Apoptotic and autophagic pathways with relevant small-molecule compounds, in cancer stem cells

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Abstract

Accumulating evidence demonstrates existence of cancer stem cells (CSCs), which are suspected of contributing to cancer cell self-renewal capacity and resistance to radiation and/or chemotherapy. Including evasion of apoptosis and autophagic cell death, CSCs have revealed abilities to resist cell death, making them appealing targets for cancer therapy. Recently, molecular mechanisms of apoptosis and of autophagy in CSCs have been gradually explored, comparing them in stem cells and in cancer cells; distinct expression of these systems in CSCs may elucidate how these cells exert their capacity of unlimited self-renewal and hierarchical differentiation. Due to their proposed ability to drive tumour initiation and progression, CSCs may be considered to be potentially useful pharmacological targets. Further, multiple compounds have been verified as triggering apoptosis and/or autophagy, suppressing tumour growth, thus providing new strategies for cancer therapy. In this review, we summarized regulation of apoptosis and autophagy in CSCs to elucidate how key proteins participate in control of survival and death; in addition, currently well-studied compounds that target CSC apoptosis and autophagy are selectively presented.

With increasing attention to CSCs in cancer therapy, researchers are now trying to find responses to unsolved questions as unambiguous as possible, which may provide novel insight into future anti-cancer regimes.

Introduction

Over the past number of decades, the cancer stem cell (CSC) hypothesis has emerged as a possible new paradigm for distinct cancers, with this cell type being proposed to contribute to chemotherapy and radiation resistance, and to represent potentially useful pharmacological targets. Conceptually akin to normal stem cells, CSCs were originally conceived to be a minority subset of malignant cells with capacity for both unlimited self-renewal and hierarchical differentiation. Hallmarks of stem cell phenotype that may contribute to CSC therapy-resistance include quiescence, evasion of apoptosis, resistance to DNA damage and expression of drug transporter pumps, all of which subsequently drive tumour repopulation, post-therapy. Evasion of apoptosis contributes to survival of CSCs, as programmed cell death is a key cell fail-safe mechanism, that protects against oncogenic events. In addition, a strong link has recently been demonstrated between EMT (epithelial-to-mesenchymal transition) and autophagy in cancer stem-like cells, and resistance of cancer cells to cytotoxic T cell-induced killing. Targeting autophagy, for example, may help avoid immune resistance in breast cancer (1).

Mechanisms of evasion of apoptosis in CSCs, have been further studied in both leukaemia and some solid tumours. Inactivation of apoptotic signalling pathways helps CSCs evade cytotoxic activity of most anti-cancer therapies. Some studies have revealed that CSCs

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typically harbour acquired defects. Inefficient signalling in either the extrinsic or intrinsic pathway of apoptosis may exist. Between these, the death receptor (DR) (extrinsic) pathway is activated in response to cross-linking of transmembrane cell surface receptors (of the DR family, including CD95 and TRAIL). Alternatively, the mitochondrial (intrinsic) pathway can be engaged by a large variety of intracellular stimuli, for example, cell stress conditions, while both extrinsic and intrinsic pathways are tightly controlled by pro- and anti-apoptotic members of the Bcl-2 protein family. DR, inhibitor of apoptosis protein (IAP) and Bcl-2 protein families play pivotal roles in regulating evasion of apoptosis in CSCs as discussed below (2).

Autophagy, type II programmed cell death, which imposes a dual effect to determine fate of a cell, is currently hypothesized to mainly contribute survival mechanisms in CSCs. Whether CSCs opt for an aggressive phenotype, choose to enter an inactive state supported by autophagy, or undergo cell death, depends on activation of different intracellular pathways and specific changes in gene expression profiles, in response to external stimuli. In addition, interaction between EMT and autophagy promotes a number of mechanisms that facilitate tumour invasiveness and high cancer cell survival under stress conditions (3).

Cross-talk between apoptosis and autophagy in CSCs has attracted a considerable amount of attention recently. Instead of functioning separately, processes of apoptosis and autophagy normally cross-talk together, providing thoroughly detailed protein–protein interaction networks. The potential of targeting cross-talk between distinct cell death types to inhibit CSCs growth, makes it promising to design multi-target drugs or combinational use of various types of drug in cancer therapy. Hitherto, including histone deacetylase inhibitors, lysosomal inhibitors and further drugs in clinical trials, many compounds have been proven to exert functions to regulate programmed cell death in CSCs. Though specific targeting of drugs to CSCs has not been reported, several studies have been conducted attempting to develop novel therapeutic strategies specifically targeting CSCs. Thus, we summarize regulation of apoptosis and autophagy in CSCs to elucidate how key proteins participate in regulating their survival and death. Also, we review currently well-studied compounds to target CSC apoptosis and autophagy, to help provide novel strategies for their targeting in cancer therapy.

Apoptotic regulators in CSCs

Distinguished by particular biochemical processes and morphological features, four distinct types of apoptosis

have been identified, which are respectively, caspase-dependent intrinsic apoptosis, caspase-independent intrinsic apoptosis, extrinsic apoptosis *via* DR and extrinsic apoptosis by dependence receptors (4). As programmed cell death by apoptosis represents a key cellular fail-safe mechanism to protect against oncogenic events, evasion of apoptosis is likely to contribute to survival of CSCs. CSCs depend on compensatory mechanisms to escape induction of apoptosis after oncogenic transformation. Otherwise, under environmental stress conditions, survival and self-renewal would not occur. Inactivation of apoptosis signalling pathways also helps CSCs evade cytotoxic activity of most anti-cancer therapies, as most of those currently used in treatment of cancers largely depend on interaction of apoptosis programmes for their anti-tumour efficacy. As discussed below, based on distinct mechanisms, regulation of apoptosis in CSCs can be differentiated from other stages of cancer cell death (Fig. 1).

Death receptors

As crucial mediators of extrinsic apoptosis, DR are members of the tumour necrosis factor (TNF) receptor superfamily which consists of more than 20 proteins with broad ranges of biological functions, including regulation of cell death and survival, differentiation and/or immune regulation (5). Eight members have been identified to date: tumour necrosis factor receptor 1 (TNFR1), CD95 (Fas), DR3, TNF-related apoptosis-inducing ligand receptor 1 (TRAILR1), TRAILR2, DR6, ectodysplasin A receptor (EDAR) and nerve growth factor receptor (6).

Two types of DR signalling complex can be distinguished. The first group is comprised of CD95 receptor, TRAILR1, TRAILR2, forming the death-inducing signalling complexes (DISCs). The second group is comprised of TNFR1, DR3, DR6 and EDAR to recruit a different set of molecules, which transduce both apoptotic and survival signals (7).

The DISC complex is responsible for transducing death ligand stimulation of DR CD95, TRAIL-R1 or TRAIL-R2 to apoptosis. DISCs consist of oligomerized receptors, DD-containing adaptor molecule Fas-associated death domain, procaspase-8 (FLICE, MACH α and Mch5), procaspase-10 and cell FLICE/caspase-8-like inhibitory protein (c-FLIP) (8). As a result of DISC formation, procaspase-8 is activated at the DISC resulting in formation of active caspase-8, which leads to activation of the apoptotic sequence (9).

Two types of cell correspond in CD95-involved DISC formation: Type I cells are characterized by high levels of CD95 DISC formation and high amounts of

Figure 1. Regulation of apoptosis in cancer stem cells (CSCs). Evasion of apoptosis contributes to survival of CSCs, which may also help them evade cytotoxic activity of most anti-cancer therapies. CSCs are sensitive to anti-TRAILR2-mediated apoptosis, and insufficient expression of c-FLIP may result in resistance to treatment using TRAIL. Moreover, overexpression of Bcl-2 can result in elevated numbers of CSCs and increased repopulation potential, which protects CSCs against a wide range of apoptotic stimuli including various chemotherapeutic agents.

active caspase-8, while in Type II cells, lower levels of CD95 DISC formation are observed. In these, an additional amplification loop is needed for effective apoptotic signalling that involves cleavage (by caspase-8) of Bcl-2 family protein Bid, to generate truncated Bid (tBid) and subsequent tBid-mediated release of cytochrome *c* (cyt. *C*) from mitochondria. Cyt. *C* binds to the cytosolic adaptor protein, apoptotic protease activating factor 1 (Apaf1), to facilitate formation of an apoptosome, followed by recruitment and activation of procaspase-9, which in turn cleaves downstream effector caspases-3, -6 and -7 (8,10). The IAP family is comprised of proteins that can bind and inactivate caspases (11,12).

As well as inducing formation of the membrane-associated DISC, stimulation of CD95 and TRAIL receptors can also induce formation of complex II, which is constructed within minutes after receptor stimulation, and potentially enhances apoptosis by amplifying

caspase activation (8). Evidence shows that TRAIL complex II activates kinase pathways which increase chemokine production, suggesting that the role of complex II may be to promote macrophage phagocytosis of apoptotic cells (13).

TNFR1 signalling differs from CD95 receptor or TRAILR1/R2-induced apoptosis. Stimulation of TNFR1 chiefly induces pro-inflammatory and immune-stimulatory activity through activation of NF-κB, mitogen-activated protein kinases (MAPKs), c-Jun amino-terminal kinase (JNK) and p38 (14). Induction of cell death by TNF is a secondary signal that is only triggered when activator signals are too weak or otherwise perturbed (15). Binding of TNF to TNFR1 induces receptor oligomerization and formation of TNFR1 complex I, followed by which regulators are recruited, thus enabling activation of NF-κB and MAPKs (16).

TNFR1-induced apoptosis is initiated solely upon formation of TNFR1 complex II, where activation of

procaspase-8 takes place, and is followed by activation of downstream death signalling. However, when complex I is involved, complex II no longer induces cell death as its signalling output is counteracted by gene-activator signals generated at complex I. Thus, if complex I does not decompose prematurely and its output reaches physiological levels, complex II-induced cell death is prevented (9).

Cancer stem cells have the ability to self-renew as well as to differentiate into heterogeneous lineages of cancer cells, and these tumour-forming cells can originate from stem, progenitor or differentiated cells (17). Due to the tumourigenic specialty of CSCs, much research concerning DR-mediated apoptosis in CSCs has been performed in recent years, shedding new light on exploiting new anti-tumour drugs for future cancer therapy.

Death receptors have been reported to be targets to induce CSC apoptosis and application of apoptosis inducers targeting DR alone, or in combination with other anti-cancer drugs, has become a new strategy for anti-cancer therapy. Basal-like breast CSCs are sensitive to anti-TRAILR2-mediated apoptosis (18). TRAILR2 is enriched in pancreatic CSCs compared to the bulk of the tumour cells. Resistance to DR-mediated apoptosis is associated with development of drug resistance. Cancer stem cells from human glioma cell lines are resistant to CD95-induced apoptosis and the monomeric form of CD95 protein can be detected predominantly in these cells, while mature cells mainly contain oligomeric aggregates of CD95 protein and are sensitive to CD95-activated apoptosis. These results suggest that glioma stem cells might be an important factor of resistance to chemotherapy requiring apoptosis through the CD95 signalling system. Other studies also show that TRAIL functions in the same role as CD95 in drug-resistance models within glioblastoma-derived stem cells (GSCs), largely due to insufficient expression of TRAIL1/2 (19).

It is worth noting that apoptotic signalling pathways, including extrinsic and intrinsic pathways, are deregulated in CSCs. In glioblastoma and lung CSCs, the DR mediating the extrinsic pathway is expressed at a high level, and up-regulation of DR4 in colon CSCs leads to chemoresistance. FLICE-like inhibitory proteins (cFLIP) are negative modulators of DR-induced apoptosis, and consist of two subtypes, long cFLIP (cFLIPL) and short cFLIP (cFLIPS). In CD133+ glioblastoma, breast cancer and T-cell acute leukaemia cells, cFLIPs are up-regulated. Silencing of cFLIPs by siRNA restores cell sensitivity to death stimuli, suppressing CSC self-renewal and tumour metastasis. Insufficient expression of DR and increased expression of c-FLIPs lead to CSCs-enriched neurosphere resistance to TRAIL (20–22).

Thus, the ability of DRs to induce apoptosis in CSC makes them a potential target for anti-cancer drug development. DR-directed therapies alone or in combination with chemotherapy may act as therapeutic options to improve current anti-cancer treatment. Moreover, expression of DRs may vary in CSCs, resulting in divergence in drug sensitivity in CSCs. Resistance to DR-mediated apoptosis (which is mainly due to insufficient expression of DR and overexpression of c-FLIP in CSCs), plays an essential role in development of drug resistance.

Bcl-2 family

Abundant evidence reveals that expression of either anti-apoptotic or pro-apoptotic Bcl-2-family genes and proteins is altered in CSCs, through translational or post-translational modifications. Importantly, there is a close link between altered expression of Bcl-2 family members in CSCs and cancer relapse, indicating promising therapeutic strategies (19,23).

B-cell lymphoma-2 gene (*Bcl-2* gene) is a crucial oncogene involved in apoptosis. Whether a cell should live or die is largely determined by the Bcl-2 family of anti- and pro-apoptotic regulators (19). One subfamily, including Bcl-2, Bcl-xL and Bcl-w, Mcl-1 are anti-apoptotic, thus protecting cells from a wide range of cytotoxic insults. While, the other sub-family, composed of Bax, Bak and Bok, promotes against apoptosis. Yet other pro-apoptotic relatives, the BH3 sub-family (exemplified by Bik, Bad and Bim), are largely unrelated in sequence to either Bcl-2 or to each other. They are conceived to be Bax- and Bak-dependent, when performing killing functions (23).

Bax-like proteins are critical for apoptosis in many cells. In response to cytotoxic signals, Bax and Bak undergo conformational change and form membrane-associated homo-oligomers. Then they are thought to disrupt outer membranes of mitochondria, thereby releasing the cytochrome c apoptotic mediator, which completes the apoptosome with function of Apaf-1, thereby activating caspase-9. Bax and Bak are also involved in apoptosis initiated from the endoplasmic reticulum (ER) in response to stress imposed by disrupted calcium homeostasis or excess unfolded proteins. In contrast, the Bcl-2 sub-family acts upstream of Bax/Bak and inactivates them by blocking their conformational changes, thereby preventing cells from becoming apoptotic. As for the BH3 sub-family, these members can bind to and inactivate Bcl-2 and other anti-apoptotic homologues, thereby activating Bax/Bak. BH3 sub-family members cannot kill without Bax/Bak, and they are believed to amplify the death signal. In general, imbalances in ratios of anti- to pro-apoptotic Bcl-2 proteins rather than expression levels of one particular

molecule of the Bcl-2 family, may tip the balance towards cell survival and regulate sensitivity to an apoptotic stimulus (24–26).

Dysregulation of the intrinsic pathway in CSCs is mainly reflected in Bcl-2 family proteins and their DNA damage response. In most tumours, anti-apoptotic Bcl-2 family proteins are overexpressed in CSCs. For instance, CD133+ glioma CSCs express high levels of anti-apoptotic proteins Bcl-2 and Bcl-XL, and high expression of Mcl-1 correlates with resistance to Bcl-2 inhibitor ABT-737, in glioma CSCs. In colon CSCs, Bcl-2 is high and inhibits apoptosis and autophagy. Down-regulation of Bcl-2 or up-regulation of Bax induces CSC apoptosis. Thus, inhibition of the mitochondrial death cascade is attractive for CSC-targeted therapeutic intervention in cancers (20–22).

Inhibitor of apoptosis proteins

Inhibitor of apoptosis proteins have also been implicated to play important roles in regulation of apoptosis in CSCs, where higher expression of IAP is expected. These comprise a family of endogenous caspase inhibitors that block apoptotic signalling pathways, at key nodes. The IAP family is made of eight human homologues, X-linked inhibitor of apoptosis (XIAP), cell IAP1 (cIAP1), cell IAP2 (cIAP2), survivin, livin/melanoma-IAP (ML-IAP), neuronal apoptosis inhibitory protein (NAIP), Bruce (apollon) and ILP-2, among which XIAP exhibits the strongest anti-apoptotic properties. It inhibits apoptotic signalling by binding to active caspases-3 and -7 and by preventing caspase-9 activation (2,27,28). One member, survivin, has been shown to regulate normal stem cell function and is associated with cell cycle progression, proliferation, and maintenance of normal cord blood CD34+ cells, in addition to its role in regulation of apoptosis (2). Remarkably, survivin is enriched in haematopoietic stem cells, neuronal precursor cells, CD34(+)/38(–) AML stem cells, and glioblastoma and astrocytoma CSCs (20–22).

Inhibitor of apoptosis protein proteins have an endogenous antagonist apoptosis-related protein in the TGF- β signalling pathway (ARTS/septin 4 isoform 2), which has been revealed in the control of stem cells. It is broadly implicated in regulating apoptotic signalling *via* direct binding and antagonizing XIAP (28–30). IAP inhibitors that neutralize aberrant expression of IAP proteins, such as XIAP, have been reported to sensitize primary glioblastoma CSCs (derived from surgical specimens) for c-irradiation-induced apoptosis, underscoring the relevance of IAP proteins as therapeutic targets. However, it remains to be determined whether

IAP inhibitors selectively target malignant versus non-malignant cells.

Autophagic regulators in CSCs

Cancer stem cells are unique in their ability to self-renew and differentiate into different cell types in malignant tissues. These are important in development, tissue renewal and a range of cancer-related cell processes. In contrast to the extensive body of knowledge available for somatic cells, the role of autophagy in maintenance and function of CSCs is only beginning to be revealed as a result of recent studies. These suggest that autophagy also plays a positive role in the origin, maintenance and systemic distribution of CSCs (31,32), besides its many functions in normal embryonic and tissue stem cells (Fig. 2).

Autophagy is a highly regulated self-digestion process, which plays a pivotal role in maintaining energy homeostasis and protein synthesis during various cell stresses through degradation of long-lived proteins and damaged organelles. There are three models of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy, among which macroautophagy is the best characterized process (referred as autophagy hereafter). Autophagy is the vesicular mode of transport for intracellular components to the lysosome, during which cytoplasm is engulfed by a specific double membrane-bound vesicle known as the autophagosome; this later fuses to the lysosome and releases its cargo of lytic enzymes (33–35).

Autophagy plays an essential role in development of drug resistance, self-renewal, differentiation and tumorigenic potentials of CSCs, while the cells have the ability to self-renew as well as to cause development of heterogeneous lineages of cancer cells which originate from stem, progenitor or differentiated sources. Thus recently, an interesting question that remains to be answered relates to possible active or suppressive contribution of autophagy to the metabolic shift of CSCs during malignant transformation and acquisition of stemness by CSC-like cell populations. In CSCs, autophagy may also play dual roles in glycolytic processes, while aerobic glycolysis is orchestrated by Akt, one of the autophagy key regulators, whose activation is achieved by membrane translocation and subsequent phosphorylation (36,37).

Hypoxia- and starvation-related autophagy functions as a cytoprotective adaptive mechanism, when CSCs deal with microenvironmental stresses. A causal relationship between activation of autophagy and enhanced cell survival of CSCs has initially been suggested by specific spatio-temporal up-regulation patterns observed

Figure 2. Regulation of autophagy in cancer stem cells (CSCs). In CSCs, oncogenic autophagic pathways play dual roles in multiple cellular processes, such as glycolysis, drug resistance, self-renewal and differentiation. Regulation of autophagy is orchestrated by two major pathways, PI3KCI–Akt–mTORC1 and Bcl-2/Bcl-XL. Furthermore, other key autophagic proteins participate in this regulation, including AMPK, Atg5 and Atg12.

for Beclin-1 in certain pre-malignant lesions of the breast. In contrast to normal breast tissues, expression levels of the autophagic effector LC3-I are also increased in lesions, and remarkably, Beclin-1-related enhancement of the autophagic flux appears to occur in tumour-founding progenitor cells (38). Also, autophagy-associated genes *DRAM1* and *SQSTM1* have been reported to regulate motility of and invasion by glioblastoma stem cells, further supporting a previously unrecognized role of autophagy in the metastatic potential of migratory CSCs.

A number of autophagy regulators are highly expressed in tumour cells carrying a mesenchymal signature, connecting autophagy to the EMT phenomenon in cancer metastasis (39). EMT is a key contributor to metastasis linked to a few CSC-like features, thus, can be considered as a crucial figure in CSC maintenance. Mesenchymal signatures of autophagy regulators intrinsically define aggressiveness and metastatic invasion, suggesting that autophagy acts positively in concert with EMT-related processes to reduce proliferation in favour of enhanced cell motility in metastasis, while supporting survival of disseminated cells with CSC-like properties (40). In breast cancer, the undifferentiated CD44+/CD24– antigenic state, is commonly attributed to breast cancer subpopulations, while CSC properties are highly enriched by EMT transcriptional factors and

mesenchymal markers. Impairment of the autophagic flux drastically reduces numbers of breast cancer cells bearing CD44+/CD24– cell surface antigens, and appears to impede ontogeny of generating invasive CD44+/CD24– mesenchymal phenotype by preventing full acquisition of a post-EMT status. Furthermore, ATG12 might be pivotal for the autophagy-regulated migratory phenotype as its genetic ablation activates transcription of the epithelial marker CD24, while concomitantly repressing the gene coding for mesenchymal filament vimentin (41).

Endothelial secreted factors suppress mitogen deprivation-induced autophagy in glioblastoma stem-like cells. *In vivo* mitogen deprivation impairs self-renewal capability and impinges on GSC survival, abolishing constitutive activation of the mTOR pathway. Moreover, pharmacological inhibition of mTOR recapitulates the mitogen deprivation scenario (42). In contrast, blocking either apoptosis or autophagy, or culturing GSCs with endothelial-secreted factors, partly restores mTOR activation and rescues GSC survival, suggesting that GSCs are attracted to mTOR, as their survival and self-renewal are profoundly dependent on this signalling axis. Thus, whether under deprivation conditions or presence of endothelial factors, mTOR function is a key regulator of GSC fate, suggesting its potential for therapeutic purposes (43).

Detailed research has validated that autophagy is essential for maintenance of CSCs. For instance, CD133+ liver CSCs (LCSCs) can be significantly enriched with higher autophagy levels after hypoxia and nutrient starvation (H/S) in hepatocellular carcinoma (HCC) Huh-7 cells. In colon CSCs, autophagy is activated through the Cdx1–Bcl-2–LC3 pathway, while in contrast, p53 exerts a major role in apoptosis and inhibits autophagy. In both colon cancer cells (CD44+/CD24+ HCT116 and CD44+/CD24+ SW1222), Cdx1 exerts a protective role over colon CSCs, which play a crucial role in chemoresistance to paclitaxel through activation of autophagy (44–46).

Moreover, breast cancer cells with low metastatic potential can be induced into a reversible state of dormancy by farnesyl transferase inhibitors (FTIs), with expression of ATG5, ATG12 and LC3B. The c-jun NH2 terminal kinase (JNK)-mediated autophagic pathway can also be up-regulated in breast CSCs with periods of FTI-induced dormancy. Knock-down of damage-regulated autophagy modulator 1 (DRAM1) may reduce p62 localization to autophagosomes and its autophagy-mediated degradation, suggesting a crucial role of DRAM1 in p62-mediated autophagy. DRAM1 expression has been correlated with activation of mitogen-activated protein kinases (MAPKs) and the mesenchymal marker c-MET in glioblastoma stem cells. In addition, irradiation of CD133+ glioma stem cells (GSCs) can induce autophagy in a short time followed by slight reduction in autophagy and viability of the cells. Gamma-radiation may also induce a large degree of autophagy in CD133+ GSCs, and CD133+ cells express higher levels of ATG proteins such as ATG5, LC3 and ATG12 (47).

Although great amounts of study have been performed, numerous questions still remain to enlighten scientists for further discoveries. For example, whether CSCs are necessarily required for activation of autophagic machinery to ensure survival and/or functioning, or how autophagy relates to self-renewal capabilities (3).

Cross-talk between apoptosis and autophagy in CSCs

Common upstream signals may trigger combined or mutually exclusive autophagy and apoptosis in CSCs, leading to complex protein–protein interaction regulation.

As mentioned above, anti-apoptotic protein Bcl-2 plays crucial roles in regulating apoptosis in CSCs, and these have validated binding to Beclin-1 preventing its interaction with PI3KCIII in autophagy. For example, prolonged exposure of breast CSCs to Rottlerin caused

apoptosis (associated with down-regulation of anti-apoptotic Bcl-2, Bcl-X(L), XIAP and cIAP-1), and autophagy-type behaviour such as suppression of phosphorylated Akt and mTOR and up-regulation of phosphorylated AMPK. A further study has shown that Rottlerin induces autophagy *via* extensive cytoplasmic vacuolization in breast CSCs. Rottlerin-induced autophagy can lead to apoptosis in breast CSCs, while Rottlerin treatment increases expression of LC3, Beclin-1 and ATG12 that accumulate during autophagy (36). Although, autophagy inhibitors suppress formation of cytoplasmic vacuolization and autophagy in CSCs, the potency of Rottlerin to induce autophagy and apoptosis might be based on its capability to activate several pathways such as AMPK and proteasome inhibition (36). In addition, the Palladium-Based Compound (Pd complex) has shown a powerful growth-inhibitory effect against both cell lines and primary cultures. More importantly, it successfully reduces viability of CSCs as first reported in this study. The Pd complex induces DNA damage and differentially induced evidence of apoptosis, as well as autophagy (36,48).

Discovering cross-talk between autophagy and apoptosis in CSCs is focused on crucial cellular processes, involving contributions from such as reactive oxygen species (ROS). Oxidative stress is one of the cell states characterized by excessive free radical production, and is one of the most important regulatory mechanisms for CSCs. In comparison to normal cells, cancer cells have higher levels of ROS, which seem to be essential for malignant initiation and progression (49,50). ROS can injure important cell components such as proteins, DNA and membrane lipids, and thus can cause cell death. Also, they have been demonstrated to be involved in various physiological and pathological processes, including DNA damage, proliferation, cell adhesion and survival (51).

Recent studies have proven that normal haemopoietic stem cells and normal mammary epithelial stem cells maintain ROS at lower levels than their mature progeny, to prevent cell differentiation and maintain long-term self-renewal. Mitochondria are the major sites of ROS production and accumulation of ROS may lead to initiation of apoptosis. The intrinsic pathway of apoptotic cell death can be triggered by many stimuli including ROS (49,50). Protein re-folding in the endoplasmic reticulum (ER) by protein disulphide isomerases can elevate oxidative stress through redox reactions involving free radicals, and mitochondrial stress and damage can also be a source of ROS in autophagy-deficient cells. ROS trigger signalling cascades, which lead to activation and phosphorylation of MAPKs, including ERK. As a consequence, transcription factors including NF- κ B and AP-1

become activated, which may lead to induction of early response genes such as *c-jun* and *c-fos*, which are involved in inflammatory influx, inhibition of apoptosis, cell proliferation, transformation, differentiation and other changes (52,53).

Targeting CSC apoptosis and autophagy as new therapeutic strategies

Compelling evidence has displayed that targeting CSCs is a promising way to investigate new anti-cancer therapies. Up to date, multiple compounds have shown anti-CSC ability by activating apoptosis and/or autophagy, as we have summarized above. Neither as enemies nor friends of maintaining cell processes in CSCs, programmed cell death, followed as apoptosis and autophagy, is involved in their regulation. Though relatively rare reports on specific targeting drugs of CSCs, many attempts have been made into developing the efficient compounds for CSCs (Table 1).

Torin-1, a highly potent and selective mTOR kinase inhibitor, has been revealed to be a powerful inhibitor, suppressing cancer stem-like cells from human metastatic CRCs (CoCSC) growth both *in vitro* and *in vivo* (54). However, considering the dual role of autophagy in cancer cell growth, and its complexity in determining detailed mechanisms of mTOR regulated autophagy under different contexts, it is troublesome to apply it to cancer drug design (30). Rottlerin (ROT) is a widely used protein kinase C-delta (PKC- δ) inhibitor. It can induce significant apoptosis in pancreatic CSCs by inhibiting phosphorylation of Akt and mTOR, expression of Bcl-2, Bcl-XL, cIAP1 and XIAP, up-regulation of Bax, and activation of caspases-3 and -9. Study has shown that ROT causes early autophagy and late apoptosis through inhibition of the PI3K/Akt/mTOR pathway in human pancreatic CSCs (55). NVP-BEZ235, a novel

dual PI3K/mTOR inhibitor, enhances radiosensitivity of human glioma stem cells *in vitro*. Combination of IR with NVP-BEZ235 significantly increases apoptosis of SU-2 cells (human glioma stem cells) (56). Similarly, recent research has shown that DT61 inhibited PI3K, mTORC1 and mTORC2 kinase activities, thus showing potent apoptosis of glioma cells and glioma patient-derived CSCs (57). Thus, mTOR, which plays a central role at the intersection of autophagy and apoptosis (along with both its upstream and downstream proteins), is a prospective target for CSCs-targeting drug design (58). Furthermore, as an anti-tumour agent has been widely investigated for over a decade, metformin can attenuate CSC phenotypes and functions, such as CSC self-renewal capacity and CSC signature, and its mediators. CSCs appear to be more sensitive to metformin due to its primary mechanism on glucose metabolism. Apoptosis is followed by activation of AMPK, a central energy sensor, in turn leading to inhibition of the mTOR signalling pathway (59) (Fig. 3a).

Histone deacetylase (HDAC), known to be a prominent target for drug development over recent years, involves miscellaneous pleiotropic effects such as cell cycle arrest and induction of apoptosis/autophagy. ITF2357 is a newly designed chemical that shows potent activity towards HDAC inhibition. Highly synergistic growth inhibition has been observed in patient-derived lung cancer stem cells (LCSC) exposed to pemetrexed followed by ITF2357. The study indicated that mechanisms of cell death induced by the combined treatment involved both autophagy and apoptosis, and provided an interesting link between these two pathways and loss of cell viability (60). Suberoylanilide hydroxamic acid (SAHA), another widely used histone deacetylase inhibitor in anti-cancer therapy, has recently been revealed to induce autophagy in glioblastoma stem cells (GSCs). SAHA specifically triggers autophagy and reduces cell

Table 1. Small-molecule compounds that modulate apoptotic and autophagic pathways in cancer stem cells

Compound	Cancer stem cell	Mechanism	Clinical trial	References
Salinomycin	Colon and breast CSCs	Apoptosis	Clinical trials	(64,66)
Curcumin	Colon CSCs	Apoptosis and Autophagy	Phase II	(68,69)
Torin-1	Colon CSCs	mTOR inhibition induced autophagy	Clinical trials	(54)
Rottlerin	Breast CSCs	mTOR inhibition induced autophagy	Clinical trials	(55)
NVP-BEZ235	Glioma stem cells	mTOR inhibition induced autophagy	Clinical trials	(56)
ITF2357	Lung CSCs	Histone deacetylase (HDAC) inhibition induced autophagy	Phase II	(59)
SAHA	Glioblastoma stem cells	Histone deacetylase (HDAC) inhibition induced autophagy	Phase II	(58)
Resveratrol	CSCs	Apoptosis	Clinical trials	(60)
Rakicidin A	CSCs	Apoptosis	/	(60)
Metformin	Breast and pancreatic CSCs	Apoptosis	Phase III	(59)
2',3'-DMF	MCF-7 breast CSCs	Apoptosis and autophagy	/	(36)

'/' means the compound is not under clinical trials.

Figure 3. Small-molecule compounds in apoptosis and autophagy of cancer stem cells (CSCs). (a) Targeting PI3K/Akt/mTOR pathway is a promising strategy to cure cancer, several compounds, including mTOR inhibitor, PI3K inhibitor and AMPK activation have been revealed to significantly suppress CSC tumour growth. (b) As crucial transcription regulators, inhibiting HDACs can down-regulate anti-apoptotic genes in CSCs, such as *Bcl-2* or *c-Myc*, thus inducing apoptotic cell death to suppress tumour growth.

viability *via* promotion of apoptosis in the late phase of GSCs. Inhibition of SAHA-induced autophagy using chloroquine has synergistic effects that further increase apoptosis. Induction of autophagy is determined by down-regulation of the Akt–mTOR signalling pathway. SAHA has been shown to be a promising agent for targeting GSCs by induction of autophagy (57) (Fig. 3b).

2',3'-DMF is highly cytotoxic to MCF-7-SC breast CSCs, by inducing extrinsic and intrinsic apoptosis. It stimulates enrichment of LC3 lipidation on intracellular membranes and increases levels of ubiquitinated caspase-8, which results in aggregation and activation of caspase-8, thus triggered apoptosis. This indicates that 2',3'-DMF might be developed as a potential treatment against breast CSCs. Interestingly, 2',3'-DMF induces dramatic increase in conversion of LC3, a well-known

marker for autophagy, yet acidic vesicular organelles, an autophagic flux marker have not been detected, suggesting that autophagy had not actually been triggered (61,36).

Besides structure-based designed small-molecule compounds, more evidence can be found concerning the relationship between stem cell or stem-like cells and natural products (Fig. 4). Resveratrol significantly reduces cell viability and mammo-sphere formation and subsequently induces apoptosis in cancer stem-like cells. Rak-icidin A, a natural product produced by a strain of micromonospora, has been shown to induce cell death in hypoxia-adapted chronic myeloic leukaemia cells, with stem cell-like apoptosis, induced by both caspase-dependent and -independent pathways (62). Both whole fruit (BMW) and skin (BMSk) extracts showed significant inhibition of CSC proliferation and colony formation *via* cleavage of LC3B (Fig. 4). Thus recently, bitter melon extracts have been revealed to help reduce numbers of DCLK1+ (identified as stem cell markers) cells in a dose-dependent manner, suggesting that the extracts may target stem cells (63).

Salinomycin was first identified as a highly effective chemical to induce apoptosis in CSCs *via* high-throughput screening. It has been observed that sensitivity of breast cancer cell lines to salinomycin correlated with relative abundance of CD44^{high}/CD24^{low} CSC-enriched subpopulations (64). Salinomycin inactivates Stat3 and down-regulates cyclin D1, which are important for self-renewal of CSCs (65). Recent study has also shown that salinomycin induces autophagy-dependent cell death in breast and colon cancer cell lines, and inhibits expression of CDH1 expression in A549 cells, concomitant to induction of ROS (66).

Curcumin, a natural phytoconstituent, is known for some anti-neoplasial activity, including signal transduction pathway modulation, cell cycle inhibition, apoptosis, generation of ROS, p53 expression and inhibition of NF-κB (67). Various independent studies have shown that combination treatment of curcumin and chemotherapeutic drugs can enhance cell sensitivity to the chemotherapeutics, indicating a promising strategy to target CSC-related chemoresistance (68). Thus, analogues or derivatives of curcumin can be developed, for instance, difluorinated-curcumin (CDF), in combination with 5-fluorouracil and oxaliplatin (5-FU + Ox), having been shown to be effective in eliminating colon CSCs (68). GO-Y030 efficiently inhibits STAT3 phosphorylation and cell viability, tumour sphere-forming capacity, and induction of apoptosis, in colorectal CSCs (69) (Fig. 3b).

Some evidence has already validated that several compounds are able to trigger combined autophagy and apoptosis, for example, both in parental and stem-like

Figure 4. Natural products and apoptosis and autophagy of cancer stem cells (CSCs). Multiple natural products have been detected to process anti-tumour abilities in CSCs, including curcumin, resveratrol, BMW and BMSk.

GBM cells, salinomycin can increase levels of autophagosomes. This correlated to increased p62 and LC3II levels, while valproate co-treatment correlated with reduced LC3II and p62 expression and increased caspase 3 cleavage. 2',3'-DMF induces ubiquitination of caspase-8, which results in an interaction between caspase-8 and LC3 and leads to aggregation and activation of caspase-8. Co-treating cells with 2',3'-DMF and 3-methyladenine (an inhibitor of LC3 lipidation), reduces activation of caspase-8. These findings provide novel insights into anti-cancer effects of distinct compounds in CSC research, by revealing that they may induce apoptosis in accompaniment with activation of autophagy, mediated by key autophagic protein conversion (36). New targets have also been found during; pp242 protects CoCSCs from effects of mTOR inhibition, and on through autophagic activation, warranting considerable attention on newly developed inhibitors (54). Dopamine receptor expression also provides a potential target of human CSCs; this was reported in 2012 (70). It is expected that further molecular mechanisms will be elucidated, following highly encouraging compound discoveries, pre-clinical experimentation and new drug development strategies.

Conclusions

Cancer stem cells have been reported to exist in a variety of cancers and are proposed to drive tumour initiation and progression (71,72). To date, chemotherapy and radiotherapy represent the two major options for cancer treatment, by for example inducing p53-dependent cell senescence and apoptosis. However, advanced cancers often develop resistance to these treatments as they often lose their p53 response due to frequent mutations to the p53 gene (73). In this scenario, developing cancer treatment strategies by boosting p53-independent senescence and/or apoptotic responses is a potential key to the success of advanced cancer treatments. Evasion of apoptosis in CSCs, as a hallmark of cancer, has been thoroughly studied both by mechanisms and by therapeutic potential. In addition, cancer cells display elevated glycolysis irrespective of presence or absence of oxygen. This warrants cancer cell proliferation and survival, making it a promising strategy for cancer therapy (49). The dual role autophagy plays in glycolysis may exert significant roles in regulation of maintaining starving CSC survival and death. Beyond evading biophysical constraints, starving CSCs must use alternative sources of energy *via* activation of catabolic processes that permit activation of autophagy to recycle intracellular components and maintain metabolic homeostasis, quiescence and cell viability, during periods of metabolic stress.

The concept of CSCs has only been widely accepted in recent years, as their discovery in various cancers has been reported. However, our understanding of cell and molecular mechanisms that underlie CSC properties is limited. Death receptors have been reported to be involved in CSCs eluding apoptosis, while roles of related caspases and the dependence receptors playing in regulation of CSCs, have not yet been fully revealed. Furthermore, studies in autophagy also still have crucial questions to be unraveled, for instance, what determines survival or death in autophagy in CSCs, and whether autophagy is widely distributed amongst all CSCs. Developments of therapeutic potentials of apoptosis and autophagy have been widely researched in recently, and many compounds have been validated to process pro-apoptotic and/or pro-autophagic abilities. However, developing specific-targeting compounds to trigger cell death in CSCs is limited. In addition, whether these compounds can exert anti-cancer abilities in a clinical setting still needs further studies in pre-clinical experimentation and clinical trials.

Besides identified apoptotic and autophagic key proteins, some other crucial proteins in distinct cell processes have been found to trigger cell death in CSCs (74). For example, retinoblastoma protein (RB) is a

classical tumour suppressor with its role in the G1/S cell cycle checkpoint, but recent data have shown RB participation in many other cell functions, including, counterintuitively, negative regulation of apoptosis (74–76). These pieces of research have inspired us to improve current understanding of signalling pathways in CSCs, as they are incomplete, and further detailed studies are needed before developing approaches for targeting cell death to cure cancers. In common with ROS, many current studies have revealed that multiple miRNAs have distinct expression levels and may regulate cross-talk between apoptosis and autophagy (77,78). In addition, microRNAs possess suppressive and ontogenetic ability to regulate tumour growth, while various miRNAs have been reported to specifically regulate in CSCs, revealing to us that much work remains to be addressed.

Increasing research has focused on regulation of cell death pathways in CSCs, other than apoptosis or autophagy. Investigation into regulation of other cell death subroutines are increasing; for instance, necrosis has been reported in melanoma, and targeting mitotic catastrophe key protein Chk1 in NSCLC stem cells during chemotherapy has been reported to be a potential therapeutic strategy in suppressing tumour cell growth (53). With augmented studies in CSCs, protein–protein interaction networks of cell death in CSCs are becoming mature, and specifically targeting PPI of cell death may be a promising future in anti-cancer drug design development. Considering the significant role CSCs play in tumour growth and distinct regulation of pathways of apoptosis and autophagy, researchers are trying to construct appropriate responses to unsolved questions as unambiguous as possible, to provide novel strategies for future cancer therapy.

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Conflict of interest

None.

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