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Role of autophagy in cholangiocarcinoma: An autophagy-based treatment strategy

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Abstract

Cholangiocarcinomas (CCAs) are diverse biliary epithelial tumours involving the intrahepatic, perihilar and distal parts of the biliary tree. The three entirely variable entities have distinct epidemiology, molecular characteristics, prognosis and strategy for clinical management. However, many cholangiocarcinoma tumor-cells appear to be resistant to current chemotherapeutic agents. The role of autophagy and the therapeutic value of autophagy-based therapy are largely unknown in CCA. The multistep nature of autophagy offers a plethora of regulation points, which are prone to be deregulated and cause different human diseases, including cancer. However, it offers multiple targetable points for designing novel therapeutic strategies. Tumor cells have evolved to use autophagy as an adaptive mechanism for survival under stressful conditions such as energy imbalance and hypoxic region of tumors within the tumor microenvironment, but also to increase invasiveness and resistance to chemotherapy. The purpose of this review is to summarize the current knowledge regarding the interplay between autophagy and cholangiocarcinogenesis, together with some preclinical studies with agents that modulate autophagy in order to induce tumor cell death. Altogether, a combinatorial strategy, which comprises the current anti-cancer agents and autophagy modulators, would represent a positive CCA patient approach.

Key Words: Autophagy; Autophagy modulators; Chemotherapy; Cholangiocarcinoma

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cells in tumorigenesis remains controversial. A grown body of research data suggests that autophagy is a promising target for several cancer types, including cholangiocarcinomas (CCAs). A novel therapeutic approach which could involve autophagy manipulation plus chemotherapeutic agents may open a new field for more beneficial therapeutic strategies for patients with CCA.

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INTRODUCTION

Cholangiocarcinoma (CCA) constitutes a highly malignant group of epithelial tumours, originated in the biliary tree, consisting of three heterogeneous entities based on their anatomical occurrence: (1) The intrahepatic (ICC), 10% of primary liver malignancies, the second most common after hepatocellular carcinoma (HCC); (2) Perihilar (PCC), the most frequent type of CCA (50%-60%); and (3) Distal (DCC) which comprises the 20%-30% of all CCA[1-5]. A rare mixed type of CCA and HCC is hepatocellular (CHC-CCA), arising from transdifferentiated hepatocytes[6,7]. CCA is a rare gastrointestinal cancer (3%), however, it exhibits a noticeably increased incidence in the last decades in Western countries (0.3-6 per 10people). It is characterized by a late diagnostic type which contributes to a high mortality rate (1-6 per 10⁵ people) and a worrisome prognosis[8,9]. The highest incidence of CCA is reported in Southeast Asia, especially in Northeast Thailand (85 per 10⁵ people) based on Age-standardized global incidence rates[10]. Except for the geographical variations that imply an interactive relationship between genetic and local environmental risk factors, CCA exhibits gender disparity with a slight male predominance (1.5 fold higher), mostly in the 5th decade of life[4,5,10], as well as racial variation based on karyotyping studies [11,12]. In endemic areas, a well-documented risk factor is the contamination with liver fluke, larvae of *Opisthorchis viverrini*, and *Clonorchis sinensis* via food consumption, and occupational exposure aflatoxins, asbestos and plutonium manual-labor and industrial work[13,14]. The majority of CCA cases in the Western world are not related to any obvious predisposing factor[13,15,16]. However, primary sclerosing cholangitis (PSC) is the most reported risk factor[17]. Pathologies related to chronic biliary inflammation account for risk factors like hepatobiliary lithiasis, chronic pancreatitis, fibropolycystic liver disease, non-alcoholic fatty liver disease (NAFLD), cirrhosis, as well as, Hepatitis B and C, viral infections, which are strongly associated with iCCA occurrence[18]. Metabolic diseases like diabetes mellitus type2 (T2DM), obesity, hypertension, as well as other inflammatory diseases may also contribute to the disease[19, 20].

Cholangiocarcinogenesis is a multistep event, resulted from deregulated signaling pathways and genomic aberrations[2,21]. Chronic biliary inflammation leads to the proinflammatory cytokine overexpression, like interleukin-6 (IL6), which has the role of growth factor in CCA[22,23]. FGFR gene fusion with MGEA5, TACC3, BICC1, PPHLN1 and ROS is reported, consisting of therapeutic targets[10,24-26]. A variety of mutations have been reported, like KRAS, TP53, RNF43, ROBO2, CDKN2A MLL3, SMAD4, ARID1A, and a recently reported in IDH, which also composes a druggable target[10,27]. KRAS and Tp53 mutations are associated with an aggressive behaviour of tumours and poor prognosis [E], while the latter is frequently coexisting with viral hepatitis B infection[28,29]. Extrahepatic CCA, are frequently associated with ERBB2, ELF3 mutations and PRKACA-PRKACB fusions, while iCCA with IDH1/2, BRAF, ARID1A and FGFR gene fusions[30]. Epigenetic and microRNAs deregulation, are also reported. The former is frequently resulted by the mutation of MLL2, ARID1A and IDH[4,12,31], involved in chromatin remodeling and DNA methylation[3,32,33]. microRNAs up or down-regulation is closely involved in cell cycle function, including autophagy, as well as in invasion, metastasis and chemoresistance[34,35], while they constitute biomarkers for survival and prognosis prediction, especially miR-10b, miR-22 and miR-551b[10,36-38].

Autophagy is a multiphasic, homeostatic, self-degenerative cellular mechanism by which non-functional, clustered or mutant proteins and impaired organelles such as Endoplasmic reticulum, peroxisomes or mitochondria, are insulated into vesicles, which are further fused with lysosomes for the degeneration process[39]. Autophagy appears to have a dual role in cancer, either promotes or suppress carcinogenesis. This peculiar capacity has created new therapeutic strategies for cancer *via* interfering in autophagy steps[40]. Despite the fact that autophagy's regulatory mechanism on tumors is still examined, many studies demonstrate propitious results of its therapeutic potency, especially in combination with other chemotherapeutic agents[41].

Based on several preclinical studies, disturbances in autophagy regulation are closely related to carcinogenesis in cholangiocytes, as well as with metastasis and dismal outcomes, while it can act as a potent anti-cancer drug target[42].

This review gathers information from the current clinical and preclinical research data, about autophagy modulation in CCA and the therapeutic strategies for this highly invasive malignancy.

MAIN ROLE OF AUTOPHAGY IN CANCER BIOLOGY

Autophagy (previously described as Macroautophagy) ensures cellular survival under stressful conditions[40]. Other less described entities of autophagy are: Microautophagy, which includes engulfment of intracellular components *via* the invagination of cell-membrane and fusion with lysosomes, as well as chaperon-mediated autophagy direct translocation of the targeted protein towards lysosomes for the degradation process[39]. In **Figure 1**, the main steps of autophagy are presented.

Despite the fact that it is a physiological mechanism, it has a dual role (as it was mentioned before), either as a tumour suppressor or promoter of tumorigenesis and metastasis[43,44]. This complex procedure includes a series of steps in order to allow the engulfment of the cellular organelles by vesicles, the formation and the expansion of phagophore, the maturation into autophagosome and the fusion of the latter with the lysosome, with the formation of autolysosome, which is responsible for the degradation and recycling of the organelles[39]. The first step of the mechanism (induction) is initiated, by the inactivation of mammalian target of rapamycin (mTOR), allowing the activation of Unc-51-like kinase1 complex (ULK1) and the cargo selection and engulfment by vacuoles. The second step (nucleation), includes the activation and phosphorylation of activated class III PI3K complex by ULK1, with the formation of PI3K -Beclin-1 complex[39]. In the third step, phagophore starts to expand *via* membrane elongation, which includes two conjugations of ATG5-ATG12 complex with ATG16 and the LC3I (soluble-form) to lipid phosphatidylethanolamine (PE), with the former recruiting more cargo for the phagophore expansion and the latter resulting into LC3II (lipid form), which locates in autophagosome-membrane for the binding of degradation-products[45]. The fourth step includes the formation of autolysosome (fusion of the autophagosome with lysosome) and the fifth, the degradation of cargo and the recycling of the products[39,46]. Autophagy is closely related to the tumour microenvironment (TME), exhibiting a protective role *via* the degradation of damaged cargo and the inhibition of tumour growth in early malignant stages[45], like damaged mitochondria, a major source of mutagenic reactive-oxygen species (ROS)[47-49]. In the late stages, it is used by the cancer cells for their adaptation and survival, in extreme micro-environmental conditions, like hypoxia and starvation, having the role of tumour growth promoter[42,45,50]. All the steps can be targeted in anti-cancer therapy *via* the induction of an autophagiccytoprotective mechanism, which can further reduce the chemo-resistance and induce cancer cell death[40,51].

ROLE OF AUTOPHAGY IN CHOLANGIOCARCINOMA

CCA is a highly diversified group of malignancies that exhibit various risk factors and an aberrant epigenetic and genetic landscape[2]. The well-established therapeutic strategies include surgical tumor resection, chemotherapy regimens, as well as locoregional therapies. Only a limited portion of patients (1/3) are eligible for tumor resection at the diagnostic time, which are further receive adjuvant chemotherapy, including either gemcitabine, cisplatin, or 5-fluorouracil (5-FU). However, they cannot put a halt to tumor recurrence and resistance. Due to the highly aggressive behavior of these malignancies, the majority of patients are diagnosed when the metastasis already occurs, or the resection is unfeasible. Palliative care is reserved for these cases, in-

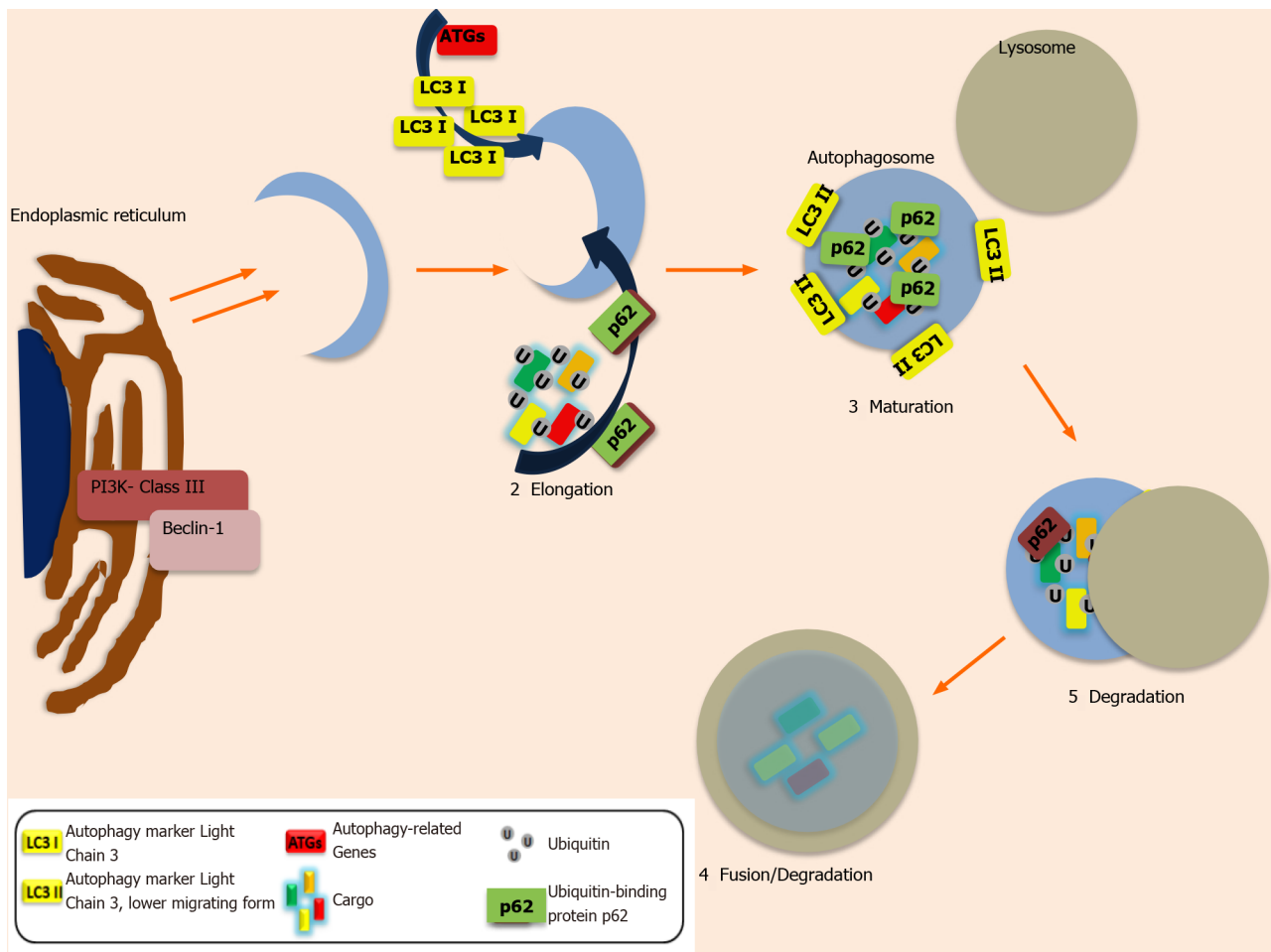


Figure 1 The stages of autophagosome formation. The autophagy process includes five distinct steps: initiation, elongation, maturation, fusion and degradation. In the first step or initiation (1), the double-membrane structure, the Phagophore, is formed after activation of PI3K-classIII – Beclin-1 complex in endoplasmic reticulum. Elongation (2) is the second step where the new-formed phagophore begin to enclose Ubiquitin-labeled cytosolic cargos such as proteins. A plethora of proteins such as LC3 (LC3-I is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate or LC3-II, responsible for the autophagosomal membrane structure), Tags (Autophagy-related genes) and p62 (an adaptor protein responsible for the docking of cargoes) have a key role in the Maturation (3) step where the Autophagosome has already formed. In the fourth step or Fusion/degradation (4) step, the Autophagosome is fused with a Lysosome in order to create the autolysosomes wherein the degradation step (5) the cytosolic cargos are digested from lysosomal enzymes.

cluding the combination of chemotherapeutic agents as gemcitabine and cisplatin, which nevertheless exhibit limited benefits[52,53]. In unsuccessful treatment cases with the former combination, another regimen is reserved, based on fluoropyrimidine [2].

Genetic and epigenetic information, as well as the knowledge of the molecular pathways in CCA, which contribute to tumor resistance, relapse, as well as metastatic behavior, open up more therapeutic approaches *via* the usage of molecular agents, although with moderate overall survival enhancement[1,54,55]. Genomic profiling of iCCA sub-classifies it into: (i) inflammatory and (ii) proliferative classes. In the former, the activation of inflammatory pathways mainly occurs, while on the latter, the activation of oncogenes demonstrates a more worrisome prognosis[12].

The heterogeneity of CCAs subtypes is also demonstrated by Next-generation sequencing analysis, which indicates different genetic mutations based on CCA's anatomical location. (iCCA *vs* extrahepatic: pCCA and dCCA). RAS mutation is more frequently exhibited in CCA, particularly in dCCA[56], however, there is a subclass of CCA, without exhibiting it. There is also emerging evidence of gene FGFR2 fusions involvement in cholangiocarcinogenesis, based on exome sequencing analysis[57]. Aberrations are also identified in the epigenetic level of gene regulation, such as histone modification, DNA hypermethylation and microRNAs (miRNAs) dysregulation, all implied in CCA Tumorigenesis[34].

As alluded to previously, a better understanding of the molecular, genomic and epigenetic affected pathways driving to CCA development and progression could give rise to new and improved generations of therapeutic approaches based on patient-

stratification. Major factors implicated in CCA establishment are chronic biliary inflammation, ductal obstruction with cholestasis and bile duct injury[58]. As a consequence of chronic inflammation, proinflammatory cytokines' overexpression occurs (TNF, IL-6, endotoxins). Persistent secretion of IL-6 by inflamed cholangiocytes and immune cells contributes to cancer establishment and progress. IL-6 oversecretion, induces nitric oxide production (*via* nitric oxide synthase), which is implied in DNA oxidation and damage[59], as well as stimulates the secretion of cyclic oxygenase (COX)-2-mediated prostaglandin, which promotes angiogenesis and disrupts the programmed cell death[60].

Autophagy has a crucial role in inflammation; however, their correlation is still being researched[44]. A large number of signaling pathways are involved in chronic inflammation, during cancer establishment, which influences the process of autophagy. An example of this interconnection is the persistent overexpression of IL6 by lung cells, as a response to arsenic exposure, that down-regulates autophagy and promotes malignant transformation[61]. IL-6 up-regulation influences the STAT3 signalling pathway *via* the inhibition of the Beclin1-Bcl2 complex, which further enables an IL-6-dependent transformation. On the contrary, Beclin1 over-stimulation enables the blockage of this transformation[61]. The above interrelation between IL-6-dependent transformation and autophagy during tumorigenesis could open up treatment opportunities in inflammatory-type iCCA. Additionally, many studies demonstrate the correlation of different pro-inflammatory signaling pathways with autophagy and stress[42].

Based on studies, many genetic mutations have been reported, implied in CCA. Harboring mutant KRAS has been identified in 40% of CCAs, particularly in dCCA with dismal outcomes[56]. Moreover, it is also related to lymphatic dissemination, lower long-term OS and higher grade, as was demonstrated in a study with a limited number of patients with iCCA and mutant KRAS gene (7.4%)[62]. In addition, based on an animal model study, concomitant mutations of KRAS and P53 are related to worse overall survival and malignant transformation in murine[63], while they constitute the most frequently reported genetic modifications[56,64]. The iCCA in murine demonstrates similar morphopathological characteristics with humans and presents an upregulation of autophagy mechanism, contributing to tumor development. The utilization of chloroquine (CQ) ceased tumor growth *via* the inhibition of the mechanism, resulting in the accumulation of LC3-II[42]. Human iCCA, with KRAS and P53 alterations, exposed as well increased mechanism of autophagy, compared with iCCA without them and the tumor progression was similarly inhibited *via* the use of CQ[42,63].

The development of KRAS axis inhibitors, such as selumetinib, opens up new therapeutic strategies, which potentially could be enhanced *via* the addition of autophagy modulators[1,65]. Mutations in MET lead to STAT modulation, Akt/PI3K and MAPK signaling pathways and are associated with aggressiveness, higher tumor stage, and reduced survival[66,67].

Furthermore, c-MET inhibition is related to an increased level of autophagy, as it was demonstrated in lung cancer[68]. Similarly, mutant EGFR and ERBB genes are associated as well with poor outcome and invasiveness[69,70]. In many cancers, treated with inhibitors of tyrosine kinase, autophagy acts as a tumor suppressor[71]. The combination of autophagy and tyrosine kinase inhibitors could potentially improve the treatment results. Moreover, the fusion of FGFR2 genes is demonstrated in CCA[72], and they are correlated with decreased autophagy levels, leading to tumorigenesis. Inhibition of the above gene induces autophagy as a tumor suppressor mechanism in breast and lung malignancies, and its effect can be enhanced with the combination of autophagy inhibitors[73,74]. All the above data support that the combination of these inhibitors could potentially increase the therapeutic potential in CCA. Alteration in the SMAD4 gene is mainly identified in dCCA[75] and in pancreatic malignancy, in which increased autophagy is associated with resistance to radiotherapy[76]. Similarly, inhibition of autophagy could also be beneficial to this type of cancer.

In the initial phase of cholangiocarcinogenesis, Adenomatous Polyposis Coli (APC) mutation has been also reported[77], with the altered mechanism of autophagy[78] and during the establishment of cancer models[79]. Aberrations in the epigenetic level, such as histone modification, DNA hypermethylation, and miRNAs deregulation, are crucial for CCA establishment and development[80] while modulating the autophagy process[81], as well. The expression and the characteristics of the cilium are influenced by the increased expression of histone deacetylase 6 (HDAC6), which reduces its length and increases its proliferation. Inhibition of HDAC6 is correlated with reduced tumor progression and restoration of cilia[82,83]. Suppression of autophagy contri-

butes to the effects of HDAC6 inhibition in many cancers such as neuroblastoma, colorectal and multiple myeloma[84].

Aggressiveness and dismal outcome of iCCA, are also reported in cases of modified HDAC1 expression[85]. Significant autophagy regulators are the methylations of histone, which decelerate it[86]. Inactivation of tumor suppressors, caused by DNA methylation, is reported in cholangiocarcinogenesis. DNA hypermethylation of IDH1/2, is identified in some iCCA cases (10%), which leads to deregulation of cellular functions, such as their differentiation[87,88]. Mutation of IDH, identified in gliomas, demonstrates the interconnection of autophagy suppression and methylations of histone[81,86], which open up therapeutic opportunities *via* autophagy inhibitors[89]. Deregulation of many non-coding RNA sequences, such as miR-21, miR-29, miR-141 and others, present either up or down-regulation and they constitute biomarkers for tumor progression, invasion, cancer cell-death and chemoresistance in CCA[90,91]. Autophagy and its components, such as autophagy-associated proteins (ATG4, ATG9), beclin1, LC3 and ULK2, are also modulated *via* miRNAs[92,93]. Induction of autophagy, *via* the action of miR-124, resulted in an altered STAT3 signaling pathway, as it was reported[94].

Autophagy modulators in combination with immunotherapy, targeted therapies and chemotherapy are positioning as a promising strategy to increase therapeutic benefits for cancer patients. Current treatment options for patients with CCA are limited to chemotherapy, thus, combinatorial scheme including autophagy modulators could offer an opportunity to increase survival of patients with CCA. Autophagy inhibition such as Hydroxy-chloroquine (HCQ) alters the mechanism of resistance and could potentially decrease CCA metastatic potential; therefore, clinical results of this study would be of great help for further design of novel therapeutic approaches involving autophagy inhibitors in CCA. Recent studies revealed the potential of the well-known autophagy marker, Beclin-1, as a prognostic factor in different cancers including CCA. It has emphasized the necessity to combine Beclin-1 expression with other autophagy-related proteins such as Bcl-2 family proteins Bcl-xL and BNIP3, HIF-1 α , PI3KC3 or ATGs to increase its clinical value for patients with CCA.

TARGETING AUTOPHAGY—A PUTATIVE THERAPEUTIC OPTION

Autophagy activators and cancer therapy

Many studies demonstrate the correlation of autophagy mechanism with the microenvironment of tumors and the antitumor immune response, in many cancers, including CRC. Major histocompatibility complex (MCH) I/II Ag presentation is closely regulated by autophagy mechanism, as well as the cellular apoptosis. The multi-roles of autophagy gave the opportunity for the development of antitumor agents that induce this mechanism. Notable activators are Rapamycin and its analog-like, deforolimus, rapalogs like temsirolimus and everolimus and mTOR inhibitors, which activate the mechanism of autophagy[95].

More particularly, it is demonstrated that therapy with Rapamycin intensifies radiotherapy effects on A549 malignant lung cells *via* autophagy activation and by expressing a dilatory effect on genome damage repairing[96]. Rapalogs, like everolimus, have been indicated that suppress the progression and the growth of malignant endometrial cells, especially when Paclitaxel is added to the therapeutic scheme[97, 98]. Both of the above autophagy activators can be added to anti-cancer therapeutic strategies, with another kind of antitumor medication. However, their use in clinical practice should be further examined[97].

Furthermore, it is reported that another anti-proliferative agent, that induces autophagy mechanism is the well-known metformin, which directs inhibition of autophagy, or *via* blocking beclin-1. Moreover, it is reported that metformin induces autophagy mechanism in the case of adenocarcinoma in the lung, as well as cell apoptosis *via* increasing tumor necrosis factor (TNF), the so-called TNF-Related-Apoptosis-Inducing Ligand (TRAIL), apoptosis[99]. In breast cancer therapy, without BRCA1 mutation, metformin is combined with another autophagy inhibitor, spautin-1, which sensitizes these tumors, for the mitochondrial-targeted disruptors. In this case, the combination of an autophagy activator and inhibitor, like metformin and spautin-1, responsively can modify the function of mitochondria differently, resulting in reducing the cell life span[100].

Induction of autophagy can be achieved *via* another agent, like Obatoclast, commonly reported in Hematologic malignant diseases[101]. This agent aims at the Bcl-2 protein family, which is closely associated with cell-apoptosis at the mitochondrion,

while is also influencing autophagosome membranes *via* necrosome congregation, resulting in necroptosis[45,102].

Alkaloids are identified as another group of autophagy inducers in malignancies [103]. Some of them are liensinine, isoliensinine and cepharanthine[48], which target AMPK phosphorylation and mTOR blockage. These agents have been utilized in cases of MEFs, in which we are presenting resistance in the cell-apoptosis mechanism[102].

In addition to the well-established antioxidant function of omega-3 polyunsaturated fatty acids (ω -3 PUFAs)[104], it has been shown that these safe natural compounds can induce 15-hydroxyprostaglandin dehydrogenase (15-PGDH) leading to inactivation of prostaglandin E2 (PGE2) that is known to drive human cholangiocarcinoma[105]. The latter, combined with the fact that ω -3 PUFAs induce autophagy-mediated cell death in cancer cells support the use of ω -3 PUFAs as non-toxic adjuvant therapeutic agents for the treatment of human cholangiocarcinoma[106].

Autophagy inhibitors and cancer therapy

A wide range of studies about autophagy and its influence on the efficacy of other cancer treatments, such as chemotherapy, radiotherapy, or immunotherapy, has been reported in the last years[107]. These studies focused on this mechanism, used by cancer cells for their energy, metabolic regulation and survival[40,108]. The dual role of autophagy, either as tumor promoter, or tumor suppressor, opened up new opportunities for anti-cancer treatment *via* autophagy-inhibitors.

The most widely known inhibitors are Chloroquine (CQ) and hydroxychloroquine (HCQ), which impede the fusion of autophagosomes with the lysosomes. Their efficiency as anti-cancer therapy has been evaluated in a variety of malignancies[43]. However, their clinical significance as monotherapy was limited due to their non-persistent inhibition[109]. The combination of other cancer therapies demonstrated better therapeutic results[41,110], such as the combination of HCQ with gemcitabine in the case of pancreatic adenocarcinoma, which resulted in a significant reduction of tumor marker 19-9 (60%)[111].

Moreover, the combination of immunotherapy and autophagy inhibitors, such as CQ with IL-2, has been proven beneficial with reduced toxicity, such as in animal-model studies of murine with hepatic metastasis. Furthermore, it was demonstrated that this dual therapeutic strategy, has a better survival rate in the long term as well as a better response by immune cells[107]. However, the response to CQ derivatives, including HCQ is variable, due to the lack of specificity, which leads to the interaction with other medical substances and the modification of tumor properties, like pH[109, 112]. Additionally, the efficacy of autophagy inhibition by the above agents, cannot be evaluated due to the absence of biomarkers, which is a significant limitation in the clinical practice. This is the reason that new inhibitors with higher specificity have been developed[41,107].

There are some new, efficacious inhibitors, such as Lys05, also described as dimeric chloroquine, which is well-tolerated and exhibits a strong antitumor action *via* the modification of lysosome enzymes[112]. Another one is SAR405, an inhibitor of kinase, which is more specific and targets Vps18 and Vps34 vacuole proteins, which have a crucial role in the initiation of autophagy-mechanism. More particularly, the initiation step is regulated by Beclin-1 and Vps34, whereas Vps34 suppression, results in the impairment of lysosomal and vesicular transport[113]. Initiation-step can also be targeted, *via* the use of Beclin-1 inhibitors, which suppress the tumor progression, intensify the antitumor activity of Natural Killer (NK) cells and induce CCL5 cytokine overexpression by cancer cells, a condition that influences the transporting of NK cells towards the malignant tumors[107].

Based on studies in various malignancies, the inhibition of ULK1 (Unc-51 Like kinase-1) by SBI-0206965, has great antitumor potential due to its higher selectivity, resulting from the suppression of ULK1-phosphorylations[114]. Some other agents, are DCMI including desmethylclomipramine, verteporfin and clomipramine, impeding the fusion of autophagosome with lysosomes or acidification lysosomes[115], whereas the addition of DCMI to doxorubicin, *in vitro*, demonstrated higher effectiveness of the latter[116]. Moreover, spautin-1, is another effective inhibitor, which impedes the initiation step of autophagy, by suppressing the crucial for the process ubiquitin-specific peptidases USP13, USP10, as well as Beclin-1, which is deubiquitinated in Vps34 complex[99].

The microenvironment of tumors, is closely related to the autophagy mechanism, as well as with the antitumor immune response. According to this fact, the inhibition of autophagy could have a negative impact on the adaptive immune response against malignant tumors. However, Starobinets et al[117] in 2016 confuted this hypothesis by proving that inhibition of autophagy does not have an adverse impact on the adaptive

Table 1 Small molecules able to induce autophagic activity

Agents	Mechanism of action
GDC-0941	Inhibitor of class I PI3K
GDC-0980	Dual inhibitor of PI3K and mTORC1
Everolimus	mTORC1 inhibitor
Temsirolimus	mTORC1 inhibitor
Rapamycin	mTORC1 inhibitor
Tat-beclin 1 peptide	Releases beclin-1 into cytoplasm-regulate autophagosome formation
Metformin	AMPK activator
Fluspirilene	Antagonists of L-type Ca ²⁺ channels
Loperamide	Antagonists of L-type Ca ²⁺ channels
Amiodarone	Antagonists of L-type Ca ²⁺ channels
Isoliensinine	Natural alkaloid
Cepharanthine	Natural alkaloid

mTORC1: Mammalian target of rapamycin complex 1; AMPK: 5' AMP-activated protein kinase; PI3K: Phosphatidylinositol 3-kinases; AKT: Protein kinase B; Beclin-1: The mammalian ortholog of the yeast autophagy-related gene 6 (Atg6).

Table 2 Small molecules able to inhibit autophagic activity

Agents	Mechanism of action
3-Methyladenine (3-MA)	Inhibitor of class III PI3K
LY294002	PI3K inhibitor
Wortmannin	PI3K inhibitor
SB202190	Cross-inhibition of the PI3K/mTOR and MAPKs pathway
MHY1485	Activator of mTOR
Azithromycin	Inhibitor of v-ATPase, inhibition of lysosomal acidification
Bafilomycin A1	Inhibitor of v-ATPase, inhibition of lysosomal acidification
Concanamycin A	Inhibitor of v-ATPase, inhibition of lysosomal acidification
Chloroquine (CQ)	Autophagosome-lysosome fusion
Hydroxy-chloroquine (HCQ)	Autophagosome-lysosome fusion
Clomipramine	Alter acidification of lysosomes
Verteporfin	Alter acidification of lysosomes
Paclitaxel	Microtubule stabilizer- inhibit phosphorylation of VPS34 at T159
Spain-1	Inhibits the activity of ubiquitin-specific peptidases, USP10 and USP13
Monensin	Inhibit autophagosome-lysosome fusion

PI3K: Phosphatidylinositol 3-kinases; mTORC1: Mammalian target of rapamycin complex 1; AMPK: 5' AMP-activated protein kinase; VPS: Vacuolar protein sorting; ATG: Autophagy-related proteins; USP: Ubiquitin-specific protease.

anti-cancer immunity in melanoma and breast cancers. For this reason, inhibitors of autophagy can be combined with another chemotherapeutic agent without negatively influencing the antitumor response of T cells towards malignant tumors[117].

Herein, we provide two summarized tables about small agents that inhibit or activate autophagy. Autophagy manipulation is already used in research to develop putative chemotherapeutic strategies with a plethora of agents for different types of cancer (Tables 1 and 2).

CONCLUSION

It is a well-established knowledge that autophagy's prominent role is strongly correlated with the degradation of dysfunctional cellular proteins and organelles. A plethora of studies in the field of cancer research and autophagy highlights the controversial role of this mechanism either as tumor suppressor or promoter mechanism in different types of cancer, including CCA. Several *in vitro* and *in vivo* studies in CCAs have associated autophagy with cholangiocarcinogenesis development and progression. Furthermore, autophagy markers such as Beclin-1 and LC3 and/or autophagy-associated proteins appeared to associate with a different CCAs stage through miRNAs expression. Current treatment options for CCA are limited to chemotherapy with limited efficacy on CCA patients. Agents that modulate autophagy in different steps in combination with the current chemotherapeutic drugs are proposed as a promising therapeutic strategy in order to increase the beneficial effect of the therapeutic expectancy of cancer patients.

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