Pharmacology & Therapeutics xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/pharmthera

Associate editor:Yongxiang Zhang

Paraptosis in the anti-cancer arsenal of natural products

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ARTICLE INFO

Keywords:
Paraptosis
Natural products
Cancer
Endoplasmic reticulum
Mitochondria
Vacuolation

ABSTRACT

Given the problems with malignant cancer cells showing innate and acquired resistance to apoptosis, we need alternative means to induce cell death in cancer. Paraptosis is a type of programmed cell death that is characterized by dilation of the endoplasmic reticulum (ER) and/or mitochondria. Although relatively little is known regarding the molecular basis of paraptosis, the underlying mechanism clearly differs from that of apoptosis. Recent studies have shown that various natural products, including curcumin, celastrol, 15d-PGJ_2, ophiobolin A, and paclitaxel, demonstrate anti-cancer effects by inducing the paraptosis-associated cell death, which was commonly characterized by vacuolation derived from the ER. Perturbation of cellular proteostasis due to proteasomal inhibition and disruption of sulfhydryl homeostasis, generation of reactive oxygen species, and/or imbalanced homeostasis of ions (e.g., Ca^{2+} and K^{+}) appear to contribute to the accumulation of misfolded protein and proteotoxicity in this process. Given the pathophysiological importance of paraptosis and the debate regarding the importance of apoptosis in solid tumor, we need to collect the available knowledge regarding paraptosis and suggest future directions in the field. Here, we review the morphological and biochemical features of paraptosis, the natural products that induce paraptosis-associated cell death, their proposed mechanisms, and the significance of paraptosis as a potential anti-cancer strategy. Such work and future clarifications should enable the development of new strategies for preventing cancer and/or combating malignant cancer.

Contents

Abbreviations: ↑, upregulation; ↓, downregulation; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2; 2-APB, 2-aminoethoxydiphenyl borate; BKCa, Ca^{2+}-activated K^{+} channel; CHX, cycloheximide; CuDIPS, Cu(II)2(3,5-diisopropylsalicylate); DMC, dimethoxycurcumin; ER, endoplasmic reticulum; GSH, glutathione; HMEC, human mammary epithelial cells; IGF-IR, insulin-like growth factor I receptor; IP_3R, inositol 1,4,5-trisphosphate receptor; JNK, Jun *N*-terminal kinase; MCU, mitochondrial Ca^{2+} uniporter; MG, megamitochondria; mito, mitochondria; MnTBAP, [5,10,15,20-Tetrakis(4-carboxyphenyl)-21*H*,23*H*-porphine]manganese(III); NAC, *N*-acetyl cysteine; NMPG, *N*-(2-mercaptopropionyl)-glycine; OP-A, ophiobolin A; PCD, programmed cell death; ROS, reactive oxygen species; RR, ruthenium red; RyR, ryanodine receptor; TAW, 8-*p*-hdroxybenzoyl tovarol; TM, tunicamycin.

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http://dx.doi.org/10.1016/j.pharmthera.2016.01.003
0163-7258/

1. Introduction

Cancer is the second leading cause of mortality worldwide, and it is expected to surpass heart diseases as the leading cause of death in the next few years (Siegel et al., 2015). Since resistance to apoptosis is closely linked to tumorigenesis and is assumed to be the cause of cellular resistance to anti-cancer therapies (Jäättelä, 1999), apoptosis has been a focus of cell death research. Although the available current data indicate that successful tumor cell clearance depends on classical apoptotic pathways, additional evidence suggests that upon failure of apoptosis, non-apoptotic cell death modalities can be triggered and may contribute to tumor cell death (Blank & Shiloh, 2007; Tait et al., 2014; Ubah & Wallace, 2014). In addition, the contribution of apoptosis to treatment success in solid tumors has been debated (de Bruin & Medema, 2008). Since the overall response of a tumor to cancer therapy is the cumulative effect of various types of cell death in heterogeneous populations of tumor cells (Kim et al., 2006), the identification and characterization of additional cell death programs may suggest strategies that could complement or offer alternatives to apoptosis-based therapeutic approaches (Mathiasen & Jäättelä, 2002).

Paraptosis is a programmed cell death mode that is accompanied by the dilation of the endoplasmic reticulum (ER) and/or mitochondria (Sperandio et al., 2000). Although we do not yet fully understand the molecular basis of paraptosis, it appears to differ from that of apoptosis, necrosis, and autophagy. Paraptosis occurs during development and neurodegeneration and is seen in cancer cells treated with various natural and synthetic anti-cancer agents (as we will describe in this article). We need to improve our understanding of the mechanisms of paraptosis-inducing natural products and their structure–activity relationships, as this could facilitate the design of novel therapeutic agents, including novel derivatives with improved abilities to induce paraptosis.

2. The historical basis of the study of paraptosis

Apoptosis is often equated with the programmed cell death (PCD) because of its widespread occurrence during development, its presence in various experimental models, and the availability of many specific markers. However, accumulating evidence indicates that many pathological conditions are characterized by modes of cell death that do not fulfill the criteria for apoptosis or necrosis. Sperandio et al. (2000) first introduced the term “paraptosis” to describe a form of PCD that is morphologically and biochemically distinct from apoptosis. The authors used human insulin-like growth factor 1 receptor (IGF-IR) to stimulate cell death in 293T cells and mouse embryonic fibroblasts and observed a unique form of cell death. The main feature of paraptosis is extensive cytoplasmic vacuolization that begins with progressive swelling of the ER and/or mitochondria. Paraptosis typically does not involve the activation of caspases, the formation of apoptotic bodies, or other characteristics of apoptotic morphologies; it is insensitive to apoptotic inhibitors (e.g., caspase inhibitors and Bcl-xL) and requires protein synthesis (Sperandio et al., 2000, 2004).

Interestingly, paraptosis-like forms of PCD have been described in neural development (Schweichel & Merker, 1973; Pilar & Landmesser, 1976; Clarke, 1990). In neurodegenerative diseases (e.g., amyotrophic lateral sclerosis and Huntington's disease) (Dal Canto & Gurney, 1994; Turmaine et al, 2000) and in other neurological conditions (e.g., ischemic damage) (Majno & Joris, 1995), at least some of the involved cell death may morphologically resemble paraptosis. Paraptosis may also play an important role in the pathophysiology of retina: it is the main mode of cell death in the corticosteroid-induced death of retinal pigment epithelial cells (Valamanesh et al., 2007) and the deaths of retinal ganglion cells in the early stages of glaucoma (Y. Wang et al., 2014b) and after retinal ischemia/reperfusion injury in rats (Wei et al., 2015).

The known paraptosis-inducing stimuli include the ligand-receptor pair of substance P and its receptor, neurokinin-1 receptor (Castro-Obregón et al., 2002), TAJ/TROY, a member of the tumor necrosis factor receptor superfamily (Wang et al., 2004), and epidermal growth factor (Fombonne et al., 2004, 2006). Moreover, human glioma cells transfected with an expression vector encoding the membrane form of macrophage colony-stimulating factor (mM-CSF) can be killed by human monocytes via paraptosis (Jadus et al., 2003; Hoa et al., 2007). The mechanisms underlying paraptosis are not well understood, but studies showed that IGF-IR-induced paraptosis required transcription and translation (Sperandio et al., 2000) and was mediated by the mitogen-activated protein kinase (MAPK) kinase, MEK-2, and Jun *N*-terminal kinase (JNK) (Sperandio et al., 2004). The same authors identified AIP-1/Alix (a protein that interacts with the cell death-related calcium-binding protein, ALG-2) as an inhibitor of paraptosis.

3. Natural products as anti-cancer agents

Natural products (i.e., chemical compounds or substances that are produced naturally by living organisms) have a long history in preventing and treating cancer (Nobili et al., 2009). Curcumin is one of the most highly studied natural products for the chemoprevention of cancer (Johnson & Mukhtar, 2007), and many successful anti-cancer drugs originated from natural sources, such as vinca alkaloids, taxanes, podophyllotoxin, and camptothecins (Safarzadeh et al., 2014). More than 3,000 plant species have reportedly been used in modern cancer therapeutics (Cragg & Newman, 2005), and ~ 30 plant-derived compounds, including curcumin and paclitaxel, have been tested to date in cancer clinical trials (Butler et al., 2014). In the 1990s, the imminent achievement of the genome project and the success of targeted therapies using “imatinib” and “trastuzumab” led to announce that natural products had become obsolete as therapeutics. However, most tumors harbor multiple signaling pathway redundancies and adaptive mechanisms rather than a single “targetable” oncogenic activation. Therefore, many cancer patients continued to die from resistant disease, and the treatment modalities (especially for solid tumors of advanced grades) remained palliative and insufficient. Over the past 15 years, therefore, pharmaceutical companies have reconsidered the potential of natural products in oncology, and they have utilized various approaches (e.g., molecular-modeling-based drug design) in the hopes of capitalizing on natural anti-cancer agents and their derivatives. With the approval of rapamycin, 12 novel natural product derivatives have been brought to market (Basmadjian et al., 2014). Many natural products can interfere with well-conserved cell signaling pathways at low concentrations. In addition, they may modulate multiple molecular targets that are frequently deregulated in cancers (e.g., transcription factors, growth factors, tumor cell survival factors, inflammatory cytokines, protein kinases, and angiogenesis factors) and thus could help overcome the resistance of cancer cells to single-target pharmaceutical drugs (Wondrak, 2009; Ahmad et al., 2012). Although apoptosis is the most well-known and dominant type of cell death induced by natural and traditional medicines (Safarzadeh et al., 2014), alternative modes of cell death (autophagic cell death, necrosis, and cell death through mitotic catastrophe) may also be induced by natural products (X. Wang et al., 2014a; Gali-Muhtasib et al., 2015).

4. Natural products that induce paraptosis-associated cell death

To date, various natural products have been reported to confer anti-cancer effects by inducing paraptosis-associated cell death. Fig. 1 presents their chemical structures and major natural sources, as reported through the end of October 2015. The different studies used terms such as “paraptosis”, “paraptosis-like cell death”, or “cytoplasmic vacuolation death”. Curcumin-induced cell death in breast cancer cells was designated as “paraptosis” (Yoon et al., 2010) because it met all the paraptotic features observed in IGF-IR-induced paraptosis (Sperandio

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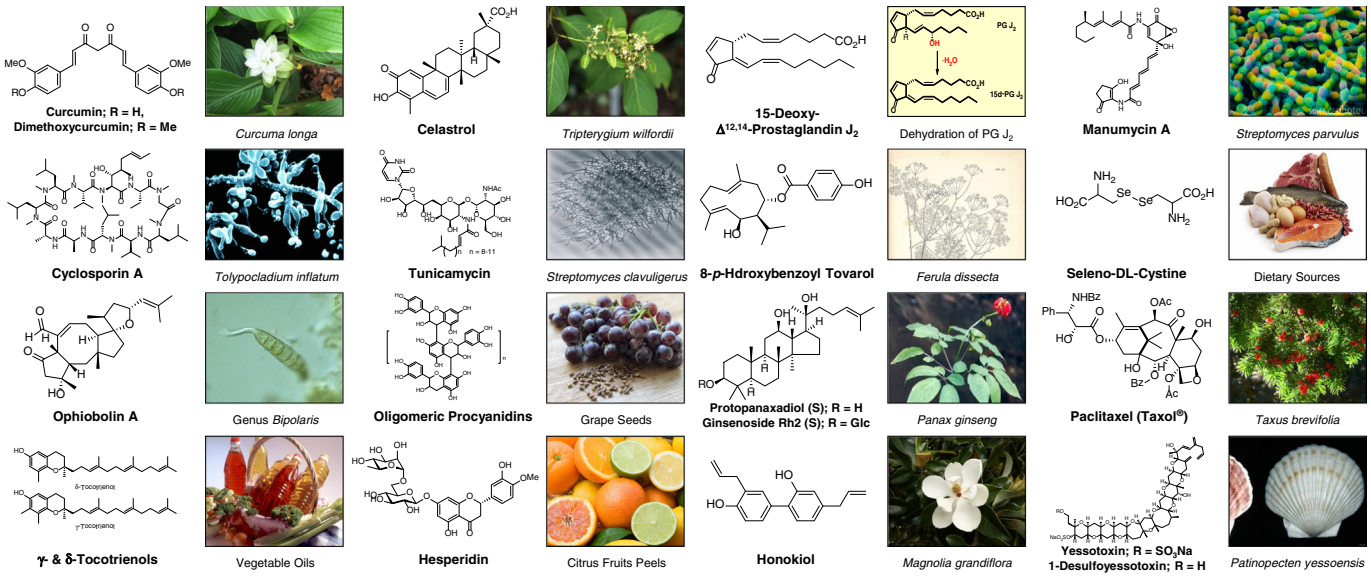

Fig. 1. Chemical structures of the paraptosis-inducing natural compounds and their major cellular sources.

et al., 2000, 2004), including dilation of the ER and mitochondria, a requirement for protein synthesis, activation of ERK1/2 and JNKs, and an inhibitory role of AIP-1/Alix in cell death. Other reports used the terms "cytoplasmic vacuolation cell death" [in the cases of that induced by15d-PGJ_2 (Kar et al., 2009), manumycin A (Singha et al., 2013), and curcumin in PC-3M cells (Lee et al., 2015)] or "paraptosis-like cell death", when cell death was accompanied by the dilation of only the ER or the biochemical characteristics were not well matched with those of IGF-IR-induced paraptosis. Notably, 15d-PGJ_2, manumycin A, and curcumin (in PC-3M cells) commonly triggered the dilation of the ER. In addition, 15d-PGJ_2-induced vacuolation and cell death were effectively blocked by cycloheximide and MEK inhibition (Kar et al., 2009). Thus, the previously reported instances of cytoplasmic vacuolation cell death may be broadly regarded as being paraptosis-associated cell death and they were included in the following discussion of the natural products that have been shown to induce paraptosis. Table 1 presents detailed information on the paraptosis-associated cell death induced by the listed natural compounds, including the concentration(s) used to induce cell death, the death mode assigned by the authors, the organelle(s) dilated during cell death, the biochemical features of death, the proposed death mechanism, and the tested cell type(s). To get a general sense of their potentials as paraptosis markers, we prioritized the involvements of ERK1/2, JNKs, and Alix in the listed cell death responses. Table 1 begins by listing the ER stress-inducing natural products, as nearly half of the listed natural products associated with paraptosis were found to induce ER stress. In this review, we will summarize and discuss the natural paraptosis-inducing products listed in Table 1.

4.1. Curcumin

Curcumin, a natural polyphenolic compound extracted from the rhizomes of *Curcuma longa,* has long been a popular dietary spice and herbal medicine in the Orient (Ammon & Wahl, 1991). This compound has attracted significant research attention due to its surprisingly wide range of beneficial properties, including anti-inflammatory (Fu et al., 2008), anti-oxidant (Sharma, 1976), chemopreventive (Park et al., 2013), chemosensitizing (Goel & Aggarwal, 2010), and radiosensitizing activities (Gupta et al., 2012b). Much of the research into the cancer-killing effects of curcumin has predominantly focused on the compound's ability to induce apoptosis (Karunagaran et al., 2005; 10–50 μM curcumin). However, curcumin has also been reported to induce non-apoptotic cell death, including mitotic catastrophe in apoptosis-resistant Bcr-Abl-expressing cells (Wolanin et al., 2006; 20 μM curcumin), autophagic cell death in malignant glioma cells (Aoki et al., 2007; 40 μM curcumin), and necrosis in prostate cancer cells (Kang et al., 2013; 100 μM curcumin). These findings suggest that curcumin may induce alternative cell death modes depending on the administered concentrations and the genetic background of the tested cells. Recently, Yoon et al. (2010) showed that 40 μM curcumin induces paraptosis in malignant breast cancer cells, but not in normal breast cells. Following the treatment of MDA-MB 435S breast cancer cells with curcumin, mitochondria swelled and fused with each other, leading to the formation of a few megamitochondria (MG) per cell; the ER was also swollen and fused, such that the cellular space became filled with ER-derived vacuoles (Fig. 2). The features of apoptosis and autophagy were not observed in curcumin-treated breast cancer cells; however, vacuolation and cell death were effectively blocked by the protein synthesis blocker, cycloheximide. In addition, activation of ERK2 or JNKs critically contributed to curcumin-induced vacuolation and cell death. Furthermore, the protein levels of AIP-1/Alix were downregulated by curcumin, and AIP-1/Alix overexpression alleviated curcumin-induced vacuolation and cell death. Yoon's group further identified a novel underlying mechanism of paraptosis by showing that the generation of reactive oxygen species (ROS), including mitochondrial superoxide, is critically involved in curcumin-induced paraptosis (Yoon et al., 2010). In addition, curcumin was shown to induce non-apoptotic and non-autophagic cytoplasmic vacuolation death in PC-3M cells (Lee et al., 2015). In this process, the curcumin-induced production of ROS triggered vacuolation-mediated cell death via increased ER stress. During curcumin-induced paraptosis, the progressive accumulation of poly-ubiquitinated proteins and inhibition of proteasomal activity was shown (Yoon et al., 2010, 2014a). Previously, curcumin was suggested to inhibit the ubiquitin isopeptidase activity that arises from the 19S regulatory subunit of the 26S proteasome, resulting in the accumulation of poly-ubiquitinated proteins (Mullally & Fitzpartrick, 2002). However, the treatment of breast cancer cells with high doses of proteasome inhibitors (1 μM MG132, 20 μM lactacystin, or 20 μM ALLN) was found to induce the formation of ER-derived vacuoles, but the mitochondria were fragmented (not dilated) in this case, and there was only slight reduction in cell viability (Yoon et al., 2010, 2012). Thus, it seems that proteasomal impairment is mainly responsible for paraptosis-related ER dilation, and that another signal is responsible for the mitochondrial dilation required for the effective

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Table 1
Involvement of MAP kinases, Alix, and ER stress and the proposed death signals in the paraptosis-associated cell death induced by natural products. Abbreviations : ↑, upregulation; ↓, downregulation; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2; 2-APB, 2-aminoethoxydiphenyl borate; CHX, cycloheximide; CuDIPS, Cu(II)2(3,5-diisopropylsalicylate)4; DMC, dimethoxycurcumin; ER, endoplasmic reticulum; GSH, glutathione; HMEC, human mammary epithelial cells; IP_3R, inositol 1,4,5-trisphosphate receptor; MCU, mitochondrial Ca^{2+} uniporter; mito., mitochondria; MnTBAP, [5,10,15,20-Tetrakis(4-carboxyphenyl)-21*H*,23*H*-porphine]manganese(III); NAC, *N*-acetyl cysteine; NMPG, *N*-(2-mercaptopropionyl)-glycine; ROS, reactive oxygen species; RR, ruthenium red; RyR, ryanodine receptor.

Name of natural product (used concentration)	Assigned cell death mode	Dilated organelles	MAP kinases and Alix	Upregulation of ER stress markers	Inhibitors of vacuolation and death	Proposed death mechanisms	Tested cancer cells and normal cells	References
Curcumin (40 μM)	Paraptosis	ER and mito.	p-ERK1/2↑, p-JNKs ↑ Alix ↓	p-eIF2α, GRP78/94, and CHOP Poly-ubiquitination	CHX SP600125 (JNK), U0126 (MEK) Alix overexpression Antioxidants (NAC, GSH, MnTBAP) RR, Ru360 (MCU) Dantrolene (RyR)	Proteasome inhibition ROS and mito. O_2^- generation Mitochondrial Ca^{2+} overload	MDA-MB 435S, MDA-MB 231, and Hs578T Safe in MCF-10A and HMEC	Yoon et al. (2010, 2012)
	Cytoplasmic vacuolation death	ER		CHOP Poly-ubiquitination	CHX NAC LC3 knockdown	ROS generation LC3 upregulation	PC-3M *In vivo* xenograft	Lee et al. (2015)
DMC (20 μM)	Paraptosis	ER and mito.	p-ERK1/2 ↑, p-JNKs ↑ Alix ↓	CHOP, ATF4, and KDEL Poly-ubiquitination	CHX U0126 (MEK) L-JNK inhibitor Antioxidants (NAC, MNTBAP, CuDIPS) CHOP knockdown	Proteasome inhibition ROS and mito. O_2^- generation	MDA-MB 435S, MDA-MB 231, Hs578T, and MCF-7, *in vivo* xenograft Safe in MCF-10A and HMEC	Yoon et al. (2014a)
Celastrol (1.2–2 μM)	Paraptosis	ER and mito.	p-ERK1/2 ↑, p-JNKs ↑, p-p38 ↑	CHOP, ATF4, and KDEL Poly-ubiquitination	CHX PD98059 (MEK), SP600125 (JNK) RR, MCU knockdown, 2-APB (IP_3R)	Proteasome inhibition Mitochondrial Ca^{2+} overload	MDA-MB 435S, MCF-7, DLD-1, and RKO	Yoon et al. (2014b)
	Paraptosis-like cell death, apoptosis, and autophagy	ER	p-ERK1/2 ↑, p-JNKs ↑, p-p38 ↑	Bip and PERK Poly-ubiquitination	CHX U0126 (MEK), SB203580 (p38)	Proteasome inhibition	HeLa	Wang et al. (2012)
15d-PGJ_2 (20 μM)	Cytoplasmic vacuolation death	ER	p-ERK1/2 ↑	Bip and CHOP Poly-ubiquitination	CHX U0126, MEK inhibitor Thiol antioxidants (NAC, NMPG) LC3 knockdown	Disruption of sulfhydryl homeostasis LC3 upregulation	HCT116, DU145, and MDA-MB 231	Kar et al. (2009)
Manumycin A (5 μM)	Cytoplasmic vacuolation death	ER	–	Bip and CHOP Poly-ubiquitination	CHX NAC	Disruption of sulfhydryl homeostasis	MDA-MB 231 Safe in HMEC	Singha et al. (2013)

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					LC3 knockdown	LC3 upregulation		
Cyclosporin A (20 μM)	Paraptosis-like	ER	Alix ↓	Bip, CHOP, p-eIF2α , IRE1α, PERK, and XBP1	CHX	Cyclophilin B ↓ (critical role)	SiHa	Ram and Ramakrishna (2014)
Tunicamycin (10 μg/ml)	Paraptosis	ER		Bip, CHOP, p-PERK and IRE1	CHX CHOP knockdown (no effect)		FRO	Kim et al. (2014)
8-*p*-Hdroxybenzoyl tovarol (18 μM)	Paraptosis-like cell death and autophagy	ER and mito.		Bip, CHOP, IRE1α, and XBP1s	CHX		HeLa	Zhang et al. (2015)
Seleno-DL-cystine (100 μM)	Paraptosis-like cell death and apoptosis	–	–	Bip and CHOP poly-ubiquitination		Misincorporation of selenocystine No ROS generation	HeLa	Wallenberg et al. (2014)
Ophiobolin A (1 μM)	Paraptosis-like	ER and mito.			CHX	BKCa inhibition	U373-MG, T98G, and GL19	Bury et al. (2013)
Oligomeric procyanidins (30 μg/ml)	Paraptosis-like	–	p-ERK1/2 ↑, p-p38 ↑		CHX	Extracellular Ca^{2+} influx	U-87	Zhang et al. (2010)
Ginsenoside Rh2 (35 μM)	Paraptosis-like cell death and apoptosis	–			CHX Removal of p53 NAC (death acceleration by inhibiting ROS and NF-kB		HCT116	Li et al. (2011)
Protopanaxadiol (35 μM)	Paraptosis-like	–			CHX NAC (death acceleration by inhibiting ROS and NF-kB)		HCT116	C. Z. Wang et al. (2013)
Paclitaxel (70 μM)	Paraptosis-like	ER (mainly) and mito. (in part)	MEK, p38, and JNK (not involved)		No effect of CHX		ASTC-a-1	Chen et al. (2008)
	Paraptosis-like	ER					A549 *In vivo* xenograft	Wang and Chen (2012)
γ- and δ-Tocotrienol (15–60 μM and 5–20 μM)	Paraptosis-like	ER and mito.				Wnt signals ↓ (β-catenin, cyclin D, c-Jun)	SW620	Zhang et al. (2011, 2013)
Hesperidin (1 mM)	Paraptosis-like	ER and mito.	p-ERK ↑		U0126 (ERK1/2, vacuolation inhibition)		HepG2	Yumnam et al. (2014)
Honokiol (20 μM for NB4; 40 μM for K562)	Paraptosis and apoptosis	ER			NAC (partially)	ROS generation	NB4 and K562	Y. Wang et al. (2013)
Yessotoxin (100 nM)	Paraptosis-like	ER and mito.	p-JNK ↑				BC3H1	Korsnes et al. (2011)
1-Desulfoyessotoxin (100 nM)	Paraptosis-like	ER and mito.	p-p38 ↑				BC3H1	Korsnes et al.(2013)

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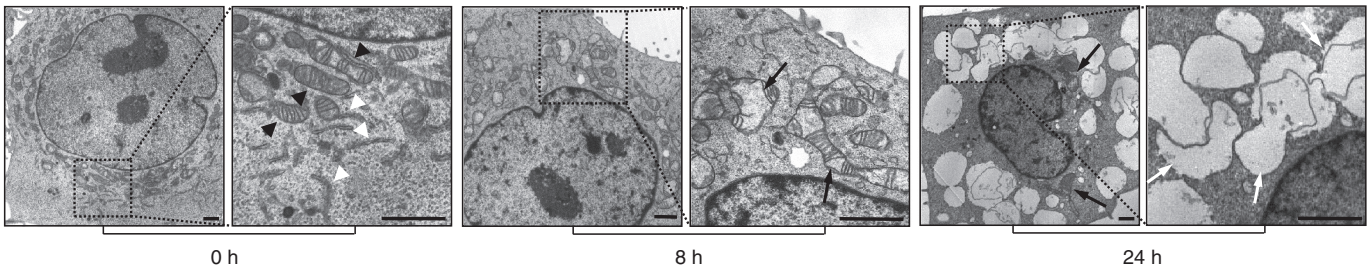

Fig. 2. Electron microscopy of curcumin-induced paraptosis. MDA-MB 435S cells were treated with 40 μM curcumin for the indicated time points and electron microscopy was performed. Black arrow heads, normal mitochondria; white arrow heads, normal ER; black arrows, megamitochondria; white arrows, swollen and fused ER. Bars, 2 μM (reprinted From *Free Radic Biol Med* 48(5); 713–726, Yoon et al. (2010) Copyright with permission from Elsevier).

induction of paraptosis. Yoon et al. (2012) found that mitochondrial Ca^{2+} overload plays a critical role in mitochondrial dilation during curcumin-induced paraptosis. More specifically, curcumin-induced stimulation of ryanodine receptors (RyRs) triggers the release of Ca^{2+} from the ER; this Ca^{2+} is taken up via mitochondrial Ca^{2+} uniporter (MCU), causing an overload that contributes to mitochondrial dilation and subsequent cell death. The importance of mitochondrial Ca^{2+} overload in paraptosis is supported by the observation that paraptosis was induced by the simultaneous inhibition of proteasomes and the mitochondrial Na^{+}/Ca^{2+} exchanger (mNCX). Although proteasome inhibition was not sufficient to induce paraptosis, co-treatment of cells with 15 nM bortezomib (a proteasome inhibitor) and 50 μM CGP-37157 (a mitochondrial NCX inhibitor), but not treatment with a single agent, effectively induced the dilations of both the ER and mitochondria by triggering sustained mitochondrial Ca^{2+} overload. Ultimately, effective induction of paraptosis appears to require both proteasomal inhibition and mitochondrial Ca^{2+} overload, and mitochondrial dilation and ER dilation seem to be interdependent during paraptosis. Although bortezomib has been successfully used to treat multiple myeloma, its anti-cancer effects are not satisfactory in solid tumors (Chen et al., 2011). Therefore, induction of paraptosis may provide a novel therapeutic strategy for overcoming the resistance of solid tumors against proteasome-inhibitor-based cancer therapy in the future.

4.2. Dimethoxycurcumin

Dimethoxycurcumin (DMC; a methylated analog of curcumin in which the phenolic-OH groups are replaced by methoxy groups) was identified to demonstrate a more potent anti-cancer effect than curcumin, with improved bioavailability and stability (Tamvakopoulos et al., 2007; Yoon et al., 2014a). Yoon et al. (2014a) reported that 20 μM DMC-induced paraptosis in breast cancer cells (similar to curcumin, but at a much lower concentration) and reduced tumor growth in a xenograft model *in vivo*. The authors proposed that DMC induces paraptosis based on the following morphological and biochemical characteristics: (a) DMC induced the swelling and fusion of the ER and mitochondria; (b) DMC-induced vacuolation and cell death was completely inhibited by cycloheximide; (c) ERK1/2 and JNKs were activated by DMC, whereas the inhibition of ERK1/2 or JNKs attenuated DMC-induced cell death; (d) AIP-1/Alix protein levels were downregulated by DMC; and (e) DMC increased mitochondrial superoxide levels, whereas antioxidants significantly blocked DMC-induced cell death. Yoon et al. (2014a) showed that the more potent anti-cancer activity of DMC (compared to curcumin) was associated with stronger inhibition of proteasome activity, providing additional evidence that proteasomal inhibition critically contributes to the paraptosis-mediated anti-cancer effect of curcumin and its derivatives. Mechanistically, curcumin and DMC are both Michael acceptors having an α,β-unsaturated β-diketone group that can react with sulfhydryl groups (Dinkova-Kostova et al., 2001) (Figs. 1 and 3A). This can induce oxidative stress by altering the cellular redox balance and potentially elevating the levels of ROS (Raja et al., 2011). Consistent with this notion, DMC was found to increase mitochondrial superoxide levels to a greater extent than curcumin during the progression of paraptosis (Yoon et al., 2014a). Furthermore, Yoon et al. (2014a) showed that CHOP plays a critical role in DMC-induced paraptosis, particularly in the context of ER dilation, indicating the possible involvement of CHOP in paraptosis-related ER dilation.

4.3. Celastrol

Celastrol is a quinone methide triterpenoid isolated from the Chinese medicinal plant, Thunder God Vine (*Tripterygium wilfordii*) (Setty & Sigal, 2005). Multiple reports have suggested that it could have anti-cancer effects (Yadav et al., 2010). Yoon et al. (2014b) recently showed that 2 μM celastrol induces paraptosis accompanied by the expansion of ER-derived vacuoles and the formation of MG in breast and colon cancer cells. Neither apoptosis nor autophagy was critically involved in this celastrol-induced cell death. Interestingly, celastrol-induced paraptosis has been shown to share the key signals involved in curcumin-induced paraptosis (Yoon et al., 2010, 2012, 2014b). For example, the celastrol-induced accumulation of poly-ubiquitinated proteins and ER stress marker proteins (e.g., ATF4, CHOP, and KDEL), vacuolation, and subsequent cell death were completely blocked by cycloheximide pretreatment. ERK1/2 and JNKs were activated by celastrol, and the inhibition of ERK1/2 and JNKs significantly attenuated celastrol-induced cell death. Celastrol treatment induced mitochondrial Ca^{2+} overload, and the resulting mitochondrial dilation was effectively attenuated by the inhibition of MCU. The celastrol-induced increases in Ca^{2+} levels arose from the ER, and the celastrol-triggered upregulation of IP_3R was found to mediate the release of Ca^{2+} from the ER. The latter finding indicates that the IP_3R-mediated release of Ca^{2+} from the ER and its subsequent MCU-mediated influx of Ca^{2+} into mitochondria critically contribute to the extensive dilation of the ER and mitochondria and subsequent celastrol-induced paraptotic cell death. Wang et al. (2012) showed that 1.2 μM celastrol-induced extensive cellular vacuolization derived from dilated cisternae of ER in HeLa cells, which thus showed a paraptosis-like morphology. Paralleling the induction of paraptosis-like cell death, both apoptosis and autophagy were induced in celastrol-treated HeLa cells. Comparing the results that paraptosis was the predominant cell death mode induced by celastrol in breast cancer cells (Yoon et al., 2014a), these results indicate that celastrol may induce different cellular fates depending on the cell types and/or cellular context. Structurally, the electrophilic character of the quinone methide substructure of celastrol allows it to react with the thiol groups of protein cysteine residues to form covalent protein adducts (Trott et al., 2008) (Fig. 3B) and promote proteotoxic stress (Boridy et al., 2014). In addition, celastrol was proposed to potently inhibit the proteasome by interacting through its conjugated ketone carbons, C2 and C6 (Yang et al., 2006). Therefore, the accumulation of misfolded proteins and failure of proteostasis may contribute to celastrol-induced paraptosis.

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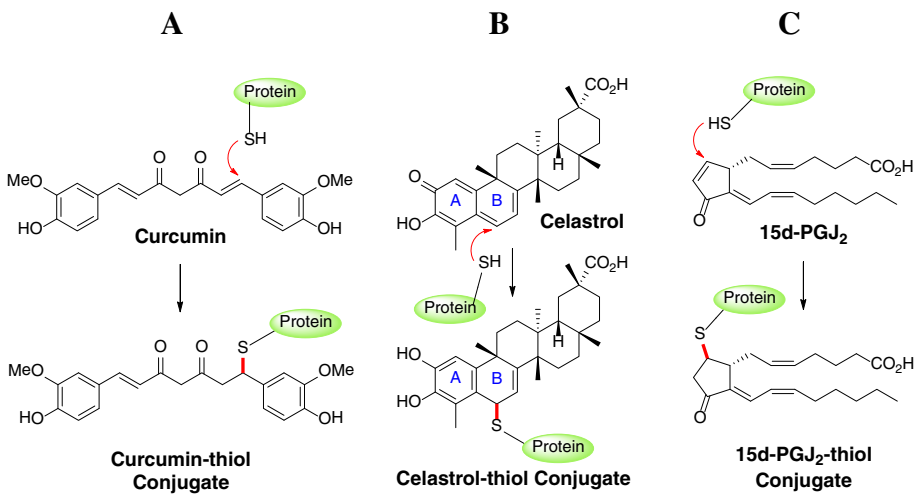

Fig. 3. Proposed conjugated addition of nucleophilic thiol group (-SH) of cysteine in protein to curcumin (A), celastrol (B), and 15d-PGJ_2 (C).

4.4. 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2

The cyclopentenone prostaglandin derivative, 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), has been found to have potent anti-proliferative activities (Butler et al., 2000). Kar et al. (2009) have shown that 15d-PGJ_2 induces non-apoptotic and non-autophagic cell death mode in breast and colon cancer cells, characterized by extensive cytoplasmic vacuolation derived from the ER dilation. These effects were completely blocked by actinomycin D and cycloheximide, which is consistent with paraptosis (Sperandio et al., 2000). 15d-PGJ_2 increased the protein levels of the I and II forms of the autophagy-related protein, LC3, and knockdown of LC3 significantly protected cells against 15d-PGJ_2-induced vacuolation and death, suggesting that LC3 plays a role in this non-autophagic, paraptosis-like cell death. The PPARγ-independent effects of 15d-PGJ_2 were shown to be mediated by either ROS production (Cernuda-Morollon et al., 2001) or covalent protein modification governed by the α,β-unsaturated ketone in the cyclopentenone ring of 15d-PGJ_2 (Perez-Sala et al., 2003) (Fig. 3C). The cytoplasmic vacuolation and cell death induced by 15d-PGJ_2 was effectively blocked by thiol antioxidants, but not by other ROS scavengers (Kar et al., 2009). This suggests that the effects of 15d-PGJ_2 may be mediated through its ability to covalently modify the free sulfhydryl groups of proteins and not through ROS production. The disruption of sulfhydryl homeostasis by 15d-PGJ_2 may lead to the accumulation of misfolded and subsequently ubiquitinated proteins. This could then trigger cytoplasmic vacuolation due to ER dilation and subsequent cell death.

4.5. Manumycin A

Manumycin A, a naturally occurring antibiotic, reduces the viability of triple-negative breast cancer cells by inducing non-apoptotic, non-autophagic cytoplasmic vacuolation cell death, while sparing normal human mammary epithelial cells (Singha et al., 2013). This cytoplasmic vacuolation and cell death was associated with the expressions of LC3, p62, and the ER stress markers, Bip and CHOP, as well as the accumulation of ubiquitinated proteins. Manumycin A contains sulfhydryl (-SH)-reactive, α,β-unsaturated ketones in its structure and NAC, a thiol antioxidant, blocked these effects of manumycin A, suggesting that this natural product may target sulfhydryl homeostasis.

4.6. Cyclosporin A

Cyclosporin A, which is a cyclic undecapeptide initially isolated from the fungus, *Tolypocladium inflatum* (Liu et al., 1991), has been widely used as an immunosuppressant. Ram and Ramakrishna (2014) showed that this agent induces non-apoptotic cell death accompanied by ER-derived vacuolation in SiHa cervical cancer cells. Cyclosporin A-induced vacuolation was prevented by cycloheximide and salubrinal, indicating that active protein synthesis is required for the formation of these vacuoles. Moreover, cyclosporin A downregulated AIP-1/Alix-1, suggesting that this treatment triggers paraptosis-like responses. The inhibition of calcineurin activity did not result in either ER stress or cellular vacuolation. However, the downregulation of cyclophilin B was found to precede cyclosporin A-induced vacuolation, and cyclophilin B knockdown was shown to induce ER stress and vacuolation. This suggests that cyclosporin A may induce paraptosis-like cell death via ER stress and vacuolation, and cyclophilin B inhibition plays a key role in this process.

4.7. Tunicamycin

Tunicamycin (TM) is a naturally occurring antibiotic that blocks the cellular biosynthesis of *N*-linked oligosaccharide (Ron, 2002). TM was shown to induce cell death by activating intrinsic apoptosis via ER stress, and to enhance autophagy-mediated cell death (Szegezdi et al., 2006; Yorimitsu et al., 2006). In addition, TM was reported to induce paraptosis (characterized by ER swelling, retention of plasma membrane integrity, a lack of apoptotic bodies and independence from caspase activity) in FRO anaplastic thyroid carcinoma cells (Kim et al., 2014). TM increased the expression of ER stress markers, but knockdown of CHOP (an effector of ER stress) did not alter cellular vacuolation or cell death, suggesting that ER stress is not a major event in the TM-induced paraptosis of FRO cells.

4.8. 8-p-Hdroxybenzoyl tovarol

8-*p*-Hdroxybenzoyl tovarol (TAW) is a germacrane-type sesquiterpenoid that can be isolated from the roots of *Ferula dissecta* (Ledeb.). TAW was shown to induce paraptosis-like cell death accompanied by extensive swelling of the ER and mitochondria in HeLa cells (Zhang et al., 2015). In these cells, TAW was found to induce ER stress, and TAW-induced vacuolation and cell death was effectively inhibited by cycloheximide treatment. Alongside this paraptosis-like cell death, TAW was also found to induce cytoprotective autophagy in HeLa cells.

4.9. Seleno-DL-cystine

Seleno-DL-cystine ((±)-3,3′-dithiobis(2-aminopropionic acid)) is a naturally occurring selenoamino acid, which plays an essential role in

the selen biochemical cycle (da Silva et al., 1997). Treatment of HeLa cells with seleno-DL-cystine at an IC_{50} concentration of 100 μM induced morphologically two distinct different types of cell death, one with apoptosis-like phenotype and the other is paraptosis-like cell death, inducing the dilation of the ER (Wallenberg et al., 2014). Treatment with seleno-DL-cystine did not induce ROS formation, but the amount of protein-bound selenium significantly increased. They proposed that random misincorporation of selenocystine into the proteins, in place of cysteine, may induce the accumulation of misfolded proteins, contributing to ER stress and cytoplasmic vacuolation.

4.10. Ophiobolin A

The sesterterpenoid phytotoxin, ophiobolin A (OP-A) belongs to a large family of over 25 natural ophiobolins that have been mainly isolated from phytopathogenic fungi of genus *Bipolaris* (Sugawara et al., 1987). Recently, Bury et al. (2013) showed that OP-A inhibited the proliferation of glioblastoma multiforme (GBM) cells and induced paraptosis-like cell death, potentially via the swelling and fusion of mitochondria and/or the ER. Cycloheximide pretreatment effectively decreased this OP-A-induced cell death, suggesting that protein synthesis is required for this process. The authors showed that the expression of big conductance Ca^{2+}-activated K^+ channel (BKCa) colocalized with both mitochondria and the ER, and that OP-A treatment inhibited BKCa channel activity. The authors hypothesized that OP-A-induced blockade of big potassium (BK) channels in the ER and mitochondria could increase intracellular K^+ concentration, causing water to enter the cell and triggering swelling/vacuolization. Recently, Dasari et al. (2015) reported that eliminating the C5, C21-1,4-dicarbonyl function of OP-A significantly reduced its anti-proliferative effects in various cancer cells. The unique reaction of OP-A with primary amines suggests that covalent modification of target proteins (i.e., through the use of active-site lysine residues to form pyrrole) may contribute to the anti-cancer activity of this agent.

4.11. Oligomeric procyanidins

F2, an oligomeric procyanidin fraction isolated from grape seeds, induced paraptosis-like non-apoptotic cell death in U-87 gliobalstoma cells (Zhang et al., 2009). Subsequently, the same authors reported that F2 triggered the activation of ERK1/2 and p38 and promoted intracellular Ca^{2+} mobilization via the G_i/G_o protein-mediated influx of extracellular Ca^{2+} (Zhang et al., 2010).

4.12. Ginsenoside Rh2 and protopanaxadiol

Rh2, a main bioactive component in steamed American ginseng extracts (Wang et al., 2009), was shown to induce both paraptosis-like cell death (characterized by the accumulation of cytoplasmic vacuoles) and caspase-dependent apoptosis in HCT116 colon cancer cells (Li et al., 2011). Although the authors did not address the origin of Rh2-induced vacuoles, they showed that vacuolization was significantly inhibited by cycloheximide and U0126 (the MEK1/2 specific inhibitor), consistent with the results shown in IGF-IR-induced paraptosis (Sperandio et al., 2004). Interestingly, removal of p53 significantly blocked both vacuole formation and cell death in Rh2-treated cells, suggesting that paraptosis-like cell death and apoptosis are mediated by p53 activity. In addition, Rh2 treatment increased ROS levels and activated the NF-κB survival pathway. Blockage of ROS by NAC or catalase inhibited the activation of NF-κB signaling and enhanced cell death in Rh2-treated cells, suggesting that the anti-cancer effects of Rh2 might be enhanced by antioxidants.

Rh2 can be converted to the triterpenoid protopanaxadiol (Bae et al., 2002), which was reported to induce paraptosis-like cell death in colon cancer cells. Moreover, protopanaxadiol-induced cytoplasmic vacuolation was effectively inhibited by cycloheximide treatment (C. Z. Wang et al., 2013a). These results suggest that, similar to Rh2, protopanaxadiol may possess the potential to act as an anti-cancer agent via paraptosis-like cell death.

4.13. Paclitaxel (Taxol®)

Paclitaxel (Taxol®), which was isolated from bark of the Pacific yew, *Taxus brevifolia*, is a potent anti-cancer drug (Ofir et al., 2002; Park et al., 2004) that shows great promise for the treatment of previously unresponsive breast, ovary, and non-small cell lung carcinomas (Crown & O'Leary, 2000; Selimovic et al., 2008). Paclitaxel can induce different types of cell death, although the exact mechanism underlying its cytotoxicity against tumor cells is still under extensive investigation. Previous reports showed that paclitaxel induces apoptosis at lower concentrations (5–50 nM) and necrosis at higher concentrations (0.1–50 μM) (Yeung et al., 1999; Pushkarev et al., 2008). At doses of 10 or 20 μM, paclitaxel was reported to induce mitotic catastrophe in HeLa cells (Michalakis et al., 2005), whereas 70 μM paclitaxel was shown to induce a paraptosis-like cell death that included cytoplasmic vacuolization derived from swelling of the ER and (to a small extent) mitochondria in ASTC-a-1 lung cancer cells (Chen et al., 2008). The authors speculated that the paclitaxel-induced disappearance of the ER dissepiment may reflect ER fusion. Moreover, the paclitaxel-treated cells underwent caspase-independent cell death without apoptotic bodies or membrane disruption, and thus fit the criteria of paraptosis. However, this paclitaxel-induced paraptosis-like cell death differed from IGF-IR-induced paraptosis in that it did not require protein synthesis and was independent of MEK and JNK (Sun et al, 2010). These results suggest that a high concentration of paclitaxel activates an alternative paraptotic cell death pathway. In addition to these *in vitro* results, paclitaxel was also found to suppress tumor growth by inducing paraptosis-like cell death via rough-ER-derived vacuolization in A549 tumor-xenograft mice *in vivo* (Wang & Chen, 2012), suggesting that induction of paraptosis-like cell death by paclitaxel could be a promising therapeutic strategy for the treatment of apoptosis-resistant cancer.

4.14. γ-Tocotrienol and δ-tocotrienol

The tocotrienols, which are members of the vitamin E family, are natural compounds that are abundant in rye, barley, oats, and palm oil (Sookwong et al., 2010). This group comprises α, β, γ, and δ homologs that differ in their numbers of methyl groups and their therapeutic functions, which include anti-cancer, anti-inflammatory, and anti-oxidant activities (Aggarwal et al., 2010). Zhang et al. (2011, 2013) showed that both δ- and γ-tocotrienol effectively induced paraptosis-like cell death in SW620 cells (a human colon cancer cell line), exhibiting IC_{50} values of 15.2 and 31.4 μM, respectively. In SW620 cells treated with δ- or γ-tocotrienol, vacuolation preceded cell death and might result from the swelling and fusion of mitochondria and/or the endoplasmic reticulum (ER). Both δ- and γ-tocotrienol inhibited the expression levels of β-catenin, cyclin D1, and c-jun in SW620 cells, suggesting that the paraptosis-like cell death induced by δ- and γ-tocotrienol may be associated with suppression of the Wnt signaling pathway.

4.15. Hesperidin

Hesperidin, a flavanone glycoside of *Citrus* fruits, was shown to induce paraptosis-like cell death accompanied by swelling of mitochondria and the ER without apoptotic features in HepG2 cells at the concentration of 1 mM, whereas the use of higher concentrations increased the apoptotic cell populations (Yumnam et al., 2014). ERK1/2 was activated during hesperidin-induced vacuolation and blockade of the ERK pathway inhibited hesperidin-induced vacuolization.

Please cite this article as: Lee, D., et al., Paraptosis in the anti-cancer arsenal of natural products, *Pharmacology & Therapeutics* (2016), http://dx.doi.org/10.1016/j.pharmthera.2016.01.003

4.16. Honokiol

Honokiol, an active compound isolated from the Chinese traditional herb *Magnolia officinalis*, has shown promise for multiple medicinal applications (including anti-inflammatory, anti-anxiety, and anti-tumor effects) in preclinical studies, without detectable toxicity at therapeutic doses (Tian et al., 2012). Although honokiol is most commonly associated with apoptosis in tumor cells, it can also induce necrosis (Li et al., 2007) and paraptosis (Wang et al., 2010). Wang et al. (2010) showed that the treatment of leukemia cell lines with lower concentrations of honokiol induced paraptosis with ER-derived vacuolization, whereas higher concentrations induced both paraptosis and apoptosis. Y. Wang et al. (2013) subsequently showed that honokiol predominantly induced paraptosis and partially induced apoptosis in NB4 cells, whereas the reverse was seen in K562 cells. Taken together, these results suggest that paraptosis and apoptosis may be complementary cell death program in leukemia cells, and that the major cell death mode induced by honokiol may depend on both the concentration and the cell type. Honokiol can therefore be considered a potential novel drug that simultaneously activates multiple death pathways, and thus could potentially help overcome multidrug resistance.

4.17. Yessotoxin and 1-desulfoyessotoxin

Yessotoxin, a polycyclic ether compound produced by dinoflagellates, can induce multiple cell death pathways at nanomolar concentrations in different model systems (Korsnes, 2012). In the BC3H1 myoblast cell line, yessotoxin treatment induced paraptosis-like cell death, which was found to be accompanied by extensive cytoplasmic vacuolation derived from dilation of the ER and mitochondria (Korsnes et al., 2011). The yessotoxin-induced death of these cells was insensitive to several caspase inhibitors. Similarly, a desulfated analog of yessotoxin, 1-desulfoyessotoxin, also induced paraptosis-like cell death (Korsnes et al., 2011, 2013). At the molecular level, JNK activation was reported during yessotoxin-induced paraptosis-like cell death (Korsnes et al., 2011), whereas p38 activation was detected in 1-desulfoyessotoxin-induced cell death (Korsnes et al., 2013).

5. Non-natural products that induce paraptosis-associated cell death

In addition to the above-described natural products, a number of chemicals and synthetic derivatives of natural products have been reported to induce paraptosis or paraptosis-like cell death in various cancer cells. The chemicals include 1-nitropyrene (Asare et al., 2008), thioxotriazole copper (II) complex A0 (Tardito et al., 2009), VER155008 (an hsp70 inhibitor) (Kim et al., 2014), and MCB-613 (an agonist of steroid receptor coactivator) (Wang et al., 2015), while the synthetic derivatives of natural products include WIN55,212-2 (a synthetic cannabinoid) (Wasik et al., 2011), everolimus (an mTOR derivative) (Baraz et al., 2014), and benfotiamine (a thiamine analog) (Sugimori et al., 2015).

6. Signals believed to be involved in natural-product-induced paraptosis-associated cell death

Table 1 shows that cycloheximide typically blocks the ER-derived vacuolation and cell death induced by most of the above-listed natural products. This is consistent with the belief that protein synthesis is required for paraptosis, although exceptional case of paclitaxel-induced paraptosis-like cell death (that was not inhibited by chcloheximide) was also reported (Chen et al., 2008). AIP-1/Alix was found to be downregulated by curcumin, dimethoxycurcumin, and cyclosporin A (Yoon et al., 2010, 2014a; Ram & Ramakrishna, 2014). In terms of the MAP kinases, the activations of ERK1/2 and JNKs critically contribute to curcumin- (Yoon et al., 2010), dimethoxycurcumin- (Yoon et al., 2014a), or celastrol- (Yoon et al., 2014b) induced paraptosis . In addition, ERK1/2 was found to be positively involved in 15d-PGJ_2-induced cytoplasmic vacuolation cell death (Kar et al., 2009) and hesperidin-induced paraptosis-like cell death (Yumnam et al., 2014). In contrast, MEK, JNK, and p38 are not involved in paclitaxel-induced paraptosis-like cell death (Chen et al., 2008), suggesting that the importance of a given MAP kinase differs depending on the stimulus.

As shown in Table 1, during paraptosis-associated cell death induced by various natural products, ER stress was commonly observed, as reflected by the upregulation of ER stress marker proteins, including Bip and CHOP (e.g., in cells treated with curcumin, dimethoxycurcumin, celastrol, 15d-PGJ_2, manumycin A, seleno-DL-cystine, cyclosporin A, tunicamycin, and 8-*p*-hdroxybenzoyl tovarol) and accumulation of poly-ubiquitinated proteins (e.g., in cells treated with curcumin, dimethoxycurcumin, celastrol, 15d-PGJ_2, manumycin A, and seleno-DL-cystine). These results suggest that paraptosis may be closely linked to ER stress due to the accumulation of misfolded proteins. Therefore, cycloheximide (as an inhibitor of protein synthesis) might thus potently inhibit paraptosis-associated cell death by reducing the burden on the homeostastic protein-folding mechanisms.

ER dilation is the most common morphological feature triggered during the paraptosis-associated cell death induced by natural products listed in Table 1. Yoon et al. (2010, 2014a, 2014b) showed that impairment of proteasome activity is the key underlying mechanism of ER-derived vacuolation during paraptosis (Fig. 4). Mimnaugh et al. (2006) proposed that overload of misfolded proteins within the ER lumen could exert an osmotic force, drawing water from the cytoplasm to distend the ER lumen. Since cancer cells highly depend on optimal proteasomal function, the inhibition of proteasomal machinery can lead to further accumulation of misfolded proteins in the ER and cytoplasm (Ustundag et al., 2007). This can overwhelm cells, leading to failure of the UPR and ER-associated degradation (ERAD), both of which protect cells from proteotoxicity. Ultimately, severe alterations in ER structures lead to irreversible functional impairments and shift the cellular balance toward death.

As the critical underlying mechanism of ER-derived vacuolization in 15d-PGJ_2-induced cytoplasmic vacuolation cell death, the disruption of sulfhydryl homeostasis was proposed (Kar et al., 2009). Interestingly, literature suggests that the pharmacological effects of 15d-PGJ_2, curcumin, and celastrol involve covalent binding (-S-C-) to cellular target proteins, although they do not resemble in structural terms

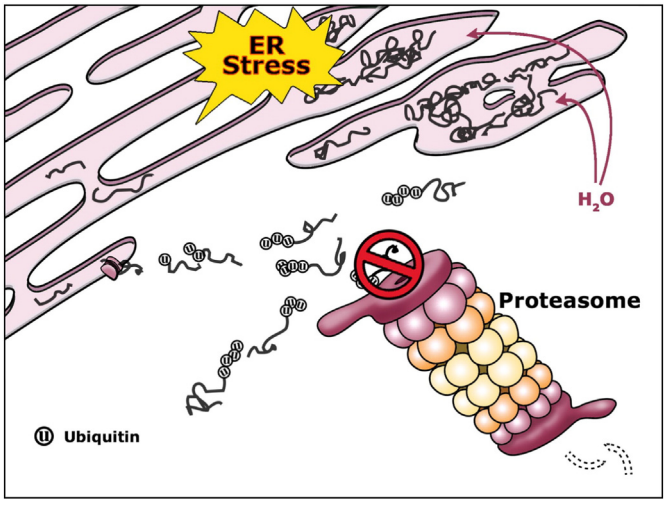

Fig. 4. Hypothetical model for ER-derived vacuolation by proteasome inhibition in paraptosis. Proteasomal inhibition by curcumin, dimethoxycurcumin, or celastrol induces the accumulation of misfolded proteins within the ER, triggering an osmotic imbalance that causes water to enter from the cytoplasm, thereby distending the ER luminal space into vacuoles.

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(Perez-Sala et al., 2003; Trott et al., 2008; Gupta et al., 2011). This presumably reflects the highly electrophilic nature of the substructures contained in these natural products, such as the α,β-unsaturated carbonyls of 15d-PGJ_2 (Perez-Sala et al., 2003), curcumin (Gupta et al., 2011), and the quinone methide within the A and B rings of celastrol (Trott et al., 2008). These substructures readily react with substances containing nucleophilic groups [e.g., the sulfhydryl (-SH) groups of proteins and glutathione (GSH), or the hydroxyl (-OH) groups of proteins] to form covalent bonds with cellular proteins via the conjugated addition reaction (Fig. 3). The disruption of sulfhydryl homeostasis and formation of covalent adducts by these natural products may trigger misfolding of target proteins and their accumulation, leading to ER-derived vacuolation. Similar to curcumin (Milacic et al., 2008) and celastrol (Yang et al., 2006), 15d-PGJ_2 was also shown to inhibit proteasome activity (Wang et al., 2006). Therefore, thiol-reactivity of these agents may be directly associated with their inhibitory activity of proteasome.

Mitochondria are also dilated during paraptosis. This highly dynamic organelle continuously fuses, divides, and moves within a cell. These dynamic morphological changes are essential for maintaining the mitochondrial DNA, ensuring respiratory activity, controlling cellular processes (e.g., calcium homeostasis and signaling), and regulating cell death (Vannuvel et al., 2013). During paraptosis, mitochondria exhibit disorganization of the cristae, take on a balloon-like shape, and then undergo vacuolation (Fig. 2). In addition, during curcumin-, celastrol-, and γ-tocotrienol-induced paraptosis-associated cell death, the formation of megamitochondria (MG) via the fusion of swollen mitochondria was reported (Yoon et al., 2010, 2014b; Zhang et al., 2013). Although the detailed mechanisms and pathophysiological meanings of MG formation are unknown, it has been postulated to be an adaptive process undertaken in response to unfavorable environmental conditions (Wakabayashi, 2002). Mitochondrial swelling, mitochondrial fusion, and an abundance of MG were also observed in axon terminals at the neuromuscular junctions of aged rats (García et al., 2013). As the fusion-mediated formation of a mitochondrial network may facilitate the buffering of Ca^{2+} released from the ER or extracellular stores (Frieden et al., 2004), mitochondrial fusion and MG formation during paraptosis may protect cells from cell death at an early phase. However, the Ca^{2+} concentration later comes to exceed the loading capacity of the MG, potentially triggering an irreversible sequence that leads to the loss of mitochondrial function and eventual cell death.

To explain the simultaneous dilation of the ER and mitochondria during paraptosis, Yoon et al. (2012) proposed that there may be positive reciprocal regulation between ROS generation and mitochondrial Ca^{2+} overload (Fig. 5). ROS are known to open ER Ca^{2+} channels (e.g., ryanodine receptors and IP_3R) to release Ca^{2+} from the ER (Cao & Kaufman, 2014). Mitochondria and the ER are interconnected physically and functionally by mitochondria-associated ER membrane (MAMs), and the Ca^{2+} released from the ER is taken up by mitochondria via MCU (Bravo et al., 2012). An increase of Ca^{2+} in the mitochondria

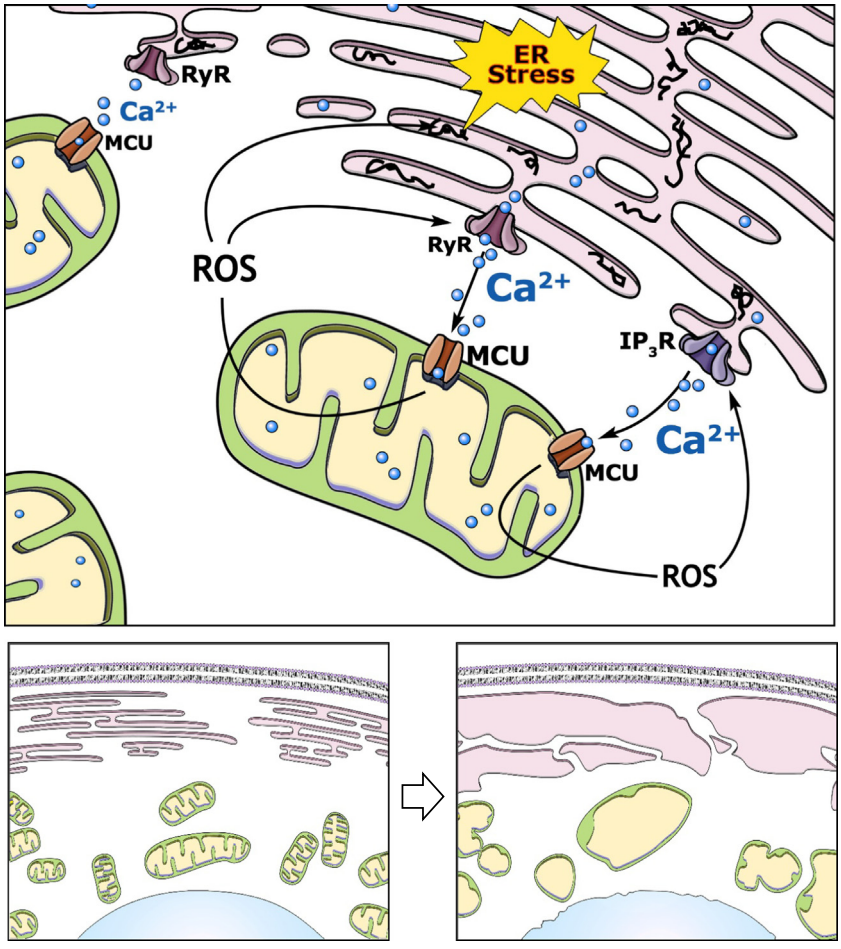

Fig. 5. Hypothetical model for the induction of paraptosis by vicious cycle between mitochondrial Ca^{2+} overload and ROS generation. Curcumin induces the RyR-mediated release of Ca^{2+} from the ER, whereas celastrol induces the IP_3R-mediated release of Ca^{2+}. This leads to an MCU-mediated Ca^{2+} influx and subsequent mitochondrial swelling. The resulting increase in mitochondrial ROS activates RyR or IP_3R to trigger further release of Ca^{2+} from the ER. In addition to affecting mitochondria, Ca^{2+} depletion from the ER enhances protein misfolding, contributing to ER dilation. In this process, a reciprocal positive-regulatory relationship may exist among Ca^{2+} influx, ROS generation, and accumulation of misfolded proteins.

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was reported to stimulate Krebs cycle dehydrogenases, further boosting O_2 consumption and ROS production (Brand, 2010). Therefore, curcumin-induced mitochondrial ROS production and release of Ca^{2+} from the ER is presumed to create a vicious cycle that simultaneously impairs the structures and functions of the ER and mitochondria, leading to paraptotic cell death. Celastrol also induces paraptosis though a similar mechanism of curcumin-induced paraptosis regarding to the involvement of Ca^{2+} and ROS, except that IP_3R is involved for the release of Ca^{2+} from the ER, instead of RyR (Yoon et al., 2014b). Therefore, paraptosis may be a cell death requiring a close communication of the ER and mitochondria via mediation of Ca^{2+} in MAM structure.

7. Paraptosis as a potential anti-cancer therapeutic strategy

Cancer drug resistance is a complex, dynamic, and elusive issue, and it has proven difficult to restore the efficacy of chemotherapy by simply reactivating apoptosis. Accumulating evidence has shown that cancer cells that are resistant to one kind of cell death mode (such as apoptosis) may be susceptible to others (Blank & Shiloh, 2007; Tait et al., 2014; Ubah & Wallace, 2014), reflecting differences in the molecular mechanisms of these cell death pathways. In this regard, paraptosis may act as an important backup cell death pathway that comes into play when the apoptotic machinery is hindered. A deeper understanding of paraptosis may provide a basis for developing new therapeutic strategies against human cancers that prove refractory to conventional chemotherapeutic drugs.

The primary challenge in cancer therapy is the need to selectively kill transformed cells, while sparing normal cells. To do this, we must understand the detailed differences between tumor and normal cells. Tumor cells exhibit deregulation of organelles, including mitochondria and the ER, and the involvement of these organelles in various cell death modes makes them attractive therapeutic targets. In rapidly proliferating tumor cells, the ER tends to be overloaded with unfolded and misfolded proteins due to the imbalance between a high metabolic demand and a limited protein-folding capacity. Therefore, tumor cells often suffer from higher ER stress than normal cells (Suh et al., 2012). In addition, it has been estimated that over 90% of human solid tumors are aneuploid and protein synthesis is often imbalanced in aneuploid cancer cells, with excess production of proteins encoded on the extra chromosomes (Deshaies, 2014). The assembly of stable protein complexes may be negatively affected in aneuploid cancer cells, making such cells highly dependent on protein quality-control mechanisms, including protein chaperones and the ubiquitin-proteasome system (UPS) (Whitesell & Lindquist, 2005; Luo et al., 2009; Williams & Amon, 2009). Furthermore, many of the mutated proteins expressed in cancer cells are likely to present folding challenges and require a high level of degradation. Therefore, the regulation of proteostasis at the ER may be an appropriate target for cancer therapy.

A common characteristic of many cancer cells is an elevation of ROS, and the hypoxic environment created by rapidly proliferating tumor cells further facilitates ROS production (Gogvadze, 2011). Cancer cells exhibit greater oxidative stress than normal cells due (at least in part) to oncogenic stimulation, increased metabolic activity and mitochondrial malfunction (Gupta et al., 2012a). Since this state of oxidative stress makes cancer cells vulnerable to agents that further augment ROS levels, the use of pro-oxidant agents is emerging as an attractive strategy for the selective targeting of tumor cells (Martín-Cordero et al., 2012).

As shown in Table 1, many natural products that induce paraptosis-associated cell death induce ER stress. Perturbation of cellular proteostasis via proteasomal inhibition and disruption of sulfhydryl homeostasis is believed to contribute to these paraptosis-inducing activities. Several natural products (including curcumin) known to induce paraptosis-associated cell death appear to enhance both ER and oxidative stress beyond the threshold levels at which they become toxic to cancer cells, which already have elevated levels of protein synthesis/folding and ROS. In addition, there may be a vicious cycle between ER and oxidative stress in cancer cells treated with paraptosis-inducing natural products that induce the dilation of both the ER and mitochondria. Therefore, therapeutic strategies aimed at using these natural products to induce paraptosis could offer a two-pronged attack strategy for selectively killing cancer cells.

8. Perspectives and future directions

Despite the broad pathophysiological relevance of paraptosis, its incidence may have been underestimated in the literature due in part to a poor understanding of its biochemical mechanisms and a lack of specific markers. Other than the general agreement that paraptosis involves dilation of the ER and/or mitochondria, there has been no clear consensus regarding its molecular basis. The nomenclature and classification criteria for this cell death mode have included terms such as “paraptosis”, “paraptosis-like cell death”, and “cytoplasmic vacuolation cell death”, which has confused the issue. Furthermore, paraptosis-like morphologies are often found along with other types of cell death (e.g., apoptosis, necrosis, and occasionally autophagy) when an inducer targets multiple cell death pathways. In such situations, it is impossible to identify common molecular markers of paraptosis. In future studies, it will be important to choose appropriate model systems that will enable researchers to selectively induce paraptosis without involving other cell death modes. Such systems should be extensively studied using genomic, proteomic, and systems biology techniques both *in vitro* and *in vivo*.

In light of the published data related to natural-product-induced paraptosis, additional studies in various models are needed to corroborate the significance of Alix (as an inhibitor) and ERK1/2 and JNKs (as mediators). Although a proteomic analysis of IGF-IR-induced paraptosis identified phosphatidylethanolamine binding protein (PEBP-1) as an inhibitor and prohibitin as a candidate mediator (Sperandio et al., 2010), their involvements in paraptosis also warrant further testing in different experimental settings. Notably, cycloheximide effectively blocked the ER-derived vacuolation and cell death induced by most of natural products listed in Table 1, suggesting that it may be a useful chemical inhibitor of paraptosis. In addition, the signals that are thought to be responsible for the paraptosis-associated cell death induced by natural products (e.g., proteasome inhibition, disruption of sulfhydryl homeostasis, ROS generation, and imbalanced homeostasis of ions such as Ca^{2+} and K^+) should be investigated further and may prove useful as candidate markers for paraptosis.

Further studies focusing on novel natural products and their derivative chemical libraries should seek to identify more effective inducers of paraptosis and clarify the common key signals that modulate this mode of cell death.

Grant support

Supported by the National Research Foundation of Korea (NRF) grants (2015R1A2A2A1006966 (Mid-Career Researcher Program) and 2011-0030043 (SRC)) funded by the Korean government (MSIP).

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgment

We would like to thank Woo-Shin Cho for drawing the figures and Chan-Mee Jeong for preparing this manuscript.

Please cite this article as: Lee, D., et al., Paraptosis in the anti-cancer arsenal of natural products, *Pharmacology & Therapeutics* (2016), http://dx.doi.org/10.1016/j.pharmthera.2016.01.003

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Please cite this article as: Lee, D., et al., Paraptosis in the anti-cancer arsenal of natural products, *Pharmacology & Therapeutics* (2016), http://dx.doi.org/10.1016/j.pharmthera.2016.01.003

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Please cite this article as: Lee, D., et al., Paraptosis in the anti-cancer arsenal of natural products, *Pharmacology & Therapeutics* (2016), http://dx.doi.org/10.1016/j.pharmthera.2016.01.003