The other caspases that have been identified include caspase-13, the caspase-4 bovine homolog, and caspase-14, which is selectively expressed in the epidermis and the hair follicles, where it contributes to epidermal differentiation.

Caspases possess proteolytic activity, cleaving proteins at aspartic acid residues. Common caspase targets are: mediators and regulators of apoptosis (Bid, Bcl-2 and Bcl-xL); structural proteins (nuclear lamins, fodrin, gelsolin, keratins 18 and 19, vimentin, plakoglobin γ-catenin and β-catenin); cellular DNA repair proteins (poly(ADP-ribose) polymerase (PARP), ATM serine/threonine kinase, Rad51, DNA-dependent protein kinase (DNA-PK)); cell cycle-related proteins (Wee1, Cdc27, Rb and the two CDK inhibitors p21CIP1 and p27KIP1) [21,22].

2.3. Other biochemical features of apoptosis

Another biochemical hallmark of apoptosis is the expression of cell surface markers ("eat me" signals) which allows the recognition of dying cells by phagocytes, leading to a quick phagocytosis without damage to the surrounding tissue. This is facilitated by the externalization of phosphatidylserine. In fact, while in normal conditions this phospholipid is retained on the cytosolic side of the plasma membrane by a flippase, it is rapidly exposed on the extracellular leaflet by a scramblase in case of apoptosis. In addition to phosphatidylserine, recent findings have evidenced that other proteins are also externalized on the cell surface during the apoptotic cascade, such as Annexin I, a protein usually implicated in the regulation of the anti-inflammatory effects of glucocorticoids, and calreticulin, a Ca^{2+}-binding chaperone that promotes protein folding and quality control in the ER lumen [23].

2.4. Apoptosis in cancer

Evasion of cell death represents one of the key events in the malignant transformation of a cell. Interestingly, there are several mechanisms through which a tumor cell can acquire resistance to apoptosis. For instance, downregulation and loss of function of different death receptors have been found in various tumor

types, leading to impairment of the apoptotic extrinsic signaling. Moreover, an altered balance of pro-/anti-apoptotic proteins has been observed in several cancers: this appears to involve not only the Bcl-2 protein family but also p53, a tumor suppressor known as "the guardian of genome" due to its ability to block the synthetic phase of the cell cycle (phase S) and promote apoptosis in cells with damaged DNA, and the inhibitor of apoptosis proteins (IAPs) [24]. Hence, triggering alternative pro-death pathways could represent an effective strategy to eliminate cancer cells unaffected by the apoptotic cascade [7]. In this context, paraptosis has recently gained increasing interest, mainly due to its involvement in the anti-tumor activity of various natural compounds.

3. PARAPTOSIS

Paraptosis was first described by Sperandio *et al.* in 2000 [8]. It is a type of programmed cell death displaying cytoplasmic vacuolation, usually consisting in mitochondrial and/or ER swelling. It requires protein synthesis and can be successfully blocked by the translation inhibitor cycloheximide. Unlike apoptosis, paraptosis does not require activation of caspases or formation of apoptotic bodies; indeed, it is not affected by Bcl-2-like anti-apoptotic protein overexpression or caspase inhibitors. On the other hand, in most cases paraptosis has been demonstrated to be dependent on mitogen-activated protein kinase (MAPK) family members, such as c-Jun N-terminal protein kinase 1 (JNK1), p38 and mitogen-activated protein kinase kinase 2 (MEK-2), and it can be inhibited by the multifunctional adapter protein AIP-1/Alix [25]. As shown in the following paragraphs of this review, it is often accompanied by an alteration of Ca^{2+} and redox homeostasis, as well as by proteostasis disruption and ER stress, a condition where unfolded/misfolded proteins accumulate in the ER lumen, culminating in the activation of pro-death processes. However, these features are not always present in cells undergoing paraptosis. Hence, the term "paraptosis-like cell death" has been coined, to describe those types of programmed cell death resembling paraptosis but lacking one or more of its common characteristics [26]. The main differences between apoptosis and paraptosis are summarized in **Figure 1**.

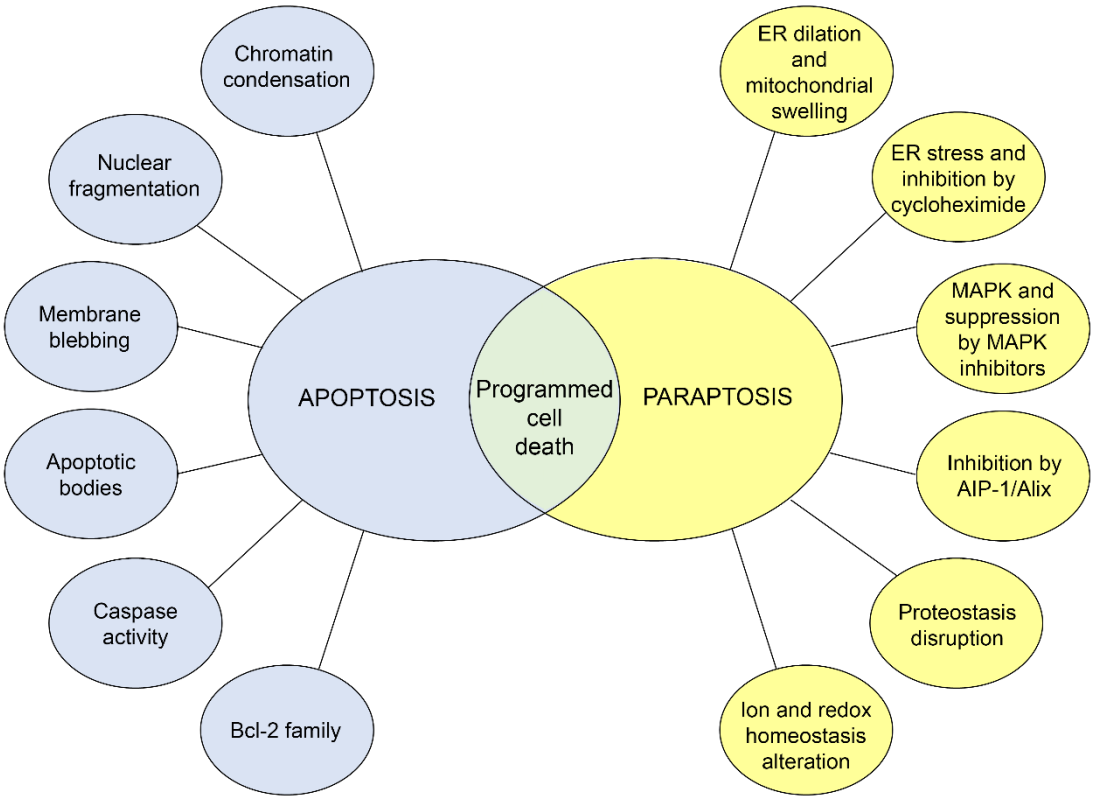


Figure 1. Main differences between apoptotic and paraptotic cell death.

3.1. Natural compounds inducing paraptosis in cancer

Paraptosis is known to occur during neural development, and it has also been observed in different neurodegenerative diseases and neurological disorders [9]. Moreover, it appears to be involved in retinal pathologies: it is activated after both glucocorticoid treatment [27,28] and reperfusion injury [29], as well as in the early phases of glaucoma [30].

Established mediators of paraptotic cell death are insulin-like growth factor 1 receptor (IGFR-1) [8], the neuropeptide substance P [31], the orphan TNF receptor TAJ/TROY [32], epidermal growth factor (EGF) [33,34] and adenine nucleotide translocase 1 (ANT1), a multitask protein implicated in cell proliferation and metabolism [35]. Moreover, both membrane cholesterol and heme homeostasis have been found to play a fundamental role in the modulation of paraptosis [36,37]. Finally, human glioma cells expressing macrophage colony-stimulating factor (mM-CSF) on their surface were reported to be eliminated by human monocytes through the paraptotic pathway [38–40].

Concerning cancer cells, many natural compounds have been demonstrated to cause paraptosis in a variety of human cancer cell lines. Among them, taxol, cyclosporine A, tunicamycin, procyanidins, curcumin, honokiol, ginsenosides, tocotrienols, celastrol, ophobiolin A, hesperidin, morusin, 6-shogaol, chalcomoracin, gambogic acid, plumbagin, 8-p-hdroxybenzoyl tovarol, *cis*-nerolidol, manumycin A, DL-selenocystine, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2, yessotoxin and 1-desulfoyessotoxin have shown promise as proparaptotic agents (**Figure 2**).

3.1.1. Taxol

Taxol is one of the most used natural-source cancer drugs worldwide. It is derived from the bark of *Taxus brevifolia* (Pacific yew) and has been approved for the treatment of several types of tumor, such as cervical, lung, breast, pancreatic and ovarian cancer [41,42].

Taxol is a well-known cytoskeletal drug that targets tubulin. In particular, it stabilizes the cell microtubules and prevents their disassembly, thus blocking mitosis progression and prolonging the mitotic checkpoint activation, ultimately leading to cell cycle arrest and apoptosis. Interestingly, it has been reported that taxol can also induce paraptosis in cancer cells. In a recent study by Guo *et al.*, low concentrations of taxol (35 nM) were shown to activate apoptosis, accompanied by nuclear fragmentation, Bax mitochondrial translocation and caspase-3 cleavage, in ASTC-a-1 human lung adenocarcinoma cells, whereas high concentrations of this natural anti-cancer agent (70 μM) triggered significant cytoplasm vacuolization, associated with ER and mitochondrial swelling [43]. These results were also confirmed by *in vivo* experiments, where taxol intratumorally injected (50 mg/kg) in A549 tumor-bearing mice successfully suppressed cancer growth, causing significant ER vacuolization without toxicity [44]. Notably, the taxol-induced paraptotic cell death was demonstrated to require neither protein synthesis nor the activation of the MEK, JNK and p38 signaling pathways, indicating that high doses of taxol can induce a non-classical paraptotic mechanism supposedly regulated by the vacuolation-related Bcl-XL translocation from mitochondria to ER [45,46].

3.1.2. Cyclosporine A

Cyclosporine A is a fungal metabolite with extensive immunosuppressive properties, generally used for the treatment of Crohn's disease, rheumatoid arthritis, nephrotic syndrome and psoriasis, as well as to prevent transplant rejection. The mechanism of action of this molecule involves its binding to the lymphocyte protein cyclophilin (immunophilin), resulting in the suppression of the calcineurin phosphatase activity and in a decreased inflammatory cytokine production by T cells [47].

Recently, Ram & Ramakrishna have demonstrated that cyclosporine A can induce calcineurin inhibition-independent paraptosis-like cell death in SiHa cervical cancer cells. In particular, unfolded protein response (UPR) and subsequent ER stress, paralleled by a reduction in the levels of cyclophilin B, were found to precede a massive cytoplasmic vacuolization. The vacuoles were confirmed to originate from the ER and their formation was accompanied by AIP1/Alix downregulation, as well as effectively prevented by cycloheximide treatment [48].

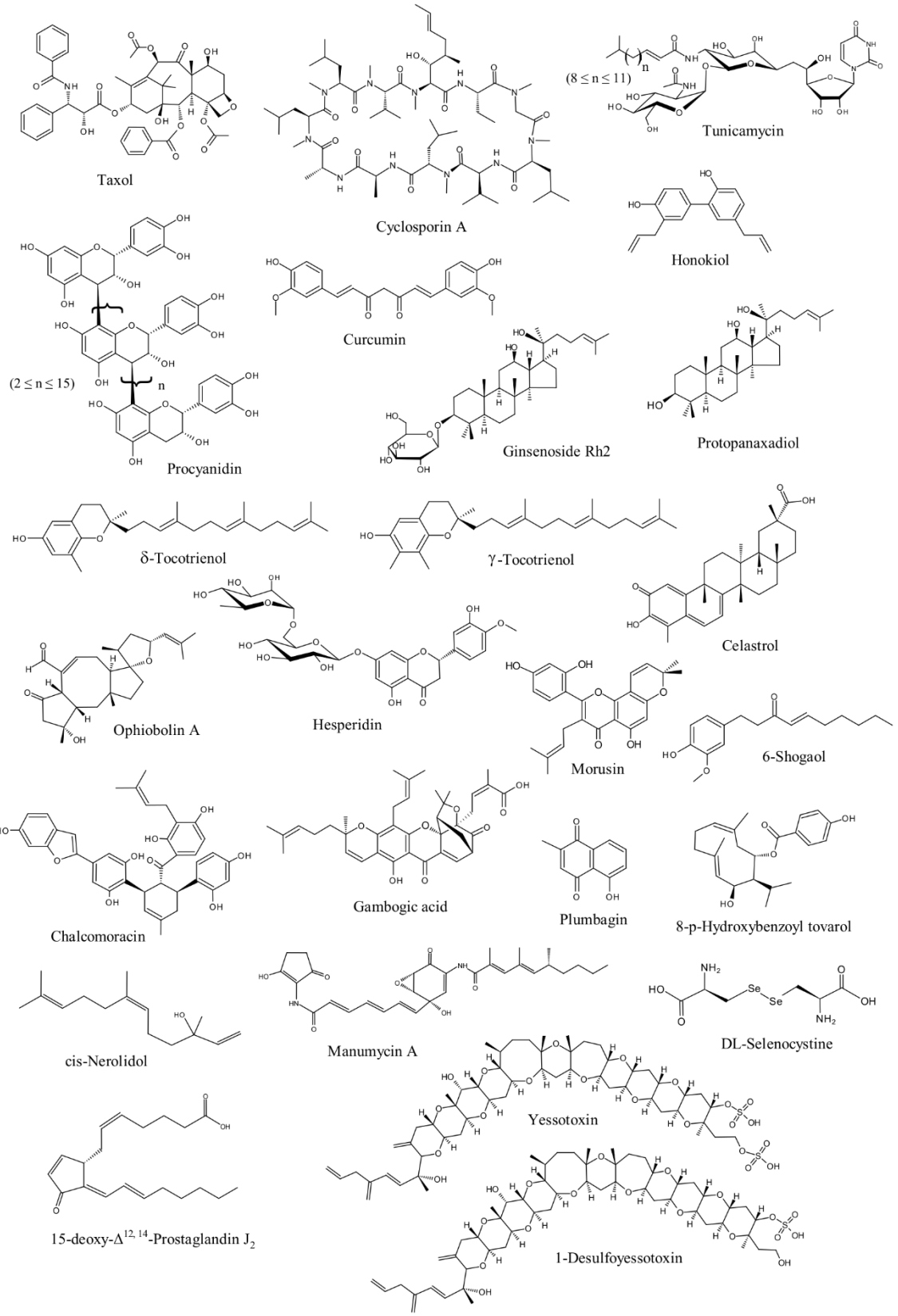


Figure 2. Chemical structures of the main natural compounds inducing paraptosis.

3.1.3. Tunicamycin

Tunicamycin, a naturally occurring antibiotic, is a well-established ER stress inducer acting via inhibition of protein N-linked glycosylation. It is known to trigger both apoptosis and autophagy in several cancer cell lines and models and, more recently, it has also been shown to mediate paraptotic cell death in FRO anaplastic thyroid carcinoma cells. Interestingly, *de novo* protein synthesis and proteasomal activity were found to be involved in the tunicamycin-mediated paraptosis, which was also potentiated by PLX4032-related $BRAF^{V600E}$ suppression [49].

3.1.4. Procyanidins

Procyanidins are members of the proanthocyanidin class of flavonoids. They are oligomeric compounds, commonly found in many plants, such as apples, maritime pine bark, cinnamon and grape seed and skin [50].

In addition to their cardioprotective and immunomodulatory actions, procyanidins obtained from grape seeds have been shown to inhibit the U-87 glioblastoma cell growth, triggering a non-apoptotic cell death phenotype resembling paraptosis [51]. In particular, dying cells exhibited extensive cytoplasmic vacuolization without membrane blebbing, nuclear fragmentation or formation of apoptotic bodies. Further experiments indicated the involvement of MAPK cascade, Ca^{2+} mobilization and new protein synthesis in the procyanidin-mediated U-87 cell death [52].

3.1.5. Curcumin

Curcumin is a bright yellow chemical that can be extracted by *Curcuma longa* plants. It is known to possess several pro-health properties, and in the last years it has gained a lot of attention for its anti-cancer effects [53].

Yoon *et al.* have shown that curcumin can induce paraptosis in different breast cancer cell lines, such as Hs578T, MDA-MB-231 and MDA-MB-435S cells, by promoting vacuolization resulting from mitochondrial and ER swelling and fusion. Blockade of protein synthesis by cycloheximide inhibited curcumin-mediated vacuolization and cytotoxicity, pointing out that protein translation is essential for this process. In curcumin-treated cells, AIP-1/Alix expression was downregulated and its overexpression reduced curcumin-mediated cell death. Extracellular signal-regulated kinase-2 (ERK2) and JNK were activated upon curcumin treatment, and mitochondrial superoxide, Ca^{2+} overload and proteasomal dysfunction were shown to be involved in paraptosis induction [54,55]. Similar results were also obtained in the A172 glioblastoma cell line, where curcumin was found to trigger cytoplasmic vacuolization, together with modulation of the inositol-requiring 1α (IRE1α) and activating transcription factor 6 (ATF6) ER stress markers, of the microRNAs (miRNAs) miR-449, miR-222, miR-27a and of the Akt protein levels [56].

Interestingly, curcumin synthetic derivatives have also been demonstrated to possess pro-paraptotic activity. In particular, dimethoxycurcumin induced paraptosis, associated with upregulation of the protein levels of CCAAT-enhancer-binding protein homologous protein (CHOP) and Noxa, in human breast cancer cells, without affecting the viability of normal mammary epithelial cells [57]. Similarly, the curcuminoid B63 triggered reactive oxygen species (ROS)-mediated ER stress and MAPK cascade in gastric cancer cells and xenografts, ultimately leading to paraptotic cell death. More importantly, B63 also caused paraptosis in 5-fluorouracil-resistant gastric cancer cells [58].

3.1.6. Honokiol

Honokiol is a lignan present in the bark, leaves and seed cones of trees belonging to the genus *Magnolia*, endowed with potent anti-inflammatory, neuroprotective, anti-oxidant and anti-tumor activities [59,60].

In K562 and NB4 leukemia cells, honokiol has been found to activate time- and concentration-dependent paraptosis, as evidenced by the presence of cytoplasmic vacuolation derived from ER swelling and by the lack of chromatin condensation, membrane blebbing and caspase activation. In particular, the mechanism of cell death has been demonstrated to be related to an increased generation of ROS [61,62].

3.1.7. Ginsenosides

Ginsenosides are a group of glycosylated triterpenes, also called saponins, naturally occurring in the plant genus *Panax* (ginseng). They exhibit a variety of biological effects, including anti-diabetic and neuroprotective actions, as well as anti-tumor properties [63].

Ginsenoside Rh2 and protopanaxadiol (PPD), a triterpenoid abundant in steamed ginseng, have been reported to induce both caspase-dependent apoptosis and caspase-independent paraptosis in HCT116 and SW480 colorectal cancer cells. Treatment of tumor cells with both these compounds resulted in ROS generation, followed by activation of NF-κB signaling; ROS blockade by N-acetyl cysteine (NAC) suppressed the upregulation of NF-κB pathway and increased Rh2-related cytotoxicity, indicating that the anti-tumor effects of Rh2 and PPD can be enhanced by antioxidants. Moreover, Rh2 treatment activated the p53 cascade; silencing of this protein significantly inhibited Rh2-mediated vacuolization, indicating that the paraptosis induced by Rh2 is dependent on p53 activity [64,65].

It should be noted that, in human colorectal cancer cells, an extract from red American ginseng has shown synergistic anti-cancer effects when given in combination with 5-fluoracil, and that this enhanced action has been associated with both paraptosis and apoptosis induction [66].

3.1.8. Tocotrienols

Tocotrienols are members of the vitamin E family. They are structurally similar to tocopherols but contain three double bonds in the carbon side chain of the molecule. Four different tocotrienols exist, α-, β-, γ- and δ-isomer, and they can be found in rice bran, palm oil, wheat germ, annatto seed, oat and barley. Tocotrienols display potent cholesterol-lowering, neuroprotective and chemopreventive activities [67–69].

Zhang *et al.* have demonstrated that both γ- and δ-tocotrienol can trigger a paraptosis-like cell death, accompanied by mitochondrial and ER swelling, in SW620 human colon carcinoma cells. Interestingly, the tocotrienol-mediated paraptosis correlated with Wnt signaling inactivation, particularly with a reduction in cyclin D1, β-catenin and c-Jun levels [70,71]. More recently, we have also shown that δ-tocotrienol can induce both apoptosis and paraptosis in prostate cancer cells. The mechanisms underlying its pro-paraptotic activity were found to be related to JNK and p38 activation, as well as to ER stress induction, since not only cycloheximide but also salubrinal, a well-known ER stress inhibitor, successfully prevents the cytoplasmic vacuolization evoked by the treatment with δ-tocotrienol [72].

3.1.9. Celastrol

Celastrol is a pentacyclic triterpenoid obtained from the root of *Tripterygium Wilfordi* plant. It exhibits anti-oxidant, anti-diabetic, anti-obesity and anti-tumor activity [73].

Celastrol has been found to trigger paraptosis-like cytoplasmic vacuolation in different tumor cell lines, such as MDA-MB-435S, A549, HeLa, PC3 and DLD-1 cells, derived from breast, lung, cervix, prostate and colon cancer, respectively [74,75]. Additional studies evidenced that, in HeLa cells, celastrol triggered the MEK and p38 pathways, whose inhibition could prevent the vacuole formation. Moreover, common markers of apoptosis and autophagy were identified, suggesting that celastrol can induce paraptotic, as well as apoptotic and autophagic cell death in tumor cells [74]. Parallelly, in MDA-MB-435S cells, celastrol treatment resulted in an increase of mitochondrial Ca^{2+} levels and ER stress activation through proteasome inhibition. The knockdown of mitochondrial Ca^{2+} uniporter (MCU), as well as the pretreatment with the MCU inhibitor ruthenium red, suppressed celastrol-mediated mitochondrial Ca^{2+} overload, mitochondrial/ER swelling, poly-ubiquitinated protein accumulation and cell death. The alteration of Ca^{2+} homeostasis mediated by celastrol was also effectively prevented through IP3 receptor (IP3R) inhibition with 2-aminoethoxydiphenyl borate (2-APB). In conclusion, the IP3R-mediated Ca^{2+} release from the ER and its subsequent MCU-dependent influx into mitochondria appear to be implicated in the paraptosis triggered by celastrol in tumor cells [75].

3.1.10. Ophiobolin A

Ophiobolin A is a sesterterpenoid fungal metabolite from *Bipolaris*, *Cochliobolus*, *Drechslera*, *Cephalosporium* and *Aspergillus* species. It exerts cytotoxic effects in leukaemia, lung cancer and melanoma cells, by inducing typical apoptotic events, including cell shrinkage, karyorrhexis and DNA laddering [76,77].

Furthermore, in human glioblastoma cells it can trigger paraptosis-like cell death, by decreasing the mitochondria- and ER-located big conductance Ca^{2+}-activated K^+ (BKCa) channel activity [78].

3.1.11. Hesperidin

Hesperidin is a flavan-on glycoside occurring in citrus fruits, displaying anti-inflammatory and tumor suppressive properties [79].

In a recent study by Yunman *et al.*, HepG2 hepatocarcinoma cells treated with crescent concentrations of hesperidin have been found to undergo paraptotic cell death, with ERK1/2-mediated mitochondrial and ER dilation and no chromatin condensation, DNA fragmentation or caspase activation [80]. Interestingly, further experiments have highlighted an increase in mitochondrial Ca^{2+} levels in hesperidin-treated HepG2 cells, and pretreatment with ruthenium red inhibited not only the mitochondrial Ca^{2+} overload but also the swelling of mitochondria and the following paraptotic cascade in these tumor cells. Moreover, Ca^{2+}-mediated ROS overproduction was observed, which was shown to contribute to the mitochondrial membrane loss and dysfunction [81].

3.1.12. Morusin

Morusin is a prenylated flavonoid that can be extracted from *M. australis* and is characterized by many biological activities, including anti-oxidant, anti-bacterial and anti-tumor properties [82].

Treatment of epithelial ovarian cancer cells with morusin resulted in paraptosis-like cell death, correlated with ER stress induction, mitochondrial Ca^{2+} accumulation, ROS formation and mitochondrial membrane potential loss. Moreover, the morusin-induced mitochondrial Ca^{2+} influx, cytoplasmic vacuolization and cell death were suppressed by pretreatment with the outer mitochondrial membrane voltage-dependent anion channel (VDAC) inhibitor 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS). Furthermore, DIDS pretreatment also blocked ER stress activation, ROS production and mitochondrial membrane potential depletion caused by morusin in tumor cells. In line with this, the ER stress-related antiproliferative effects exerted by morusin were also counteracted by the co-treatment with DIDS in ovarian cancer xenograft models. In conclusion, morusin has demonstrated promising anti-tumor potential in epithelial ovarian cancer cells, by triggering paraptosis via VDAC-mediated mitochondrial Ca^{2+} overload both *in vitro* and *in vivo* [83].

3.1.13. 6-Shogaol

Shogaols are ginger constituents, structurally similar to gingerol. The most common of the group is 6-shogaol [84]. In A549 non-small lung cancer cells and in MDA-MB-231 breast cancer cells, it has been demonstrated to trigger paraptosis, accompanied by protein light chain 3B (LC3 I and LC3 II) and polyubiquitin binding protein p62 upregulation, as well as by an increase in the levels of ER stress markers, such as binding immunoglobulin protein (BiP) and CHOP. More importantly, the pro-paraptotic effects exerted by 6-shogaol have been reported to correlate with the inhibition of the 26S proteasome [85].

3.1.14. Chalcomoracin

Chalcomoracin is a potent antibacterial compound obtained from *Morus alba* [86]. It promotes the formation of ER-derived vacuoles in PC3 and MDA-MB-231 cells. Further studies revealed that chalcomoracin treatment not only downregulated AIP-1/Alix levels but also upregulated PINK1 (PTEN-induced kinase 1), a key marker of mitophagy, in these cell lines. Moreover, LNCaP prostate cancer cells were shown to be more responsive to chalcomoracin pro-paraptotic effects after ectopic expression of Myc-PINK1, highlighting the crucial role of PINK1-driven mitophagy in the paraptosis induced by this phytochemical. Finally, both mitophagy and cytoplasmic vacuolization were inhibited by NAC, suggesting the ROS involvement in these two processes. These results were also confirmed in MDA-MB-231 tumor-bearing mice [87].

3.1.15. Gambogic acid

Gambogic acid, a xanthonoid isolated from the resin of the tree *Garcinia hanburyi*, has been recently reported to possess different anti-cancer properties [88]. Seo *et al.* have demonstrated that thiol proteostasis alteration and subsequent paraptosis could be responsible for the anti-tumor effects of this compound in various breast cancer cell lines. In particular, gambogic acid is able to form Michael adducts by reacting with cysteinyl thiols, pointing out that its capacity to covalently modify the protein nucleophilic cysteinyl groups may generate misfolded proteins accumulating within the ER and mitochondria, thus inducing paraptosis-associated ER stress and dilation, as well as mitochondrial swelling and fusion [89].

3.1.16. Plumbagin

Plumbagin is an active secondary metabolite obtained from the roots of *Plumbago rosea*, exhibiting potent anti-septic, anti-inflammatory and anti-tumor effects [90]. It has been demonstrated to trigger paraptosis-associated cytoplasmic vacuolation in different cancer cell lines, such as A549 non-small lung cancer, MDA-MB-231 breast cancer and HeLa cervical cancer cells, but not in WI-38 normal fibroblasts. In particular, the empty vacuoles observed after plumbagin treatment were found to originate from the ER, where polyubiquitinated proteins were shown to accumulate, due to the inhibition of the 26S proteasome chymotrypsin-like activity. The vacuolization and cell death were not correlated with ROS production, but they were successfully blocked by thiol antioxidants, indicating that cell sulfur homeostasis could be affected by plumbagin treatment. Plumbagin also caused mitochondrial dysfunction and energy depletion [91].

3.1.17. 8-p-Hdroxybenzoyl tovarol

8-p-Hdroxybenzoyl tovarol is a germacrane-type sesquiterpenoid extracted from the roots of *Ferula dissecta (Ledeb.) Ledeb*. In HeLa human cervical cancer cells, it has been reported to induce paraptosis involving extensive cytoplasmic vacuolization without caspase cleavage. In particular, the paraptotic cell death evoked by this compound has been demonstrated to be associated with UPR and ER stress. Notably, 8-p-hdroxybenzoyl tovarol also activated autophagy, which was found to antagonize paraptosis; rapamycin, an autophagy inducer, and 3-methyladenine, an autophagy inhibitor, increased and decreased this effect, respectively. In addition, the knock down of the autophagic regulator beclin-1 significantly promoted ER stress. Collectively, these results suggest that 8-p-hdroxybenzoyl tovarol can trigger paraptosis and protective autophagy in tumor cells [92].

3.1.18. Cis-Nerolidol

Nerolidol, also called peruviol and penetrol, is a sesquiterpene alcohol abundant in the essential oils of many plants, including jasmine, ginger, neroli, lavender, lemon grass, *Cannabis sativa* and tea tree. Two nerolidol isomers exist, *cis* and *trans*, that differ in the ligand arrangement around the central double bond [93]. The *cis*-isomer has been found to activate caspase-independent paraptotic cell death in the HepG2/C3A human hepatocellular carcinoma cell line. In particular, the paraptosis induced by this compound has been shown to be accompanied by EIF2AK3, ERN1, CYP2C19 and CYP1A2 upregulation, suggesting its correlation with ER stress and increased activity of cytochrome P450 enzymes [94].

3.1.19. Manumycin A

Manumycin A is a natural microbial metabolite able to specifically target and inhibit the farnesyl protein transferase, thus inducing cancer cell death. Notably, it has been demonstrated that it can trigger cytoplasmic vacuolation, paralleled by upregulation of ER stress (BiP and CHOP) and autophagy (LC3 and p62) markers, in triple-negative breast cancer cells. In particular, it appears to severely alter sulfhydryl homeostasis, thus leading to accumulation of ubiquitinated proteins and subsequent paraptosis activation. These paraptotic effects were also observed in xenograft models, together with PTEN, p21 and p27 upregulation and Akt dephosphorylation. Intriguingly, normal human mammary epithelial cells did not undergo cytoplasmic vacuolation and paraptotic cell death when treated with manumycin A [95].

3.1.20. DL-Selenocystine

DL-Selenocystine is a diselenide-bridged amino acid displaying both anti- and pro-oxidant actions. Treatment of HeLa cells with a 100 μM dose of this redox-active selenium compound resulted in the activation of two morphologically distinct cell death processes, one with apoptotic phenotype and the other resembling paraptosis, accompanied by UPR, ER stress and cytoplasmic vacuolization [96].

3.1.21. 15-deoxy-Δ^{12,14}-Prostaglandin J$_2$

15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2 (15d-PGJ2) is a cyclopentenone prostaglandin derivative obtained by free radical-mediated arachidonic acid peroxidation. It has potent tumor growth-suppressing properties mediated by both PPARγ dependent and independent mechanisms, and in colon, breast and prostate cancer cell lines it has been found to trigger a novel form of cell death, characterized by sulfhydryl homeostasis disruption and ubiquitinated protein accumulation, culminating in ER dilation and extensive cytoplasmic vacuolation. In addition, 15d-PGJ2-mediated cell death is prevented by pre-treatment with actinomycin D or cycloheximide, indicating that new protein synthesis is essential for its modulation. Furthermore, LC3 upregulation appears to represent a crucial event in the cell death mediated by 15d-PGJ2, since its knockdown conferred significant protection against cytoplasmic vacuolation and ER dilation [97].

3.1.22. Yessotoxin and 1-desulfoyessotoxin

Yessotoxin is a polyether toxin produced by different dinoflagellates [98]. In the BC3H1 myoblast cell line, this marine toxin and its desulphated analog 1-desulfoyessotoxin can activate paraptotic cell death, correlated with JNK activation and p38 phosphorylation, respectively [99,100].

3.1.23. Other compounds

Withaferin A is a steroidal lactone, present in *Withania somnifera*, *Acnistus arborescens* and other members of *Solanaceae* family [101]. It has been observed to trigger oxidative stress-mediated paraptosis in MCF-7 and MDA-MB-231 human breast cancer cell lines [102]. Similar results have also been obtained by treating MDA-MB-231 cells with DETD-35, a sesquiterpene lactone analog of deoxyelephantopin, a natural compound from *Elephantopus scaber* [103].

Treatment of leukemia cancer cells with xanthohumol, a prenylated chalcone derived from hops (*Humulus lupulus L.*) [104], resulted in p38-related paraptotic cell death, involving both UPR and ER stress [105].

A549 and NCI-H1299 human lung cancer cells treated with prenylated bibenzyls from the Chinese Liverwort *Radula constricta* have been shown to undergo mitochondria-derived paraptosis [106]. Similarly, in A549 and K562 cells, a complex formed by copper and hinokitiol, a natural monoterpenoid found in the wood of trees belonging to the *Cupressaceae* family, inhibited the activity of the 19S proteasomal deubiquitinating enzymes, thus leading to ubiquitinated protein accumulation and subsequent ER stress-related paraptotic cell death [107].

A purified resin glycoside fraction from *Pharbitidis Semen* has been reported to activate chloride intracellular channel-1 (CLIC1) and to increase the cytosolic Cl^- concentration in human colon cancer cells, ultimately leading to cytoplasmic vacuolization and paraptosis [108].

3.2. Overview of the paraptotic mechanisms activated by natural products

As shown in **Table 1**, several mechanisms have been proposed to be implicated in the phytochemical-mediated paraptotic cell death.

While MAPK activation has been positively associated with the induction of paraptosis by different nutraceuticals, AIP-1/Alix was demonstrated to be inhibited after cyclosporin A, curcumin, morusin and chalcomoracin treatment, highlighting the key role played by these signaling pathways in the modulation of paraptotic cell death. However, MEK, JNK and p38 upregulation was not observed in taxol-induced paraptosis, indicating that involvement of MAPKs may be dependent on the type of the pro-death stimulus. Importantly, many other molecular pathways have been shown to be modulated during paraptotic cell death, such as Bcl-XL, p53, Wnt, CYPs, PTEN, p21, p27 and Akt; however, their specific role has not been elucidated yet.

Table 1. Natural compounds and their pro-paraptotic mechanisms of action.

Natural compound	Dilated/swollen organelles	MAPK and AIP-1/Alix	Proteostasis alteration	Ion and redox homeostasis alteration	Cross-talk with other cell death modes	References
Taxol	ER and mitochondria	MEK, JNK and p38 not activated	_	_	_	[43-46]
Cyclosporine A	ER	AIP-1/Alix downregulation	ER stress Inhibition by cycloheximide	_	_	[48]
Tunicamycin	ER	_	Proteasome inhibition Inhibition by cycloheximide	_	_	[49]
Procyanidins	_	ERK1/2 and p38	Inhibition by cycloheximide	Ca^{2+} overload	_	[51,52]
Curcumin	ER and mitochondria	ERK2 and JNK AIP-1/Alix downregulation	Proteasome inhibition ER stress Inhibition by cycloheximide	Ca^{2+} overload ROS	_	[54-58]
Honokiol	ER	_	_	ROS	_	[61,62]
Ginsenosides	ER and mitochondria	MEK1/2	Inhibition by cycloheximide	ROS	_	[64-66]
Tocotrienols	ER and mitochondria	JNK and p38	ER stress Inhibition by cycloheximide	_	_	[70-72]
Celastrol	ER and mitochondria	MEK1/2, ERK1/2, JNK and p38	Proteasome inhibition ER stress Inhibition by cycloheximide	Ca^{2+} overload	Autophagy	[74,75]
Ophiobolin A	ER and mitochondria	_	Inhibition by cycloheximide	Changes in K^{+} levels	_	[78]
Hesperidin	ER and mitochondria	ERK1/2	_	Ca^{2+} overload ROS	_	[80,81]
Morusin	ER and mitochondria	AIP-1/Alix downregulation	ER stress Inhibition by cycloheximide	Ca^{2+} overload ROS	_	[83]

6-Shogaol	ER	_	Proteasome inhibition ER stress Inhibition by cycloheximide	_	Autophagy	[85]
Chalcomoracin	ER	AIP-1/Alix downregulation	ER stress Inhibition by cycloheximide	Ca^{2+} overload ROS	Mitophagy	[87]
Gambogic acid	ER and mitochondria	ERK1/2 and JNK	Sulfhydryl homeostasis disruption Proteasome inhibition ER stress Inhibition by cycloheximide	ROS	_	[89]
Plumbagin	ER	_	Sulfhydryl homeostasis disruption Proteasome inhibition ER stress Inhibition by cycloheximide	ROS	_	[91]
8-p-hdroxybenzoyl tovarol	ER and mitochondria	_	ER stress Inhibition by cycloheximide	_	Autophagy	[92]
Cis-nerolidol	ER	_	ER stress	_	_	[94]
Manumycin A	_	_	Sulfhydryl homeostasis disruption ER stress Inhibition by cycloheximide	_	Autophagy	[95]
DL-selenocystine	ER	_	ER stress	_	Autophagy	[96]
15-deoxy-$\Delta^{12,14}$-prostaglandin J2	ER	_	Sulfhydryl homeostasis disruption ER stress Inhibition by cycloheximide	_	LC3 upregulation	[97]
Yessotoxin and 1-desulfoyessotoxin	ER and mitochondria	JNK and p38	_	_	_	[99,100]

In terms of morphological features, ER dilation commonly occurs during paraptosis. In particular, it has been associated with proteostasis disruption, involving sulfhydryl homeostasis alteration and proteasome inhibition. Indeed, celastrol, gambogic acid, plumbagin, manumycin A and 15d-PGJ2 have been proposed to exert their anti-cancer effects by covalently binding (-S-C-) to specific target proteins in the tumor cell. Interestingly, despite differing in their chemical structure, these compounds share a common highly electrophilic nature, due to the α,β-unsaturated carbonyls of gambogic acid, plumbagin, manumycin A and 15d-PGJ2 and to the quinone methide within the A and B rings of celastrol. These substructures rapidly react with the protein sulfhydryl (-SH) and hydroxyl (-OH) groups to form covalent bonds via the conjugate addition reaction. Notably, the thiol-reactivity of these agents may be directly associated with their inhibitory effects on the proteasomal activity, since most of them were shown to specifically target the proteasome. Both the disruption of sulfhydryl homeostasis and proteasomal inhibition may then trigger protein misfolding and accumulation in the ER lumen. This could generate an osmotic force responsible for the ingress of water from the cytoplasm into the ER, thus causing its dilation. Parallelly, alteration of protein homeostasis could lead to failure of the UPR and ER-associated degradation (ERAD): this could also explain why ER stress is often activated during paraptosis. As mentioned above, ER stress is a pathological condition characterized by the accumulation of unfolded/misfolded proteins in the ER lumen and by the BiP-mediated activation of three stress sensors named double-stranded RNA-dependent protein kinase PKR-like ER kinase (PERK), IRE1α and ATF6 [109]; notably, the upregulation of these pro-death programs has been found in different tumor cell lines treated with the above natural compounds. Therefore, pre-treatment of paraptotic cells with the protein synthesis inhibitor cycloheximide could prevent their death by reducing proteotoxicity.

Mitochondria also undergo swelling during paraptosis, exhibiting cristae disintegration and loss. Moreover, during paraptotic cell death induced by ginsenosides, tocotrienols and several other natural products, mitochondrial fusion and subsequent megamitochondria formation have been observed. This could represent an adaptive response aimed at facilitating the removal of Ca^{2+} released from the ER and at delaying the onset of cell death. In fact, mitochondria can uptake the cytosolic Ca^{2+} via VDAC channel and the Ca^{2+} accumulating in the ER through mitochondria-associated ER membranes (MAMs), finally storing it in the mitochondrial matrix via MCU-mediated transport [110]. Thus, phytochemical-induced Ca^{2+} release from the ER may exceed the loading capacity of mitochondria, causing their swelling and dysfunction. This may also lead to the mitochondrial metabolism impairment and the following ROS generation frequently observed in case of pro-paraptotic Ca^{2+} overload. In this regard, it should be noted that, while oxidative stress generally positively correlates with paraptosis, suppression of ROS production by antioxidants further increased ginsenoside-related cytotoxicity, pointing out that free radicals my play a dual role in the modulation of paraptotic cell death, by promoting cytoplasmic vacuolation on one hand and by triggering NF-κB and other survival pathways on the other hand.

Cytoplasmic vacuolation can also be caused by BKCa inhibition, as evidenced after ophiobolin A treatment. In particular, BKCa inactivation could presumably induce K^+ accumulation in the ER and mitochondria, giving rise to the osmotic pressure responsible for the formation of vacuoles. Furthermore, impaired K^+ homeostasis leads to the activation of Ca^{2+} channels in the plasma membrane, thus promoting Ca^{2+} influx into the cell and subsequent induction of the paraptotic cascade.

Regarding the link between paraptosis and other cell death modes, ER dilation mediated by celastrol, 6-shogaol, 8-p-hdroxybenzoyl tovarol, manumycin A and DL-selenocystine was found to be paralleled by the induction of autophagy, as a strategy presumably adopted by the tumor cell to degrade the accumulating unfolded/misfolded proteins. However, cytoplasmic vacuolation was shown to be dependent on LC3 expression but not on the activation of the autophagic flux in 15d-PGJ2-mediated paraptotic cell death. In addition, after chalocomoracin treatment, PINK1-regulated paraptosis was observed, indicating that mitophagy may play a crucial role in the degradation of swollen mitochondria. Similarly, despite a direct correlation between phytochemical-induced paraptotic and immunogenic cell death has not been elucidated yet, an interesting study by Hoa *et al.* suggests that the paraptosis triggered by prolonged BKCa activation enhances tumor immunogenicity [40]. In particular, when macrophages encounter the mM-CSF-expressing glioma cells, ROS are produced, stimulating BKCa function and K^+/N^+ level alteration, culminating in paraptotic cell death; notably, during this particular type of paraptosis, specific “danger signals”, such as Hsp70, Hsp90 and GRP94/gp96, are released by dying cells, thus recruiting antigen-presenting cells and eliciting a strong anti-

tumor immune response. Since immunogenic cell death is also tightly regulated by ER stress, which is involved in paraptosis, the cross-talk between these two cell death programs should be further investigated.

4. Conclusion

Despite its involvement in the anti-cancer activity of several novel anti-tumor agents, the number of studies focusing on the role of paraptosis in cancer treatment is still scanty, probably due to a lack of specific markers and a limited understanding of its molecular mechanisms. However, emerging evidence has highlighted that apoptosis-resistant tumor cells could be susceptible to alternative programmed cell death modes; in this context, paraptosis may be specifically triggered in case of chemoresistance associated with apoptosis inhibition [111]. Moreover, paraptotic cell death is known to involve both ER and mitochondria, whose function is often altered in malignant cells but not in normal cells. Indeed, in overproliferating cancer cells an unfolded/misfolded protein overload in the ER lumen, due to an imbalance between protein folding capacity and metabolic demand, and an elevation of ROS, following increased metabolic activity and mitochondrial dysfunction, are usually observed; in this regard, specific targeting of these organelles via ER stress- and ROS-mediated paraptosis induction could represent an effective novel strategy to improve the therapeutic outcomes and reduce the toxicity of conventional treatments [112]. Thus, further studies focusing on new natural compounds and their synthetic derivatives, aimed at identifying more effective paraptosis inducers and at clarifying the molecular mechanisms underlying this form of cell death, are urgently needed.

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