THE INHIBITION OF AUTOPHAGY AND HEDGEHOG PATHWAY LEADS TO A DECREASE IN THE PROLIFERATION OF CHOLANGIOCARCINOMA

A MASTER'S THESIS

By

Nihan Aktaş

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THE INHIBITION OF AUTOPHAGY AND HEDGEHOG
PATHWAY LEADS TO A DECREASE IN THE
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// ABSTRACT

THE INHIBITION OF AUTOPHAGY AND HEDGEHOG PATHWAY LEADS TO A DECREASE IN THE PROLIFERATION OF CHOLANGIOCARCINOMA

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June, 2019

Cholangiocarcinoma (CCA) is the second most common liver cancer type. The median survival rate of CCA patients is really low. Aberrant signaling pathways such as PI3K/AKT/mTOR pathway could be main drivers in CCA pathogenesis. Hedgehog (Hh) pathway is also dysregulated in several carcinomas including CCA. It regulates and crosstalks with autophagy, which is a lysosomal degradation process. There is no study showing the crosstalk between Hh pathway and autophagy in the context of CCA. Since both autophagy and Hh pathways are dysregulated in CCA, better understanding of how they crosstalk with each other and contribute to CCA pathogenesis is important. Considering this crosstalk between Hh pathway and autophagy, we conducted a combination treatment comprising Hh and autophagy pathway inhibitors in EGI-1 and TFK-1 CCA cell lines. In our study, we firstly checked anti-proliferative effects of Hh pathway inhibitor, GANT61, and different autophagy blockers using MTT and Annexin V assay and cell cycle analysis. After determination of IC30 of GANT61 (15 uM), chloroquine (25 uM for TFK-1 and 50 uM for EGI-1), and nocodazole (0.2 uM for EGI-1 and 0.4 uM for TFK-1), we conducted combination experiments. When we inhibit Hh pathway with targeting different steps of autophagy, we observed that proliferation of both EGI-1 and TFK-1 cells decreased compared to single treatments. After that, we checked the expression of autophagy-related LC3B protein and Akt, a negative regulator of autophagy, using western blotting after single treatments and combinational treatments. Based on the change in LC3B and Akt expression, we also concluded that, inhibition of autophagy with Hh pathway either induce or inhibit autophagy depends on the administered treatments. This study highlights the importance of deciphering the exact mechanisms that control autophagy in CCA, thus leading to better treatment.

Keywords: Hedgehog, autophagy, combination, cholangiocarcinoma

ÖZET

KOLANJİYOKARSİNOMA PROLİFERASYONUNUN OTOFAJİ VE HEDGEHOG SİNYAL YOLAKLARININ İNHİBİSYONU İLE AZALTILMASI

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Kolanjiokarsinoma (KKA) ikinci en yaygın karaciğer kanseri türü olarak bilinmektedir. KKA hastalarında hayatta kalma oranları olduça düşüktür. KKA patogenezinde PI3K/AKT/mTOR gibi sinyal yolaklarındaki bozuklukların temel mekanizmalardan biri olduğu düşünülmektedir. Hedgehog sinyal yolağının da KKA dahil birçok karsinogenezde bozulduğu daha önceki çalışmalarla gösterilmiştir. Hh sinyal yolağının lizozomal yıkım sürecinde rol alan otofaji sinyal yolağı ile de etkileşim halinde olduğu bilinmektedir. KKA patogenezinde Hh ve otofaji yolaklarındaki etkileşimin rolü bilinmemektedir. Bu yolaklarının KKA'da deregule edildiği de düşünülerek, bu iki sinyal yolağı arasındaki etkileşimin ve bu etkileşimin KKA patogenezindeki rolünün anlaşılması oldukça önemlidir. Bu çalışmada Hh ve otofaji yolakları arasındaki etkileşimi göz önünde bulundurarak EGI-1 ve TFK-1 hücrelerine otofaji ve Hh sinyal yolağı inhibitörlerini içeren bir kombinasyon muamelesi yapılmıştır. Çalışmamızda ilk olarak bir Hh inhibitörü olan GANT61'in ve farklı otofaji inhibitörlerinin antiproliferatif etkileri MTT, Annexin V ve hücre siklusu analizi ile ortaya konulmuştur. GANT61 (15 uM), chloroquine (TFK-1 için 25 uM ve EGI-1 için 50 uM), ve nocodazole (EGI-1 için 0.2 uM ve TFK-1 için 0.4 uM)'ün IC30 konsantrasyonları belirlendikten sonra, kombinasyon denemeleri yapılmıştır. EGI-1 ve TFK-1 hücreleri Hh inhibitörü ve otofaji yolağının farklı basamaklarını inhibe eden inhibitörlerle muamele edildiğinde, her iki kombinasyonda da hücre proliferasyonunun azaldığı gözlemlenmiştir.Hem tek bir inhibitörle hem de kombinasyonla muamele edilmiş hücrelerin otofaji bağlantılı LC3B ve otofajinin nefatif regülatörü olan Akt ifadesi western blot yöntemi ile incelenmiştir.LC3B ifadesi, Hh inhibisyonu ile birlikte otofajide hangi basamağın inhibe edildiğine bağlı olarak kombinasyonun otofajiyi tetiklediği ya da baskıladığını ortaya koymaktadır. Bu veriler CCA'da otofajiyi kontrol eden mekanizmaları ortaya koyarak, tedavisi için umut vadetmektedir.

Anahtar kelimeler: hedgehog, otofaji, kombinasyon, kolanjiokarsinoma

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1. Introduction

Cholangiocarcinoma (CCA) is one of the most common liver cancer types. The median 5-year survival rate of CCA patients is ranging between 15% and 30% [71]. It has been shown that aberrant signaling pathways such as PI3K/AKT/mTOR and Notch could be the main drivers of the CCA pathogenesis [35,177, 187, 253]. Hedgehog (Hh) pathway is one of these pathways, which has role in tissue differentiation and stem cell maintenance (Cilloni & Saglio, 2012; Zuo et al., 2015). Aberrant activation of this pathway has been observed in several carcinomas including cholangiocarcinoma (El Khatib et al., 2013; Zuo et al., 2015). Hh pathway is known to crosstalk and regulate autophagy, which is a lysosomal degradation process involved in the regulation of several metabolic processes, organelle turnover and cellular homeostasis [1-5]. Although some studies indicated that aberrant Hh signaling pathway and autophagy activation is observed in chemoresistance and, there is no study showing the crosstalk between Hh pathway and autophagy in the context of CCA [2,6]. There is only one study which shows expression of Hh pathway components and markers of autophagy in the Primary sclerosing cholangitis (PSC) patients but the link between Hh pathway and autophagy pathway has not been studied until now [7].

1.1. Anatomy of Liver and Biliary Tract

For a full understanding of the development and pathogenesis of CCA, it is essential to understand the anatomy of liver and biliary tract. Liver is an essential organ, playing a role in metabolism, homeostasis and systemic immunity regulation [8,9]. It is composed of parenchymal and non-paranchymal cells including hepatocytes, liver sinusoidal endothelial cells, as well as hepatic stellate cells and resident immune cells. Hepatoblasts are bipotent liver progenitors, which differentiate into hepatocytes and cholangiocytes [10]. While hepatoblasts differentiate into hepatocytes in the liver parenchyma, they give rise to cholangiocytes in the periportal area. For that reason, hepatoblasts require periportal area in order to differentiate into cholangiocytes [11]. Hepatocytes are polarized epithelial cells and they are the most predominant type of cells in the liver [12]. Cholangiocytes are another type of epithelial cells that play a role in supporting and determining biliary secretions [13,14]. While the lineage specification in the developmental stage of liver is studied, differentiation of hepatoblasts in the adult liver is still not properly described. During the differentiation of hepatoblast into

hepatocytes, the hepatocyte growth factor (HGF) receptor, and c-Met are known to be involved but the underlying mechanism is still not known [15]. Notch and Wnt signaling pathways are also shown to be involved in differentiation of hepatoblasts. It is shown that the Notch1 and Notch2 receptors are expressed by jagged 1 interaction during hepatoblast activation in mouse and human biliary diseases, respectively. Notch2 cleavage is triggered when jagged 1 bind to Notch2 and this cleavage cause the translocation of this fragment into the nucleus [16,17]. The Notch signaling pathway is inhibited during hepatocyte regeneration by the ubiquitin ligase Numb and this pathway is also important for differentiation of hepatoblasts into cholangiocytes [17,18].

The biliary system is composed of two different parts, which are intrahepatic and extrahepatic parts. The intrahepatic part creates a network, which is bound by canals of Hering to the bile canaliculi. The extrahepatic duct system includes gallbladder in addition to hepatic, cystic and common bile ducts and transfer the bile from the liver to duodenum [19].

1.2. Primary Liver Cancers

According to the American Cancer Society, incidence of liver cancer has tripled after 1980 and deaths caused by liver cancer have multiplied by around 3% per year since 2000. It has been estimated that in USA, 42.030 new liver cancer cases will be diagnosed in 2019 and around 31.780 of them are expected to die [20].

Primary liver cancer includes several neoplasms, among which are hepatocellular carcinoma (HCC), intrahepatic bile duct cancer (cholangiocarcinoma), hepatoblastoma, angiosarcoma and hemangiosarcoma [20].

1.2.1 Hepatocellular Carcinoma (HCC)

Th incidence rate of HCC has increased in the last 40 years and the mortality rate caused by this disease is still increasing in United States and Canada [21]. American Cancer Society estimates that three-fourths of liver cancer, which are expected to be observed in 2019, will be hepatocellular carcinoma [20]. HCC has been characterized by several growth patterns. The first type of HCC starts as a single tumor and enlarge over time. This type of disease spreads to the other liver part only at later stages. The second type of HCC begins as several small cancer nodules throughout the liver, not

only as a single tumor. This type of HCC is the most common type in US and it has generally been seen people with cirrhosis [20].

The development of HCC is generally triggered by oxidative stress, and inflammation, Hepatisis B Virus (HBV) and Hepatisis C Virus (HCV) infections, overconsumption of alcohol and tobacco products. In addition to that, diabetes and obesity are included in the risk factors for HCC [22-24]. In the underdeveloped countries without sanitation, the most common risk factor for HCC is chronic HBV [25, 26]. In several developed countries, non-alcoholic stearohepatisis (NASH) increases the risks factor that lead to the development of HCC since it induces fibrosis and cirrhosis [26-28]. Additionally, a mycotoxin, aflatoxin B causes mutations in DNA especially the TP53 gene, thus, decreasing the tumor suppressive function of p53 [26, 29]. Normally, the parent molecule of aflatoxin is not harmful but, the cytochrome p450 superfamily members convert it to mutagenic and carcinogenic intermediates [22, 29-33].

1.2.2 Cholangiocarcinoma (CCA)

Cholangiocarcinoma (CCA) is the second most common type of liver cancer after HCC. This disease is an epithelial cell carcinoma, which arises at several locations in the biliary tree and it also presents cholangiocyte differentiation markers [34,35].

1.2.2.1 Epidemiology of CCA

CCA is the second most common hepatic malignancy after HCC [36-39]. The incidence age for CCA is around 70 in most of the world especially in Western countries, and it affects both women and men but it is slightly more common in males compared to females [37, 40, 41]. CCA with its subgroups represents approximately 3% of malignancies in gastrointestinal system [37, 39, 42]. The incidence and mortality rates due to CCA vary depending on the geographical location and the incidence rate of CCA is higher in Eastern compared to Western countries [37].

1.2.2.2 Etiology

Even though the majority of cases of CCA are de novo, there are several other risk factors that trigger the development of CCA [43]. One of these risk factors is hepatobiliary flukes including *Opisthorchis viverrini* and *Clonorchis sinensis*, which are really common in East Asia, where the consumption of pickled, undercooked or raw fishes is high. In East Asia, the percentage of intrahepatic cholangiocarcinoma (iCCA)

is around 85 % of the primitive liver cancer in this area [44-48]. These parasites live in the bile duct and feed on biliary epithelial cells resulting in chronic inflammation, which is associated with an increased risk of CCA [49]. The relationship between hepatobiliary flukes and CCA has also been confirmed by animal studies [50, 51]. Primary sclerosing cholangitis (PSC) is a common disease of chronic cholestatic liver and biliary tract and this disease is one of the well-known risk factors of CCA [52].Hepatolithiasis is another risk factor for CCA, which is characterized by the presence of gallstones in the bile ducts of liver. Hepatolithiasis is more common in Asia than Western countries [53-55]. The strong association between CCA and cholelithiasis especially choledocholithiasis and iCCA have also been revealed in studies conducted in different geographic locations [57-59]. Some malformations in congenital biliary tract including bile duct cysts and Caroli disease have been considered as important risk factor in CCA. These malformations are found in 10-15 % of CCA patient [60-62]. Moreover, increased incidence rates of CCA observed in obesity patient shows the relationship between CCA and metabolic syndrome [63, 64]. Increased leptin levels are also seen in obese patient and it is known to stimulate CCA cell growth [65]. In addition to that, HBV, HCV and cirrhosis are also well known risk factors for CCA [66, 67]. A lot of toxic and environmental factors which are nitrosamine, thorotrast, vinyl chlorides, asbestos, dioxins as well as food contaminations have a role in the development and the pathogenesis of the disease [43]. In addition to these factors, consumption of alcohol and tobacco products has been considered as risk factors for CCA.

The relationship between metabolic syndrome and iCCA development has been revealed. Non-alcoholic fatty liver disease (NAFLD) contributes to NASH progression by cirrhosis. After which, NASH promotes the development of iCCA due to fibrosis [68]. Moreover, the role of hypertension has also been studied as a risk factor but it exact role is still not clear.

1.2.2.3 Classification of CCA

According to anatomical location, CCA is classified as intrahepatic CCA (iCCA), perihilar CCA (pCCA) and distal CCA (dCCA) [69, 70].

a. Intrahepatic Cholangiocarcinoma (iCCA)

Cholangiocarcinoma originating in the smaller bile duct branches of liver is known as intrahepatic cholangiocarcinoma [71]. According to morphology and its

growth pattern, iCCA has been classified into 4 types, which are mass-forming, periductal-infiltrating, superficial spreading and undefined iCCA [35, 72-74]. The superficial spreading and intraductal type of iCCA have been related to the best prognosis. However, periductal and mass-forming types have worse prognosis [35, 75]. In addition to that, mass-forming iCCA represent 78% of iCCA and it is a gray to grayish-white solid carcinoma, which appears as a mass lesion in the hepatic parenchyma. Mass-forming iCCA might be quite larger and fibrosis is really common in this type of iCCA. Periductal-infiltrating iCCA represents around 16% of iCCA. This type of iCCA is characterized by the extension of the carcinoma throughout the portal tracts, infiltrating into the hepatic parenchyma. Moreover, thickening and strictures in the affected bile ducts are observed in this type of iCCA. Lastly, intraductal growth is the most rarely seen type of iCCA, which is 6 % of all iCCA. While iCCAs arising from the intrahepatic small bile ducts or bile stem cells are generally mass-forming, those arising from intrahepatic larger bile ducts could be either periductal-infiltrating or intraductal growth [76-78]. In the previous studies, the poorest prognosis has shown to be in periductal and mass-forming iCCA [35].

b. Distal Cholangiocarcinoma (dCCA)

Distal cholangiocarcinoma (dCCA) is a type of extrahepatic cholangiocarcinoma (eCCA) which start to form outside of the liver. The incidence age in this type of CCA is around 50-70 years [71, 79]. Histologically more than 90% of are well-differentiated [80].

c. Perihilar Cholangiocarcinoma (pCCA)

Like dCCA, perihilar (hilar) cholangiocarcinoma (pCCA) which is also known as Klatskin tumors is a type of extrahepatic CCA (eCCA). It has been classified based on its morphological growth into mass-forming, exophytic and intraductal types. Intraductal pCCA are also classified into subtypes as periductal infiltrating, which is the most common type of pCCA, mass pCCA and nodular pCCA. Intraductal tubulopapillary neoplasm is the most recently found type of pCCA with better prognosis compared to exophytic type of pCCA [35, 81].

1.2.2.4 Diagnosis and Treatment

iCCA is difficult to diagnose and treatment of iCCA is challenging.This carcinoma has been characterized by a malignant mass lesion in non-cirrhotic liver but this intrahepatic lesion solely is not enough to identify this type and further diagnostic steps are required for differentiation [35, 82]. During the diagnosis of iCCA, the probability of the malignancy has increased due to the existence of liver capsule retraction. Also, biliary dilatation in the vicinity of intrahepatic lesion increases the probability of the malignancy. Although positron emission tomography (PET) scan might be a good method to diagnose metastatic disease, most of CCAs are PET negative with ^{18}F-fluorodeoxyglucose [35, 83, 84]. Magnetic resonance imaging (MRI) and computed tomography (CT) scans are correlated with low misdiagnosis compared to contrast-enhanced ultrasound [35, 85]. Carbohydrate antigen 19-9 (CA19-9) has been used as a serum biomarker for diagnosis of iCCA for a long time. However, better biomarkers for iCCA are necessary for better diagnosis and detection because CA19-9 levels also is elevated in different malignancies such as ovarian and colon tumors as well as benign biliary obstruction and cholangitis [35, 86].

Surgical candidacy of patient, biochemical characteristics and size of lesion have been taken into consideration to decide on an appropriate treatment option for iCCA. The tumor burden should be checked by imaging of the abdomen and chest in addition to lymph nodes, which are larger than 2 cm. Curative surgical resection achievements are around 30% in the patient with negative tumor margins which is the border of the tissue resected in surgery and if there is no cancer cells in the margin it is called as negative tumor margin [35, 36]. Poor outcomes have been observed in patients with positive tumour margins, portal hypertension, lymph node metastasis as well as cirrhosis [35, 36, 87]. On the contrary to pCCA, liver transplantation is not a recommended option for iCCA because after transplantation, there is 42-65% risk for recurrence even in the patients with mixed hepatocellular-cholangiocellular carcinomas [35, 88].

Since iCCA is a metastatic disease of liver, locoregional therapy has been considered as a palliative treatment approach but the effectiveness of this approach is still under question. There are also some limitations in the treatment of iCCA with radiofrequency ablation. Moreover, high tumor recurrence rates have been observed

after this process [35, 89]. Although transarterial chemoembolisation (TACE) is not used in the iCCA therapy as a standardized method, it might increase the survival rates of iCCA patients [35, 89-91]. A phase III clinical study have analyzed 410 patients and the researchers observed that the median survival rates of these patients increased from 8.1 months to 11.7 months after receiving gemcitabine alone and cisplatin and gemcitabine combination, respectively. Moreover, 80 of these patients with good outcomes are iCCA which indicates a backbone for further studies in iCCA treatment [92]. In addition to these, radiation therapy is used for iCCA treatment and intra-arterial usage of radioactive ^{90}Y in the radiotherapy has been reported for iCCA [35, 91]. Till now, several studies have focused on targeting molecular aberrations in iCCA but there are no approved targeted therapies for iCCA treatment (Table 1).

Molecular Aberrations	Inhibitors	References
FGFR2 fusion (45 %)	FGFR small molecule kinase inhibitors	[42, 367-370]
IDH 1 /2 (23-28%)	Mutant IDH inhibitors	[169, 371, 372, 42, 170]
BAP1 (9%), ARID1A (36 %)	HDAC inhibitors	[42, 373]
Mcl-1 (16-21%)	Mcl-1 selective inhibitors	[42, 373]
KRAS (11-25%)	MEK inhibitors	[42, 374]
PI3K-AKT-mTOR (4-8 %)	AKT inhibitors, mTOR inhibitors	[42, 375]

Table 1.2.2.4.1 Molecular aberrations and potential inhibitors in iCCA

The main symptoms of dCCA are obstructive jaundice, abdominal pain, as well as cholangitis [94]. Increased cholestatic parameters are also observed in the blood tests of dCCA patients [95, 96].Although different tumor markers including CA19-9 and/or carcinoembryonic antigen (CEA) have been used to diagnose dCCA but specificity and sensitivity of these markers are moderate [97]. Also, extrahepatic bile duct thickening and/or stricture are used for dCCA diagnosis. CT and MRI with magnetic resonance cholangiopancreatography (MRCP) are useful for detection of tumor burden. An important therapeutic tool, ERC, and intraductal ultrasonography aid in diagnosis of dCCA. Moreover, for the proper analyses of lymph node metastasis and vascular structures, endoscopic ultrasound (EUS) with fine needle aspiration (FNA) is useful techniques. There are still doubts about the function of intraductal visualization with cholangioscopy in dCCA diagnosis [95, 96].

Molecular Aberrations	Inhibitors	References
ERBB2/ERBB3 (11-14%)	EGFR inhibitors	[42, 373, 376, 377]
KRAS (58-68%)	MEK inhibitors	[42, 373, 378]
PI3K-AKT-mTOR (4%)	HDAC inhibitors	[375, 379]

Table 1.2.2.4.2 Molecular aberrations and potential inhibitors in dCCA

Size, location and morphology of tumor as well as, involvement of lymph nodes and portal vein and metastasis can be described well if endoscopic ultrasonography and cross-sectional imaging studies are performed carefully. However, the number of imaging technique studies for detection of pCCA and quality of the available techniques are moderate [35, 98, 99]. Magnetic resonance imaging (MRI) and computed tomography (CT) scan techniques are used to detect perihilar mass. Magnetic resonance cholangiography (MRC) provides better description of the extent of the bile duct lesion. Usage of MRI and MRC together improves the sensitivity and accuracy of diagnosis [35,100]. Lymph node fine-needle aspiration, which is a type of biopsy procedure, is another precious method for pCCA diagnosis. Increased CA 19-9 serum levels alone are not effective diagnostic criteria for pCCA, that's why, serum levels of Immunoglobulin G4 (IgG4) should also be checked to obtain IgG4-related cholangiopathy [35,101].

In the treatment of pCCA, liver transplantation together with neoadjuvant chemoradiation is a good option, but most of the patients do not have the necessary criteria, which are 3 cm unresectable tumor or less radial diameter without metastases [35, 102].

If surgery of liver transplantation is not an option for the patient, gemcitabine and cisplatin combination therapy has been taken into consideration in the treatment of pCCA but efficiency of this combination therapy is not significantly higher than gemcitabine alone so there is no standard treatment for pCCA [92]. Metal stents can be a palliative option for pCCA treatment if the life expectancy of the patient is lower than 4-6 months [35,103-105]. Additionally, endoscopic intraductal radiofrequency ablation has been considered as another palliative therapy for pCCA [35, 106].

Molecular Aberrations	Inhibitors	References
EGFR/HER2 (4-25%)	EGFR inhibitors	[42, 373, 381]
Protein kinase A (PKA) pathway (10%)	Small molecule PKA inhibitor	[42, 380]

Table 1.2.2.4.3 Molecular aberrations and potential inhibitors in pCCA

1.2.2.5 Pathogenesis of CCA

There are multiple factors which contributes pathogenesis of CCA. One of them is cancer stem cells (CSCs) in CCA. The correlation between the presence of cancer stem cell markers, which are SOX2, CD133, CD90 and CD13 and poor diagnosis of CCA have been revealed in the previous studies [107-109]. Most of cancer stem cells of CCA express epithelial-mesencymal transition (EMT) markers and contribute to chemoresistance, desmoplasticity as well as aggressiveness of this cancer [40, 110,111]. In the previous studies, the cells expressing CD133 were shown to resist chemotherapy and also, recurrence of tumor has been observed in these cells. In addition to that, the relationship between CD44 and vascular invasion in CCA cells also indicate the importance of stem cells in the pathogenesis of CCA [107]. Some of these stem cell markers, which are leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5), CD133 and epithelial cellular adhesion molecule (EpCAM) are also expressed by normal stem cells localized in liver and biliary tree but chronic damage of liver activates and expand these compartments [112,113]. Additionally, tumor microenvironment (TME) consisting of cancer associated mocarophages (CAMs), cancer associated fibroblasts (CAFs) and vascular cells, which all constitute the cancer stem cell niche, contributes to stemness and resistance to chemotherapy [110,114,40,111]. Inflammation is one of the key factors in CCA pathogenesis [39,112,113]. Inflammation and cholestasis support tumor formation by creating an environment in which several mechanisms are aberrated including DNA repair proteins, enzymes, tumor suppressor genes and proto-oncogenes [115]. Interleukin-6 (IL-6) is one of the key cytokines, which is also a mitogen, involved in CCA pathogenesis and is known to affect proliferation of CCA cells [116,117]. Also, IL-6 inhibits telomere shortening by increasing telomerase activity in CCA cell, which contributes to the escape from cell senescence [117-121]. Crosstalk between IL-6 and other pathways has been shown in the previous studies. IL-6 causes the overexpression of epidermal growth factor receptor (EGFR) and the inhibition of EGFR signaling, which stimulate a decrease in CCA

proliferation [117,122,123]. EGFR is known to increase cyclooxygenase-2 (COX-2) expression, which has role in CCA development by promoting growth and survival of CCA cells [124-127]. Inducible nitric oxide synthase (iNOS) is a mediator of COX-2 induction. iNOS induces COX-2 expression in immortalized CCA, not in nonmalignant cells and this might suggest a relationship between iNOS and COX-2 expression in CCA development [128]. In addition to these factors, overexpression of mitogenic hepatocyte growth factor and its receptor results in sustainable induction of cell growth in CCA [117,129].

A variety of somatic mutations in oncogenes like KRAS, chromatin modifying genes including PBMR1 and BAP1 and tumor suppressor genes like TP53 and SMAD4 has also been identified in CCA. Whereas BAP1 and IDH1/2 mutations have been commonly observed in non-liver fluke associated CCA, TP53 and SMAD4 mutations are more frequent in liver fluke associated CCA [130-133]. Mutations observed in oncogenic pathway components' genes are shown to be found in CCA [132,134]. Additionally, gene fusions have been identified in CCA. Fibroblast growth factor 2 (FGFR2) fusion protein formations, found in up to 45% of iCCA, is an example of these fusions [132, 136, 137]. Overexpression of IL-6R, c-MET and EGFR, transactivation between receptors (MET/EGFR and COX-2/IL-6), and inactivation of feedback mechanisms causes dysregulation of apoptosis, cell cycle and proliferation [132].

Many hormones and growth factors have anti-apoptotic effect on neoplastic cholangiocytes and promote cell proliferation [40,137].Overexpression of estrogen receptors, which are estrogen receptor α (ER-α) and estrogen receptor-β (ER-β), is detected in CCA and renders CCA sensitive to estrogen [40,138]. It is shown that activation of ER-α promote cell proliferation in CCA, whereas ER-β stimulation results in anti-neoplasticity by the induction of apoptosis [40, 139]. The inhibition of CCA growth has been demonstrated by the administration of tamoxifen and KB9520, which are ER antagonist and ER-β-selective agonist, respectively [40,140]. Additionally, stimulation of VEGF and IL-6 by estrogens has been shown in CCA studies [40,141,142]. Estrogens modulate CCA growth and development by cooperating with insulin-like growth factor 1 and insulin-like growth factor 1 receptor [143].

Unlike HCC, CCA has been characterized by a desmoplastic TMEtme, which includes several types of cells and some extracellular components supporting and

promoting the progression of the tumor [144,145]. The patient survival can be estimated by checking the tumor-infiltrating cell composition of CCA. Poor prognosis of CCA patients, gemcitabine resistance and decrease of survival are associated with the increase of the innate immune response components comprising the tumor associated neutrophils (TANs) and TAs in addition to the lack of adaptive immune response, which includes CD4+ and CD8+ [145,146].

Tumor associated macrophages (TAMs) are known to be a key component of TME and they are involved in supporting the initiation and growth of tumor with the help of CSCs [145, [145,148]. Polarization of macrophages to M2 state, which is tumor promoting has been related to poor prognosis and metastasis of CCA [40,148,149]. Additionally, TAMs are known to modulate TME and support EMT as well as metastasis and tumor growth by secreting tumor promoting factors such as, tumor necrosis α (TNF-α), Interleukin 10 (IL10), IL-6, transforming growth factor β (TGF-β) and vascular endothelial growth factor A (VEGFA) [145,150]. C-X-C Motif Chemokine Ligand 5 (CXCL5) signaling controls neutrophil recruitment in CCA inducing migration by phosphoinositide 3-kinases/AKT (PI3K-AKT) and mitogen-activated protein kinases (MAPK) pathways [145,151].

TME is known to inhibit acquired immune response, which includes activation of cytotoxic response with the help of CD8+ and CD4+ T cells thorough limiting infiltration of T cells, decreasing antigen presentation and leading to activation of lymphocyte. Although tumor infiltrating lymphocytes (TILs) including CD4+, CD8+ and FoxP3+ has been related to good outcomes in CCA, TILs have been involved in TME modulation, which is correlated with chemoresistance in CCA and escaping the immune system [145,146].

TNF-α, which is a pro-inflammatory cytokine promoting apoptotic cell death has been considered as a pro-tumorigenic mediator together with nuclear factor kappa light chain enhancer of activated B cells (NF-κB). Expression of TNF-α by Kupffer cells and macrophages in TME has an important role in CCA progression and invasion [145,152,153]. Soluble and/or transmembrane (tm) forms of TNF-α can either bind to TNFR1 receptor or TNFR2 receptor. Despite TNFR-associated death signaling domain, these two receptors could stimulate tumor-promoting signals and contribute to tumor progression [145, 152,154,155].

TGF-β has a dual role in CCA progression. Although overexpression of TGF-β has resulted in poor prognosis, metastasis and recurrence in CCA, tumor suppressor properties of TGF-β has shown an inhibition of tumor at the early stages of CCA [77, 145, 156-158].

1.2.2.6 Molecular Aberrations in CCA

CCA pathogenesis is related to alterations in DNA repair mechanisms (TP53), epigenetic changes (IDH1 and IDH2). In addition to that, some signaling pathways are dysregulated in this malignancy for example the WNT-CTNNB1 signaling pathway, tyrosine kinase signaling, protein tyrosine phosphatase (PTPN3) [40, 16, 131, 133, 136, 151, 159-163].

Alterations in PTEN, a tumor suppressor gene, with the activation of AKT or mTOR have been shown to be associated with poor outcomes in microaaray analysis of 221 eCCA patient but good outcomes have been reported in iCCA patient [35, 164,165].

Formation of the fusion gene products some of which are FGFR2- TXLNA, GFFR2-BICC1 and FGFR2-TACC3 have been highlighted in CCA [40, 162, 166]. These fusions trigger fibroblast growth factor receptor (FGFR) kinase activation resulting in changes in cell morphology and an increase in cell proliferation [40]. Suppression of FGFR fusion protein's oncogenic ability both *in vitro* and *in vivo* by different FGFR kinase inhibitors including pazopanib and BGJ398 has been considered as a promising therapy for CCA in different studies [40, 167].

Survival advantages can be acquired by epigenetic changes. Hot-spot mutations in IDH1 and IDH2 genes have been connected to development of iCCA [35, 168-170]. These genes have drawn attention to CCA because 2-hydroxyglutarate, the product of IDH1 and IDH2 can be used as a biomarker detected in the serum of CCA patients [35, 171]. Inhibition of gain of function mutations in IDH genes has been shown to cause the dysregulating hepatocyte nuclear factor 4α, which blocks the differentiation of hepatocytes and promotes CCA [35, 40, 170, 172, 173].

Moreover, overexpression of histone deacetylase 6 (HDAC6) has been shown in CCA. This promotes an increase in proliferation followed by primary cilium shortening.

The targeting of HDAC in CCA causes decrease in cell proliferation and restoring of the primary cilium [40, 174].

Besides genetic aberrations, CCA pathogenesis can be controlled by aberrant signaling pathways including Ras-MAPK, PI3K/AKT/mTOR, Notch and Hedgehog (Hh) pathways [175-180]

Ras-MAPK pathway has been reported as an important pathway for CCA development. One study has shown that the poor outcomes and clinicopathological traits were obtained with integrative molecular analysis technique in 119 iCCA patient [35, 178]. In the same study, proliferation and inflammatory classes were identified as gene signature classes. In the inflammatory class, they observed cytokine overexpression and activation of both STAT3 and inflammatory signaling pathways, while they characterized the proliferation class by oncogenic pathway activation and KRAS and BRAF mutations. They also showed that different copy number variations in a lot of oncogenes in the proliferation class representing 62 % of iCCA patient. The proteins, which are encoded by these oncogenes, are the members of RAS-RAF-MEK-ERK signaling cascade stimulating cell proliferation or PI3K-AKT-mTOR signaling promoting survival. The second class, inflammatory class has been shown to stimulate the inflammatory pathways resulting in cytokine and transcriptional factor STAT3 overexpression, thus, modulating growth and survival of cell, which are observed in several carcinomas [35, 178-181].

Notch signaling is another important signaling pathway controlling cell-fate determination during embryonic development of many organs including the biliary tree. In addition to that, it has been involved in CCA development and progression [183,184]. Dysregulation of Notch is related to inflammation and carcinogenesis. The interaction between Notch ligands and their receptors activates the pathway by promoting γ-secretase-dependent Notch receptor cleavage and the release of Notch intracellular domain [183,185]. Activation of Notch pathway differentiates mature adult hepatocytes into iCCA precursors in different preclinical models [35, 186,187]. In addition to those pathways, autophagy pathway is also dysregulated in CCA.

1.2.3 Autophagy in CCA

Recently, CCA cells have been shown to modulate autophagy as a survival and escape mechanism and the detection of high expression of autophagy genes and proteins indicates the importance of autophagy in terms of CCA pathogenesis [188]. Autophagy, which is literally known as self-eating is a lysosomal degradation process that plays a key role in protein and organelle turnover. Autophagy has several physiological and pathophysiological functions. It has an extremely important function in maintaining homeostasis. Autophagy creates different molecular building blocks including amino acids, fatty acids, and nucleotides and they are recycled to bio-energetic pathways after their release into the cytosol [189-191]. Autophagy is involved in many pathological conditions including neurodegenerative diseases such as Huntington disease, Parkinson disease as well as Alzheimer disease and hereditary muscle diseases. Moreover, it can induce or maintain tumor growth depending on tissue or cellular conditions [1, 2, 4, 5, 170, 192-195].

The rate of autophagy is low under normal conditions but it is activated in some physiological conditions such as stress and starvation or chemically by rapamycin and trehalose [2, 3, 195]. Autophagy is a really complex process and several proteins are involved in autophagy. A family of proteins, which are known as ‘AuTophaGy-related’ (ATG) proteins have been identified in the *Saccharomyces cerevisiae* and these proteins are recruited hierarchically and regulate autophagic steps [2, 196]. The autophagy pathway is comprised of different stages, which are induction, autophagosome nucleation, elongation and completion, lysosomal fusion and degradation [2, 197]. The first steps of autophagy are the induction and nucleation of the membrane forming autophagosome that is called an isolation membrane or phagophore. In the elongation step, the newly formed membrane grows and fuses the edge of the membrane resulting in formation of double membrane vacuole called autophagosome. Fusion of the mature autophagosome with the lysosome creates autolysosome structure termed autophagolysosome. In the final step, acid hydrolases degrade the luminal content in the autophagolysosomes and acid hydrolases are recycled by permeases [2, 198-200].

Two regulators of autophagy are mTORC1, mammalian target of rapamycin complex 1, and AMPK (AMP-activated protein kinase) in eukaryotic cells and both of them control induction phase of autophagy [2, 200]. mTORC1 consist of mTOR,

mammalian target of rapamycin, Raptor, mTOR's regulatory associated protein, mLST8, mammalian Lethal-with-Sec-Thirteen 8, as well as Deptor, DEP-domain containing mTOR inretacting protein, PRAS40, proline rich Akt substrate of 40-kDA, and FKBP38, FK-506 binding protein 38 [2,201]. Growth factors, stress signals, amino acids, as well as, glucose and energy status are up-stream signals, which are collected by MTORC1 pathway [2, 202]. mTORC1 is an important down-stream target of PI3K/Akt pathway and this pathway is shown to be activated in several malignancies [2, 203-205]. After activation, mTORC1 transduce anti-autophagic signals by binding to ULK, UNC52-like kinase multi protein complex, which is consisting of ATG101, FIP200, focal adhesion kinase family-interacting protein of 200 kDA, as well as ATG13 and the Ser/Thr protein kinase ULK1 (or ULK2) and this complex has key role in phagophore formation. ULK1 and ATG13 are directly phosphorylated by mTORC1 resulting in downregulation of autophagy [206-209]. ULK1 is also involved in the autophagy independent regulation of mTORC1 since it is a target of AKT and it can be modulated by insulin stimulation [2,210]. Additionally, dissociation of mTORC1 from ULK1/ULK2 complex results in ULK1/ULK2 formation and phosphorylation of ATG13 and FIP200, which is important for autophagic activity. This dissociation has been activated by nutrient deprivation and mTORC1 inhibition pharmacologically such as treatment with rapamycin [189, 2, 211, 212]. Phosphorylation of Raptor by ULK1/ULK2 at several sites causes inhibition of mTORC1. The aim of this negative feedback by autophagy activation is maintaining mTORC1 inhibition in nutrient limited conditions [2, 214]. AMPK also serves as intracellular energy sensor and it has been activated by LKB1, the tumor suppressor liver kinase B1, resulting in downregulation of ATP-consuming processes and upregulation of ATP-creating process [2, 4]. AMPK upregulates the activity of ULK1 by directly phosphorylating multiple sites of ULK1 indicating the importance of AMPK in regulation of autophagy [2, 213]. In the nucleation step, formation of autophagosome membrane requires the recruitment of proteins and lipids and the formation of Beclin-1 core complex by Beclin-1, the class III PI3K/hVps34, and p150/hVps15 [2, 214-216]. Moreover, activation of Beclin-1 core complex forms PI-3 phosphate, which promotes the nucleation of autophagosomal membrane. Direct interaction between Beclin-1 and its binding partners cause either activation of autophagy by ATG14L, autophagy and Beclin 1 regulator 1 (AMBRA1) or UV radiation resistance-associated gene (UVRAG) but binding of Bcl-2, Bcl-xL or Mcl-1 cause inhibition of autophagy [2, 217, 218]. The phases of vesicle elongation and

completion are controlled by two ubiquitin-like systems. The first system consisting of ATG7 and ATG3, which controls lipid modification such as lipid PE, phosphatidylethanolamine, of Light Chain 3 (LC3) and lipidation of LC3 which step need initial LC3 cleavage by ATG4B protease [2, 219]. Unlipidated LC3, which is LC3-I, is generally cytosolic. Lipidation results in conversion of LC3-I to LC3-II which is related to autophagosome membrane [2, 189, 219]. LC3 recruits cargo proteins and organelles into the autophagosome together with p62 and NBR1, neighbor of BRCA1 gene 1 protein [2, 220]. Conversion of LC3-1 to LC3-II as well as presence of LC3 in autophagosomes is used as autophagy markers [2, 197]. The second system is consisting of ATG7 and ATG10 regulating ATG12 to ATG5 conjugation. Effective promotion of LC3 lipidation requires membrane binding with the help of Atg12–Atg5–Atg16L complex. It is shown that Atg12 conjugation negatively affect the binding of Atg5, however, Atg16 activates it. Promotion of Atg8 lipidation requires membrane binding with the help of Atg12-Atg5-Atg16 complex [2, 189, 221]. After that, fusion of autophagosomes with lysosomes forms autophagolysosomes in which engulfed content is degraded and recycled [2, 189, 216]. Autophagy can be terminated by reactivation of mTORC1 by nutrients. This is an important feedback mechanism, which inhibits autophagy activation during starvation [2].

Regulation of cancer by autophagy is still under question and whether autophagy cause cell death or survival is still unknown. Considering the tumor suppressor and promoting effects of autophagy, it looks like a paradox. Additionally, there are some studies indicating that the knock-down of autophagy related genes either cause or prevents cancer cell death [222]. Dual role of autophagy paradox can be enlightened if the role of autophagy in different steps of tumorigenesis is understood. The basal autophagy might prevent initiation of carcinogenesis and serves as tumor suppressor. Actually, autophagy removes damaged organelles including mitochondria, which potentially produces reactive oxygen species (ROS) in high amounts and decrease probability of genomic instability and inflammation, thus, causing the initiation of cancer. Impaired autophagy has been related to high DNA damage, and aneuploidy. Moreover, increased levels of ROS and aberrant p62/SQSTM1 and ER chaperone accumulation are associated with autophagy dysregulation. These factors indicate a key role of autophagy in tumor suppression [2, 223]. On the contrary, autophagy could serve as a pro-survival mechanism. Upregulation of autophagy has been observed in

carcinomas depending on stress such as radiotherapy, chemotherapy, deprivation of nutrient and growth factors as well as hypoxia and DNA damage [2, 224]. In these situations, autophagy is used as an escape mechanism from death stimuli and it helps carcinomas to manage metabolic stress [2, 224-227]. Considering abovementioned information, targeting and inhibiting autophagy selectively could be a potential therapeutic agent restoring chemosensitivity and it can stimulate cell death. Autophagy can be inhibited by some autophagy inhibitors such as chloroquine and hydroxycholroquine which are approved by the U.S. Food and Drug Administration (FDA) for clinical usage [228] (Table 1).

Name of the Inhibitor	**Target**	**References**
Nocodazole	autophagosome-lysosome fusion	[229-231]
Ammonium chloride	autolysosome formation	[232]
Hydroxychloroquine	inhibit lysosomal acidification and prevent the degradation of autophagosomes	[233-235]
Chloroquine	Lysosome	[235, 237, 238]
3-Methyyladenine	Autophagosome formation	[236]
Wortmannin	Autophagosome formation	[236, 239]
LY294002	Autophagosome formation	[236, 240]
SBI-0206965	Autophagosome formation	[233, 241]
Spautin-1	Autophagosome formation	[233, 242-244]
SAR405	Autophagosome formation	[233]
NSC185058	Autophagosome formation	[233]
Verteporfin	Autophagosome formation and accumulation	[245, 246]
ROC325	Lysosome	[247, 248]
Lys05	Lysosome	[233, 249, 250, 251, 252]

Table 1.2.2.7.1 Autophagy Inhibitors

As mentioned before, one of the most important aberrant pathways in CCA is the PI3K/AKT/mTOR, which is activated in nearly 80% of eCCA patients and 60% of iCCA patients [177, 253]. That's why, targeting of the PI3K/AKT/mTOR pathway might be a good option for CCA treatment. Inflammatory environment stimulates this pathway in CCA. It has been shown that cytokines, CXCL5 growth factor as well as VEGF and leukemia initiating factor (LIF) controls the PI3K/AKT/mTOR activation, which is associated with poor prognosis in CCA patients [177, 143, 151, 254-256]. Considering sensitization of CCA patients to chemotherapy by the PI3K/AKT/mTOR inhibition, autophagy can be suggested as an escape mechanism in CCA by

chemoresistance. Some combinational therapies comprising autophagy and mTOR inhibitors are used to overcome this [254, 257]. Clinical trials using autophagy inhibitors in combination therapies result is promising outcomes. Thus, a better understanding of autophagic pathway and the crosstalk between autophagy and other pathways might increase the efficiency of treatments in CCA [2].

1.2.4 Hedgehog Pathway

One of these pathways crosstalking with autophagy is Hh pathway. Firstly, Wieschaus and Nussland-Volhard described this evolutionary conserved pathway and they got Nobel Prize in 1980 using *Drosophila melanogaster*. This pathway is known to play significant roles in several cell-determining processes during embryogenesis and development as well as maintenance of stem cells and polarity and differentiation of tissues [258, 259, 260, 261]. In many studies, dysregulation of the Hh pathway is shown to function as oncogenic driver in several carcinomas like hematologic malignancies, glioblastoma, pancreatic cancer, and CCA [175, 260]. Hh pathway can be manipulated by inhibitors. There are some clinical trials for the Smo inhibitors, such as erismodegib and saridegib [262, 263]. FDA approved another Smo inhibitor, vismodegib, for unresectable or metastatic basal cell carcinomas of the skin treatment [263, 264]. In another study, it is shown that vismodegib decrease the migratory ability and invasion of cancer cells *in vivo* [2].

There are three identified subgroups of Hh which are Sonic Hh (Shh), Indian Hh (Ihh) and Desert Hh (Dhh) [265].

1.2.4.1 Canonical Hh Pathway

Four major components of Hh pathway in *Drosophila melanogaster* are Hh (the ligand), patched (the receptor), smoothened (the signal transducer) and Gli, which is the effector transcription factor. This signaling pathway controls many target genes such as, *Ptch1* and *Gli1* [266].

Primary cilium (PC), which is a small immoveable cilium, consisting of polymerized tubulin is required for the canonical Hh pathway in mammals [267,268]. The PC is essential for the activation of Hh pathway. It regulates the entrance and exit of components of Hh pathway from primary cilium to the nucleus [268, 269].

Hh ligand is a morphogen and it is secreted by many cells [268, 270]. Hh is produced as a 45 kDa precursor protein with N- and C-terminal domains. After formation of N-terminal domain (Hh-N) by cleavage, this newly formed fragment is prenylated [268]. This prenylation, which is also known as lipidation, is crucial for the function of Hh-N domain [268, 269]. This hydrophobicity helps Hh to diffuse into the extracellular matrix [268, 271].

Unlike several membrane spanning receptors, Hh receptor, the 12-pass transmembrane protein patched (Ptch) could block the activity of Hh pathway. In the absence of Hh ligand, Ptch constitutively inhibits the Hh pathway by negatively affecting Smo (downstream effector of smoothened) [268, 272]. Binding of Hh to Ptch repress Ptch by preventing inhibitory effect on Smo thereby it allows signaling by the pathway [268, 269]. When Hh connects to Ptch, this complex is internalized and degraded [268, 273]. Two forms of Ptch which are Ptch-1 and Ptch-2 exist in mammalian cells, Ptch-1 is more expressed and more critical than Ptch-2 for the activity of Hh pathway [268, 274]. For the activation of the pathway, Hh has to bind to co-receptors, which are CAM-related downregulated by oncogenes (Cdo),brother of Cdo (Boc) and growth arrest-specific (GAS)-1 to enhance the connection between Hh and Ptch [268, 275]. Hedgehog interacting protein (Hip) conversely affects binding of Hh to Ptch by binding to Hh and blocking Hh-Ptch binding and negatively regulating the Hh pathway [268, 276]. A 7-pass transmembrane G protein-coupled receptor (Smo) serves as a signal transducer for Hh signaling pathway, which is used as a target for Hh pathway modulation. Smo is blocked by Ptch to enter the PC in the absence of Hh and it is localized in the intra cytoplasmic vesicles [268, 277]. Binding of Hh-Ptch allows Smo to enter the PC [268, 278].

Gli transcription factors belonging to the Kruppel-like family involve a zinc-finger DNA-binding domain [268, 279]. Gli-1, Gli-2 and Gli-3 are the types of Gli proteins found in mammals. In the absence of Hh ligand, Smo binds to a suppressor protein complex consisting of Fu (fused kinase), SuFu (suppressor of fused) and Cos (Costal-2). Since Smo is inactivated, Gli cannot enter the nucleus [268, 280]. When Smo is activated, Gli-2 functions as transcription enhancer inducing Gli-1 expression [268, 281]. In addition to that Gli-1 degradation is promoted by AMPK mediated phosprylation of Gli-1 [282, 283]. Additionally, Smo activation might result in dissociation of Gli. After that, Gli moves back to cytoplasm and goes into nucleus. This

causes the transcription of many genes such as VEGF, a-smooth muscle actin (a-SMA), Nanog, Sox-2, Sox-9 as well as IL-6 [268, 284, 285].

1.2.4.2 Non-canonical Hh Pathway

Two types of non-canonical Hh pathway have been studied which are Ptch-dependent but Smo independent (type 1) and Smo-dependent but Hh ligand and Ptch-independent (type 2). Apoptosis by caspase-3 activation might be triggered through Ptch in type 1 signaling in the absence of Hh ligand [268, 286]. Additionally, Patch-1 could prevent proliferation through cyclin D nuclear localization in the absence of Hh ligands [268, 287]. Teperino et al. showed that Smo could trigger a Warburg-like effect, which promote glycolytic metabolism in adipose and muscle tissues by a calcium-AMPK kinase axis [268, 280].

1.2.4.3 Crosstalk between Hh and Other Pathways

As a member of complex signaling network, Hh pathway crosstalks with other signaling pathways including TGF-β and WNT and orchestrate cellular response in Hh-responsive cells in the liver [288-290]. Members of Gli family modulate transcription of TGF-β target genes including SNAIL, and regulate WNT signaling by modulating Wnt5a and soluble frizzled receptor-1 [291]. Indian Hh downregulation activates WNT signaling by APC mutation and contribute to colorectal tumorigenesis [292, 293]. Hh signaling is upstream to WNT and it negatively regulating WNT signaling by frizzled-related protein 1 (SFRP1). Dysregulation of the balance between these two pathways is a crucial factor in tumorigenesis. For example, increased level of Hh molecules is detected in squamous cell carcinoma of the uterine cervix but, SRP1 expression is downregulated in the same carcinoma. This shows that WNT and Hh pathways are differentially activated in this carcinoma [292]. The same results have also been shown in gastric carcinoma. The Sonic Hh pathway components are high in gastric carcinoma, however, expression of WNT signaling molecules is low [292, 294].

Additionally, Gli transcription factor accumulation is regulated by different ligands [290, 295]. Insulin-like growth factor inhibits Gli1 phosphorylation in PKA dependent manner in Hh-responsive cells. This results in the prevention of proteosomal degradation of Gli by glycogen synthase kinase 3 (GSK-3) and activation of Hh pathway [290, 296].

Gli activity is also enhanced by activation of receptor kinases including EGFR and Platelet Derived Growth Factor Receptor Alpha and their downstream effectors, such as RAS/RAF/MEK/ERK and PI3K/AKT signaling [297, 302]. Hh and PI3K/AKT/mTOR pathway crosstalk has been studied in several carcinomas such as chronic myeloid leukemia [299, 232]. Hh is known to inhibit autophagy by negatively affecting the phosphorylated extracellular signal-regulated kinases including eukaryotic transcription factor alpha (pERK- eIF2α) [3, 128, 303, 263]. Down-regulation of Gli trigger autophagy through regulation of mTOR phosphorylation by ERK1/2 (Sun et al., 2014). Inhibition of Hh is shown to cause autophagy induction in neuroblastoma and pancreatic cancer [304, 305, 5].

Hh is shown to regulate autophagy by activation of Gli transcription, which is controlled by PI3K/AKT/mTOR [177, 304]. PI3K/AKT/mTOR axis is also shown to regulate activity of Gli by PP2A (protein phosphatase 2A), serving as mTOR complex antagonist [306, 206]. Additionally, it has been shown that the PI3K/AKT and the MEK/ERK pathways crosstalk support EMT, angiogenesis as well as metastasis by activation of Gli [299, 306].

1.2.4.4 Hh Pathway in Pathogenesis of CCA

It is known that, Hh ligands and cholangiocyte-derived Hh ligands are synthesized and secreted by cholangiocytes during liver fibrosis. Hh signaling is involved in the dysfunctional repair promotion and causes fibrogenesis, carcinogenesis and hepatic inflammation [290]. Hh is well documented factor in pathogenesis of several solid tumors. Hh signaling is also involved in the survival and growth signal regulation in cholangiocytes indicating the key role of Hh pathway in CCA carcinogenesis [93,307]. El Khatib et al showed that Hh is activated in 50% of human CCA samples. The activation of Hh ligand by fibrosis induces tumorigenesis in CCA. Additionally, Hh pathway inhibition is shown to attenuate CCA carcinogenesis and stimulate necrosis of CCA cells [175, 261]. The key role of noncanonical Hh pathway is shown to be inhibited by cyclopamine. For example, it inhibits the expression of PTCH1 and cause cell cycle arrest in breast cancer cells [261, 6, 35].

CCA cells are shown to express TRIAL which induces apoptosis through its receptors, death receptor 4 (DR4) and death receptor 5 (DR5), binding. It is also shown that Hh signaling pathway directly controls mRNA and protein levels of polo-like

kinase 2, polo-like kinase 2 promotes stabilization of Mcl-1 and regulates cell death resistance by TRIAL in CCA [309].

Dense despolastic stroma is characteristic of CCA and cancer-associated myofibroblasts (MFBs) are the main component of the stroma [161, 310]. MFBs have key role in CCA carcinogenesis by promoting cell proliferation, migration and invasion. Platelet-derived growth factor (PDGF)-BB is an MFB-derived survival factor, which stimulates TRIAL cytotoxicity of CCA cells. This resistance is controlled by trafficking of SMO to the cytoplasm [309].

80% of human cancers highly express serine/threonine kinase polo-like kinase (PLK) [311, 312]. Gli1 and Gli2 transcription factors bind to PLK2 promoter, thus, Hh signaling is directly involved in regulation of PLK2 expression [309].

Although some studies have indicated that aberrant Hh signaling and autophagy activation promotes resistance to chemotherapy, there is no current study indicating this in CCA [2, 6]. Caprino et al, have just demonstrated expression of Hh and autophagy markers by PSC patients but they did not study the crosstalk between Hh and autophagy [7].

The Hh pathway is a really complex pathway and it crosstalks with multiple other pathways for that reason, Hh pathway targeting alone is challenging and did not show many promising results. Additionally, activation of autophagy by Hh pathway serves as survival and escape mechanism in the context of CCA. Moreover, the crosstalk between Hh and autophagy pathways requires better understanding in the context of CCA pathogenesis. Considering these challenges in the sole targeting of Hh pathway and the correlation between autophagy and Hh pathway, we propose a combinational targeted therapy for both autophagy and Hh pathway as a good treatment option to be able to abrogate CCA.

2. MATERIAL AND METHOD

2.1 Maintenance of cells

Two different type of CCA cells, which are TFK-1 and EGI-1 were chosen to perform the experiments on.

TFK-1and EGI-1 cell lines were obtained from the German National Resource Center for Biological Material (DSMZ), and they were cultured under the recommended conditions.Both cell lines were cultured in RPMI medium (Sigma, cat. no. R8758) supplemented with 10 % FBS and 100 U/mL penicilin/streptomycin at 37 C in 5% CO_2 incubator·

TFK-1 cells were seeded out as $20X10^6$ cells /10 mL and EGI-1 cells were seeded out $10X10^6$ cells/ $20cm^2$. The confluent cultures of both EGI-1 and TFK-1 were splitted 1:2 every 2 days using trypsin/EDTA. For trypsinization, 1X trypsin (10 min for TFK-1 and 25 min for EGI-1) was used after washing the cells with 1X PBS. After trypsinization step, the cells were collected with culture medium in order to inactivate trypsin enzyme and centrifuged at 900 rpm for 5 min. The pellet was dissolved with fresh media and the cells were seeded out into corning 100mm X 20mm style dishes.

2.2 Detection of Protein Expression by Western Blotting

TFK-1 and EGI-1 cells were plated into the 6-well plates ($5X10^6$cells/mL). After overnight incubation, the cells were treated with drugs for 48h. After trypsinization, the cells were collected and washed using cold phosphate-buffered saline (PBS). The harvested cells were lysed with RIPA lysis buffer system (ChemCruz, cat.no. sc-24948) after addition of 10µL protease inhibitor cocktail, 10µL PMSF solution and 10µL sodium orthovanadate solution per ml of 1X RIPA lysis buffer. The extracted protein concentration was calculated with DC protein assay kit (Biorad/USA cat. no. 500-0113, cat.no. 500-0114, cat. no. 500-0115) under manufacturer's instruction. The absorption of proteins was measured at 750 nm with Varioskan™ LUX multimode microplate reader (Thermo Scientific™). The cell lysates were loaded as 20-60 µg per well. Gel

electrophoreses (10-15% acrylamide gels) were performed (Biorad, München). The PVDF membranes were blocked using 5 % dried milk in 1X TNT containing 5M NaCI, 2M Tris pH 7,5 and 10 % Tween20 1 hr at room temperature on the shaker and they were incubated overnight at 4°C with the primary antibodies which are ULK1 (1:1000; cat. no. 4773S; Cell signaling), Akt (1: 1,000; cat. no. 4691S; Cell signaling) as well as LC3B (1:1000; cat. no. 2775S; Cell signaling), GAPDH (1:1,000; cat. no. 2118S; Cell signaling) and Anti-β-Actin (1:1000; cat. no. A1978; Sigma Aldrich) antibodies. After overnight incubation with primary antibodies, membranes were washed with 1X TNT buffer for 10min and this washing step was repeated 3 times. After washing steps, the membranes were incubated with the following secondary antibodies for 1hr at room temperature: Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L) (1:10,000 - 1:200,000 for Western blotting with ECL substrates; cat. no. 111-035-003); Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L) (1:10,000 - 1:200,000 for Western blotting with ECL substrates; cat. no. 115-035-003) (both from jackson immunoresearch europe ltd). The washing steps with 1X TNT buffer were repeated and the signals were detected by Pierce™ ECL Western Blotting Substrate (cat. no: 32106; UK) with ChemiDoc™ Imaging Systems (Biorad).

2.3 Drug Preparation and In Vitro Cell Viability Assay

The cells were plated 5000 cells/100 uL for cell viability assays. After overnight incubation the cells were treated with DMSO, Hh pathway inhibitors, autophagy pathway blockers and combination of Hh pathway inhibitors and autophagy pathway blockers in order to check whether the Hh and autophagy pathways are activated in CCA.

In order to inhibit Hh pathway, Gli inhibitor GANT61 [[dihydro-2-(4-pyridinyl)-1,3(2H,4H)-pyrimidinediyl]bis (methylene)]bis[N,Ndimethyl benzenamine] (Stanton et al., 2009; Desch et al., 2010; Ozone et al., 2010; Queiroz et al., 2010; Amakye et al., 2013; Kakanj et al., 2013; Zahreddine et al., 2014; Han et al 2015; Infante et al., 2015; Kern et al 2015; Samanta et al., 2015; Falkenberg et al 2016; Vlčková et al., 2016) were dissolved in DMSO and their stock solutions were prepared. After treatment of the cells with DMSO and GANT61 (5µM-50µM) for 24, 48, and 72 hours their IC50 concentrations were calculated using Graphpad prism 7 program based on the

proliferation curve [175, 259]. In order to manipulate autophagy pathway PP242 (25 to 500 nM) was achieved. For autophagy pathway inhibition follows were used: autophagosome-lysosome fusion using vinblastine and nocodazole (0,1 to 10μM); autophagolysosomal degradation using NH4Cl (10 to 20mM), chloroquine and hydroxychloroquine (5 to 100μM).

Proliferation of cells was calculated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) Cell Viability Assay, which has been used to determine cell viability since 1980's [314]. First of all, cells were seeded in triplicate in 96-well plates as $5X10^3$ cells per well. After 24, 48 or 72h incubation, 10 μl of MTT solution (cat. no. M2128; Sigma Aldrich) was added to each well and the cells were incubated for 2-4h at 37°C. After incubation, the formazan crystals were solubilized with 100 μl of DMSO. The plates were incubated for 15 min on the shaker and the absorbance was measured with Varioskan™ LUX multimode microplate reader (Thermo Scientific™) at 570 nm.

2.4 Autophagy Modulation by Autophagy Blockers

The manipulation of autophagy pathway was performed by different autophagy blockers. For activation of autophagy pathway PP242 in DMSO (25 to 500 nM) was used and for inhibition of autophagy the following were used: autophagosome-lysosome fusion using vinblastine in water and nocodazole in DMSO (0,1 to 10 μM); autophagolysosomal degradation using NH4Cl in water (10 to 20mM), chloroquine and hydroxychloroquine in water (5 to 100μM). Autophagic activity was determined according to the recently updated guidelines [313]. The protein expression was checked using the following autophagy markers: LC3B, ULK-1, Akt, GAPDH and Anti-β-Actin and the aforementioned experimental procedure for western blot was followed for the detection of these autophagic markers' expression.

2.5 Cell Death Assay

For apoptosis assay, 2 million cells/mL were plated, treated and incubated in 6 well plates for 48h. After 48h incubation, the cells were collected after trypsinization and they were centrifugated at 1700 rpm for 5 minutes. The cells were rinsed with PBS twice and centrifugated and dissolved in 200 μl 1X binding buffer (diluted with ultra-pure water). The untreated control cells were dissolved in 800 μl 1X binding buffer and

separated as $PI^+/FITC^+$, $PI^-/FITC^-$, $PI^+/FITC^-$, $PI^-/FITC^+$. For detection of apoptosis 5 µL of fluorochrome-conjugated Annexin V (eBioscience™ Annexin V Apoptosis Detection Kit APC, cat. no. 88-8007-72) was added to 100 µL sample. After 10-15 min. incubation at room temperature, the cells were resuspended with 200 µL of 1X binding buffer and 5 µL of Propidium Iodide staining solution (eBioscience™ Annexin V Apoptosis Detection Kit APC, cat. no. 88-8007-72) was performed and analyzed with BD LRFortessa™ Cell Analyzer flow cytometer.

2.6 Cell Cycle Analysis

For cell cycle analysis, 1 million cells/well were plated and incubated overnight. The cells were treated and after 48h incubation, the cells were trypsinized and centrifuged at 260 G for 10 min at 4 °C. The supernatant was removed and the cells were dissolved with 1 mL cold PBS. After centrifugation at 260 G for 10 min at 4 °C, the supernatant was removed and this washing step was repeated 2 times. The cells were dissolved with 1 mL cold PBS and 4 mL cold ethanol (70%) was added on it and vortexed gently and incubated overnight at -20 °C for fixation. Next day, the supernatant was removed completely after centrifugation at 260 G for 10 min at 4 °C. The cells were dissolved with 1 mL cold PBS, centrifuged at 260 G for 10 min at 4 °C and supernatant was removed. The pellet was homogenized with 1 mL 0.1% Triton-X (Merck Millipore 1.08603.1000) in PBS and 100 uL RNase (200ug/mL) (Sigma R5503) was added on it and incubated at 37 °C for 30 min. The untreated control was separated into two as propidium iodide (PI) (Sigma P4170) stained and PI unstained. 100 uL PI (1 mg/mL) was added to all samples and PI stained untreated control. After 15 minutes incubation at room temperature, the samples were analyzed with BD LRFortessaTM Cell Analyzer flow cytometer.

2.7 Combination Experiments

The combination experiments were performed using Table 2.7.1 based on IC20 and IC30 drug concentrations.

Drugs	EGI-1	TFK-1
GANT61+ chloroquine	15 μM+ 25 μM	15 μM+ 50 μM
GANT61+ nocodazole	15 μM+ 0,2 μM	15 μM+ 0,4 μM

Table 2.7.1 Concentrations of drugs for combination experiments

2.8 Statistical analysis

The statistical analyses were done by unpaired Student's t-test. Comparisons of more than two groups were performed by 2way ANOVA. The level of significance was set at $P<0.05$. All data were presented as mean ±s.d. Calculations were performed by using GraphPad Prism7.

3. RESULTS

In this study, the crosstalk between autophagy and Hh signaling pathways were reconstructed using specific modulators and inhibitors. In accordance with this purpose, two cholangiocarcinoma cell lines, which are EGI-1 and TFK-1 were selected and all experiments were performed on these cells *in vitro.*

3.1. Autophagy Signaling Manipulation and Its Effect on the Proliferation of CCA Cell Lines

In order to check the importance of the activation or inhibition of autophagy on the proliferation of CCA cell lines, we have treated TFK-1 and EGI-1 cells with different modulators of autophagy. Zhou et al., revealed that autophagy can be activated through suppression of mTOR through starvation or mTOR inhibitors such as PP242 and Torin1 [315]. In order to check the effect of autophagy activation on CCA, the TFK-1 and EGI-1 cells were treated with PP242 (25-500 nM) and number of cells/control were calculated. It is observed that cell proliferation decreased by 40% for EGI-1 and TFK-1 cells at the highest dose of 500nM.

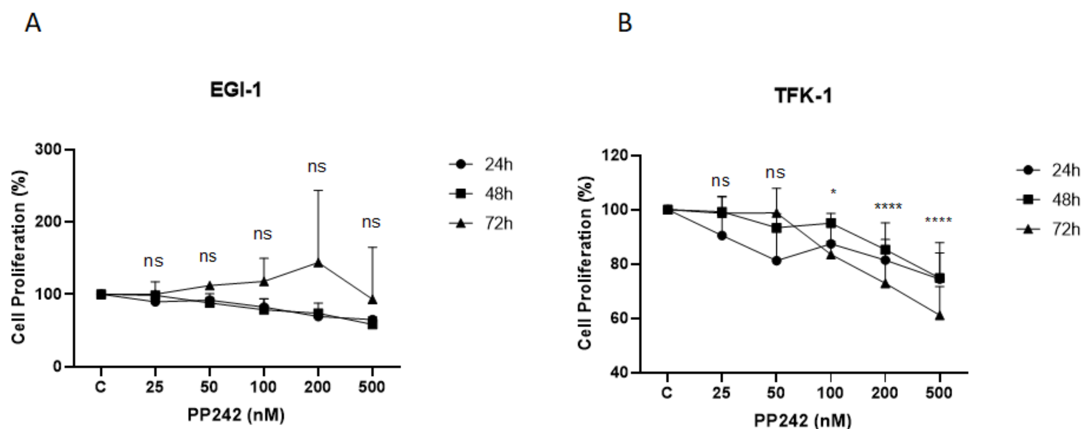


Figure 3.1.1 The effect of PP242 treatment on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 24h, 48h and 72h. The proliferation was normalized per control shown as percentage of proliferation cells. The number of replicates was 3 in each experiment and the average of the replicates performed during each experiment were imported to GraphPad, where the statistical analysis was done on different independent experiments (n=3) and, (ns=P>0.05, *= P ≤ 0.05, **= P ≤ 0.01, ***= P ≤ 0.001, ****= P ≤ 0.0001).

It is shown that number of autophagosomes and LC3-II level are elevated after treatment with vinblastine, which is the microtubule-depolymerizing agent [316-320]. This increase inhibits autophagosome fusion with lysosome by disturbing the activity of microtubules [316]. In our study, we optimized the concentrations of vinblastine as 0.25-50 μM for EGI-1 and 0.5-50 μM for TFK-1 (Figure 3.1.3). We observed that treatment of EGI-1 and TFK-1 cells with vinblastine diminished cell number 50% (Figure 3.1.1) and 60% (Figure 3.1.1), respectively.

In kidney cells, nocodazole, which is another microtubule-depolymerizing agent increase autophagosome number and cause an inhibition of autophagy-mediated protein degradation and endosome autophagosome fusion [229, 316, 318]. When we treated EGI-1 and TFK-1 cells with Nocodazole in both time-dependent and dose-dependent manner, the results showed that proliferation of cells/per control decreased by 50% (Figure 3.1.2).

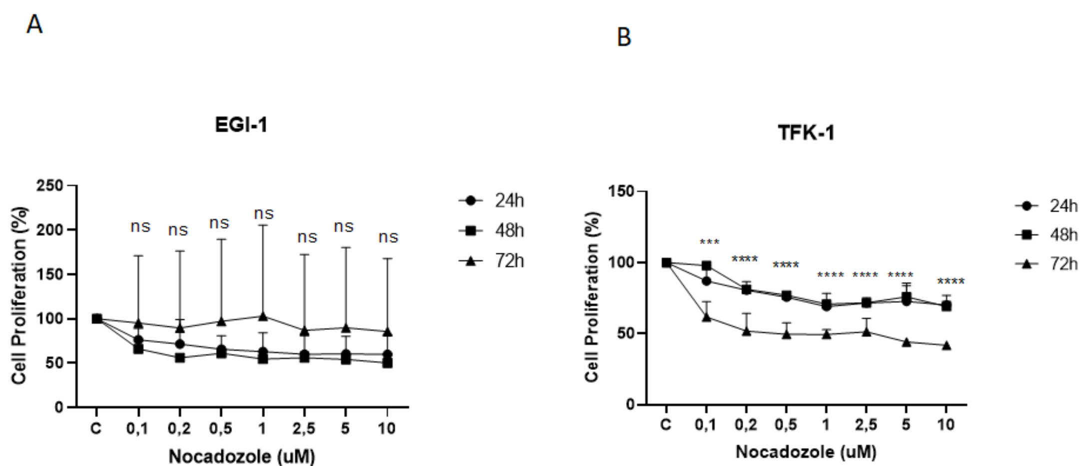


Figure 3.1.2 The effect of Nocodazole treatment on the cell proliferation of (A) EGI-1 and TFK-1 cells for 24h, 48h and 72h. The proliferation was normalized per control and shown as percentage of proliferation cells. The number of replicates was 3 in each experiment and the average of the replicates performed during each experiment were imported to GraphPad, where the statistical analysis was done on different independent experiments (n=2) and, ns=P>0.05, *= P ≤ 0.05, **= P ≤ 0.01, ***= P ≤ 0.001, ****= P ≤ 0.0001.

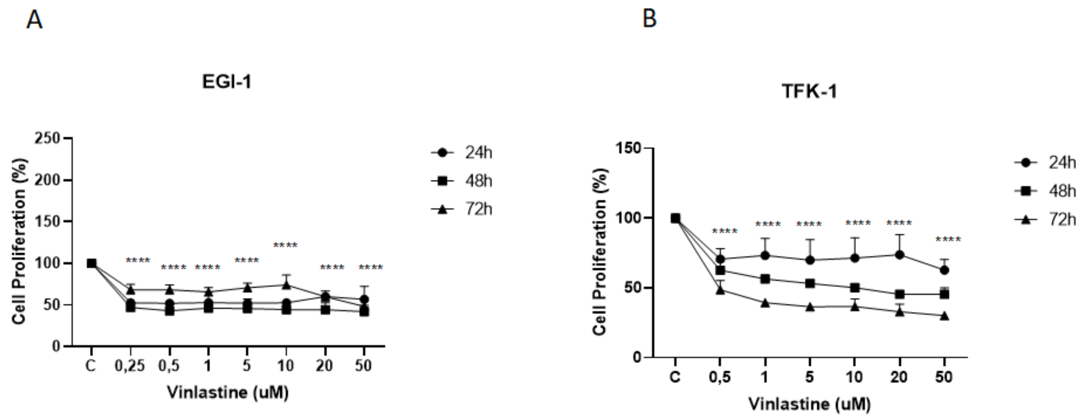


Figure 3.1.3 The effect of Vinblastine treatment on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 24h, 48h and 72h. The proliferation was normalized per control and shown as percentage of proliferation cells. The number of replicates was 3 in each experiment and the average of the replicates performed during each experiment were imported to GraphPad, where the statistical analysis was done on these replicates (n=3) and, ns=P>0.05, *= P ≤ 0.05, **= P ≤ 0.01, ***= P ≤ 0.001, ****= P ≤ 0.0001.

Chloroquine and hydroxychloroquine are shown to block autophagosomal degradation and inhibit autophagy by blocking lysosomal acidification [321-323]. We observed that the treatment of EGI-1 cells with both hydroxychloroquine and chloroquine decreased the proliferation of these cells 80-90% per control. In addition to that, 70% of TFK-1 cells died after hydroxychloroquine and chloroquine treatment (Figure 3.1.4 and Figure 3.1.5).

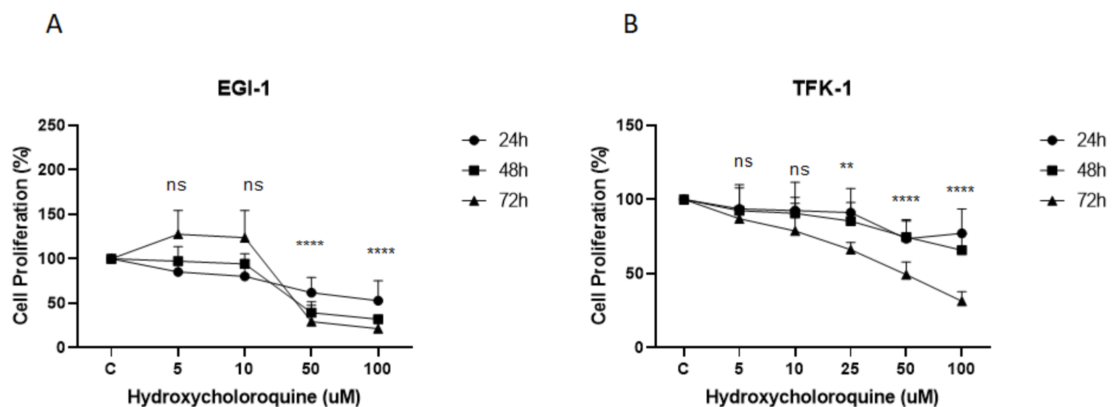


Figure 3.1.4 The effect of Hydroxychloroquine treatment on the cell proliferation of EGI-1 and TFK-1 cells for 24h, 48h and 72h. The proliferation was normalized per control and shown as percentage of proliferation cells. The number of replicates was 3 in each experiment and the average of the replicates performed during each experiment were imported to GraphPad, where the statistical analysis was done on different independent experiments (n=2 for EGI-1 cells and n=3 for TFK-1 cells) and, ns=P>0.05, *= P ≤ 0.05, **= P ≤ 0.01, ***= P ≤ 0.001, ****= P ≤ 0.0001.

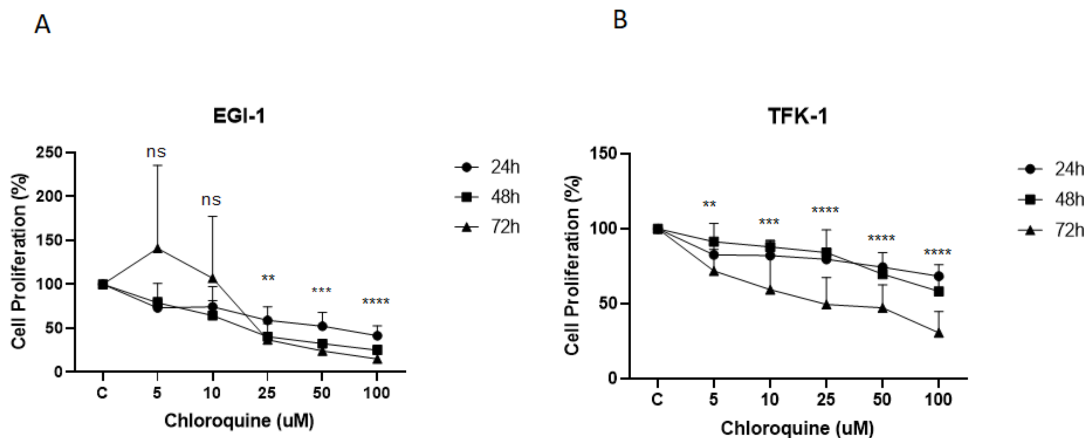


Figure 3.1.5 The effect of Chloroquine treatment on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 24h, 48h and 72h. The proliferation was normalized per control and shown as percentage of proliferation cells. The number of replicates was 3 in each experiment and the average of the replicates performed during each experiment were imported to GraphPad, where the statistical analysis was done on different independent experiments (n=3) and and, ns=P>0.05, *= P ≤ 0.05, **= P ≤ 0.01, ***= P ≤ 0.001, ****= P ≤ 0.0001.

We also showed that 20 mM ammonium chloride inhibit the proliferation of TFK-1 (Figure 3.1.11) and EGI-1 cells (Figure 3.1.1.12).

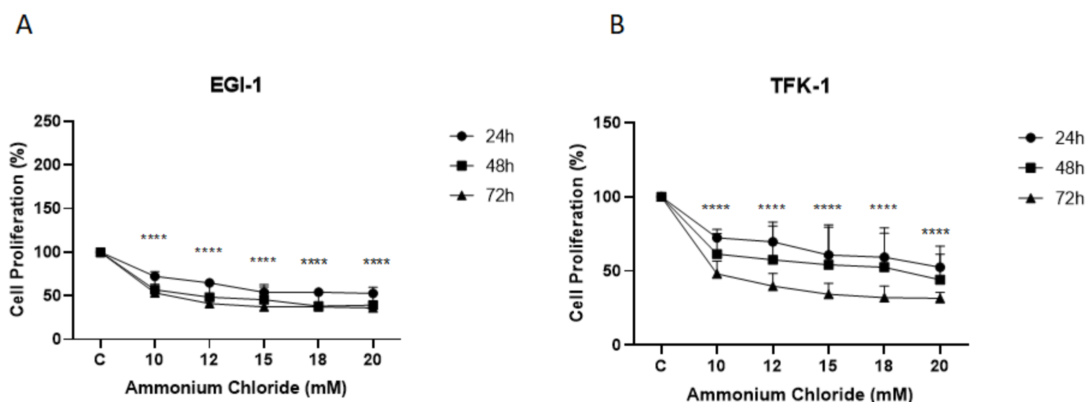


Figure 3.1.11 The effect of ammonium chloride treatment on the cell proliferation of EGI-1 and TFK-1 cells for 24h, 48h and 72h. The proliferation was normalized per control and shown as percentage of proliferation cells. The number of replicates was 3 in each experiment and the average of the replicates performed during each experiment were imported to GraphPad, where the statistical analysis was done on different independent experiments (n=3) and, ns=P>0.05, *= P ≤ 0.05, **= P ≤ 0.01, ***= P ≤ 0.001, ****= P ≤ 0.0001.

These data indicate that autophagy is an important mechanism that is essential for the survival and proliferation of CCA cell lines and inhibiting it can decrease the proliferation of this aggresive cancer.

3.2 The Effect of Hedgehog Pathway Inhibition on the proliferation of CCA Cell Lines

More than 50 compounds have been studied in order to inhibit Hh signaling in several carcinomas [324, 325]. Particularly clinical usage of SMO antagonist, GDC-0449, was approved by Food and Drug Administration in 2012 [325-328]. GANT61 is the other agent, which has been considered as a promising agent for Hh signaling. GANT61 directly binds Gli transcription factor and blocks Hh signaling pathway (Lauth et al., 2007; Benvenuto et al., 2016). In order to see cytotoxic effects of GANT61 on EGI-1 and TFK-1 cells and to assess the importance of Hh pathway on the suvrvival of these cells, we treated these TFK-1 and EGI-1 with different increasing concentrations of GANT61. It was observed that 50 μM of GANT61 treatment lead to more than 90% decrease in the proliferation of EGI-1 for 48h and TFK-1 for 72h(Figure 3.2.1).

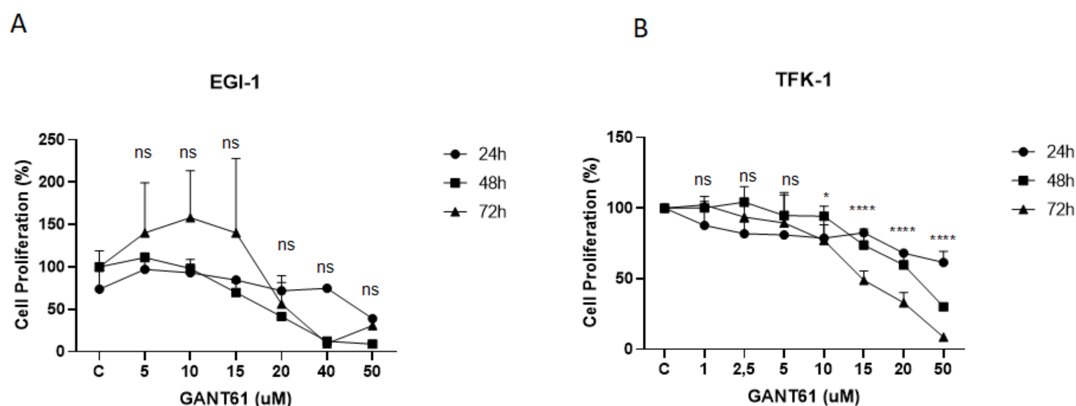


Figure 3.2.1 The effect of GANT61 treatment on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 24h, 48h and 72h. The proliferation was normalized per control and shown as percentage of proliferation cells. The number of replicates was 3 in each experiment and the average of the replicates performed during each experiment were imported to GraphPad, where the statistical analysis was done on different independent experiments (n=3 for EGI-1 cells, n=2 for TFK-1 cells) and and, ns=P>0.05, *= P ≤ 0.05, **= P ≤ 0.01, ***= P ≤ 0.001, ****= P ≤ 0.0001.

3.3 Combatting CCA by the Dual Inhibition of Hh and Autophagy

In order to check the synergistic effect of combination therapy on the proliferation of EGI-1 cells, the cells were treated with GANT61 alone,or chloroquine alone and combination of GANT61 and chloroquine for 48h. IC30 doses of both GANT61 (15 µM) and chloroquine (25 µM) were determined by cell proliferation assays for the further combination experiments. When the cells were treated with GANT61 and chloroquine alone, the proliferation of cells decreased by around 30% of cells compared to DMSO control (C) and water control. On the other hand, the combination treatment of GANT61 and chloroquine lead to further decrease in cell proliferation compared to GANT61 or chloroquine alone (Figure 3.3.1).

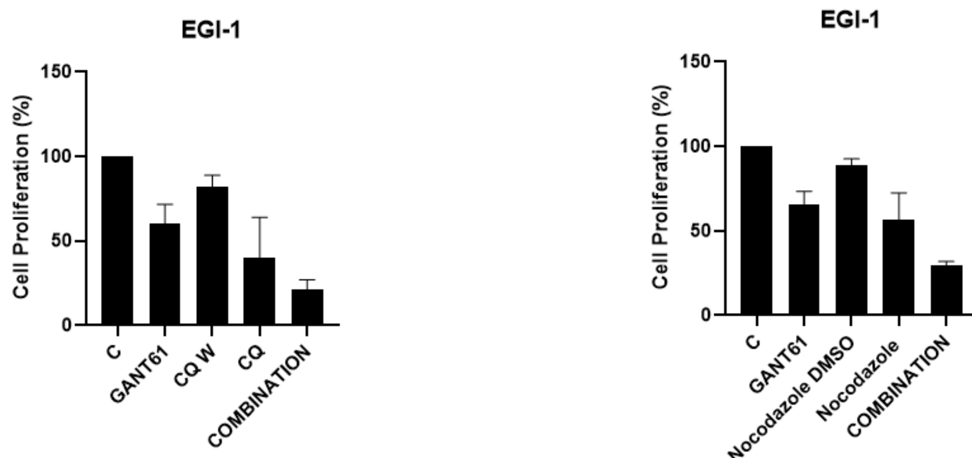


Figure 3.3.1 (A) The proliferation of EGI-1 cells after treatment with GANT61 alone (15 µM), chloroquine alone (25 µM) and combination of GANT61 and chloroquine for 48h. The proliferation of cells was normalized to the control. The standard deviation was performed on the number of replicates (n=3) (B) The proliferation of EGI-1 cells after treatment with GANT61 alone (15 µM), nocodazole alone (0,2 µM) and combination of GANT61 and chloroquine for 48h. The proliferation of cells was normalized to the control.The standard deviation was performed on the number of replicates (n=3).

In the similar manner, after determination of IC30 doses of both GANT61 (15 µM) and nocodazole (0.2 µM) for the EGI-1 cells, the cells were treated with GANT61 alone, nocodazole alone and combination of GANT61 and nocodazole for 48h. It is observed that treatment of EGI-1 cells with both GANT61 and nocodazole decrease cell proliferation around 40% and 20% higher more than GANT61 and or nocodazole alone, respectively (Figure 3.3.1).

When we treated TFK-1 cells with GANT61 alone, chloroquine alone and combination of both, we observed that the proliferation of TFK-1 cells decreased in the combination compared to single treatments (Figure 3.3.2).

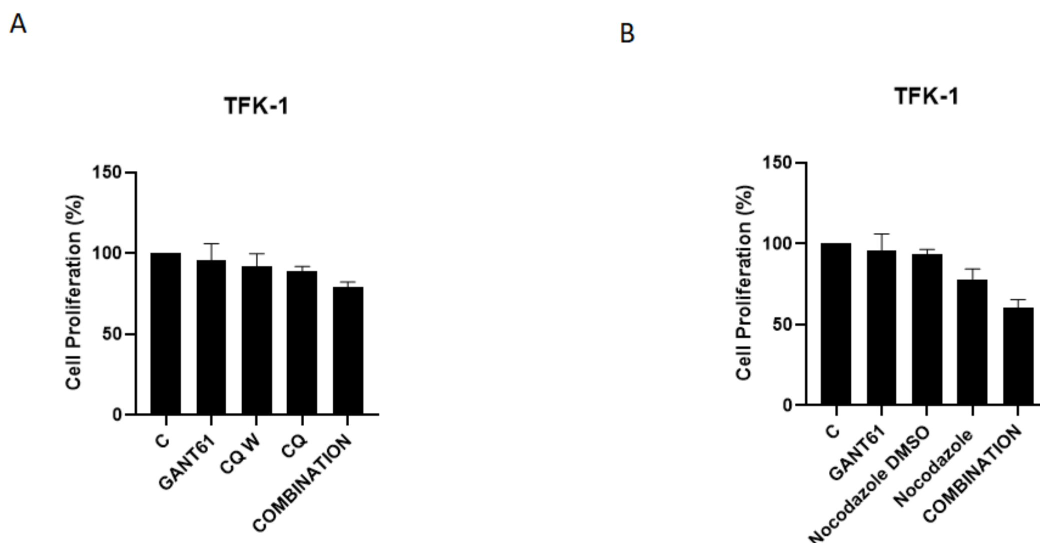


Figure 3.3.2 (A) The proliferation of TFK-1 cells after treatment with GANT61 alone (15 µM), chloroquine alone (50 µM) and combination of GANT61 and chloroquine for 48h. The proliferation of cells was normalized to the control.The standard deviation was performed on the number of replicates (n=3) (B) The proliferation of TFK-1 cells after treatment with GANT61 alone (15 µM), Nocodazole alone (0.4 µM) and combination of GANT61 and chloroquine for 48h. The proliferation of cells was normalized to the control.The standard deviation was performed on the number of replicates (n=3).

Combination of GANT61 and nocodazole is also shown to decrease TFK-1 cell proliferation to 60% compared to DMSO control (Figure 3.3.4).

This suggest that dual treatment of Hh inhibitors and autophagy blockers leads to a futher decrease in the proliferation of CCA cell lines compared to individual treamtments. Thus, suggesting that these treatments can sensitize CCA.

3.4 Autophagy inhibition Lead to an Increase in Cell Death in TFK-1 and EGI-1 CCA Cell Lines

After determination anti-proliferative effects of inhibitors, using proliferation test results, the 100 µM and 200 µM of hydroxychloroquine and chloroquine were determined and the TFK-1 cells were treated with hyroxychloroquine and chloroquine for 48h. After 48h treatment with both chloroquine and hydroxychloroquine, 2.5 fold

increase in the percentage of necrotic cells/control was observed at the highest dose (Figure 3.4.1).

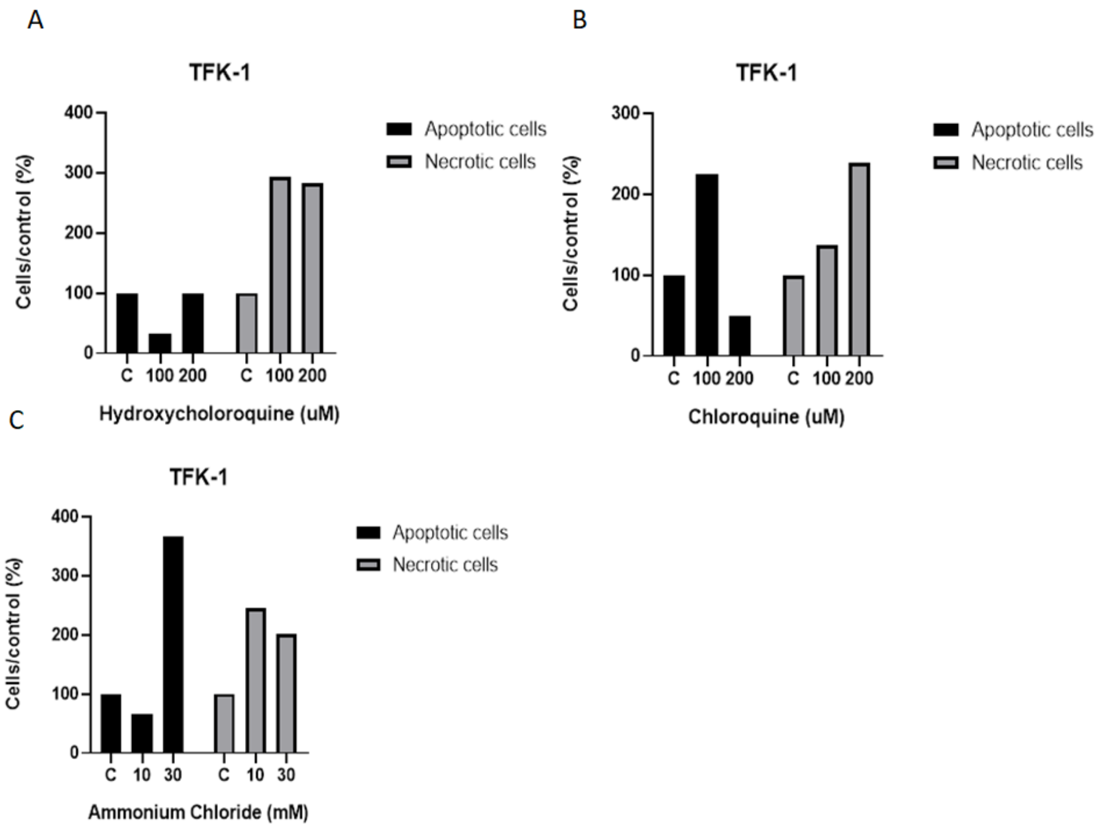


Figure 3.4.1 (A) Determination of apoptotic and necrotic cell percentage after 48h hydrochloroquine treatmeat. The graph shows the quantification of apoptotic and necrotic cell percentage of TFK-1 cells per water control after hydroxychloroquine treatment. (B) Determination of apoptotic and necrotic cell percentage of TFK-1 cells after 48h chloroquine treatment. The graph shows the quantification of apoptotic and necrotic cell percentage of TFK-1 cells per water control after chloroquine treatment. (C) Determination of apoptotic and necrotic cell percentage of TFK-1 cells after 48h ammonium chloride treatment. The graph shows the quantification of apoptotic and necrotic cell percentage of TFK-1 cells per water control after ammonium treatment.

Using 10 mM and 30 mM concentrations of ammonium chloride, the TFK-1 cells were treated for 48h. A 3.5 fold increase in the percentage of apoptotic cells was observed compared to control (Figure 3.4.1).

48h chloroquine treatment is shown to increase necrotic cell percentage of EGI-1 cells 2.5 folds at the 100 µM (Figure 3.4.2).

After that, the EGI-1 cells were treated 10 mM and 30 mM of ammonium chloride. Ammonium chloride increased the necrotic cell percentage more than 3 folds compared to the control (Figure 3.4.2).

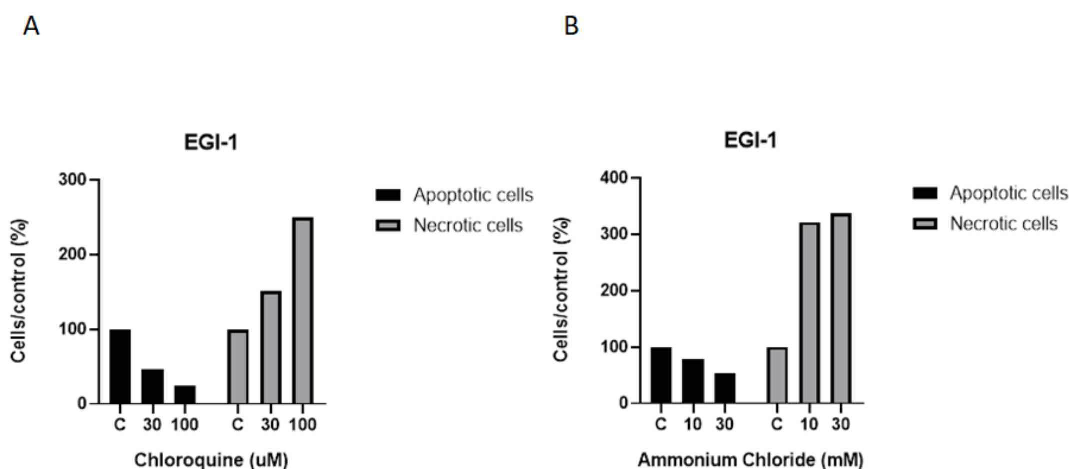


Figure 3.4.2 (A) Determination of apoptotic and necrotic cell percentage of EGI-1 cells after 48h chloroquine treatment. The graph shows the quantification of apoptotic and necrotic cell percentage of TFK-1 cells per water control after chloroquine treatment. The experiment was repeated 2 times. (B) Determination of apoptotic and necrotic cell percentage of EGI-1 cells after 48h ammonium chloride treatment. The graph shows the quantification of apoptotic and necrotic cell percentage of TFK-1 cells per water control after ammonium treatment.

To further understand and explain the decrease in cell proliferation that is observed after autophagy inhibition, we sought to check the cell cycle profile of the cells. Some flow cytometric techniques can be used in order to follow changes in the cell cycle after environmental factors, radiation or drug treatments [329-331]. PI flow cytometric assay is one of these methods. PI is a fluorogenic dye, which binds nucleic acids stoichiometrically and DNA content of the cell can be detected by fluorescence emission [332-334].

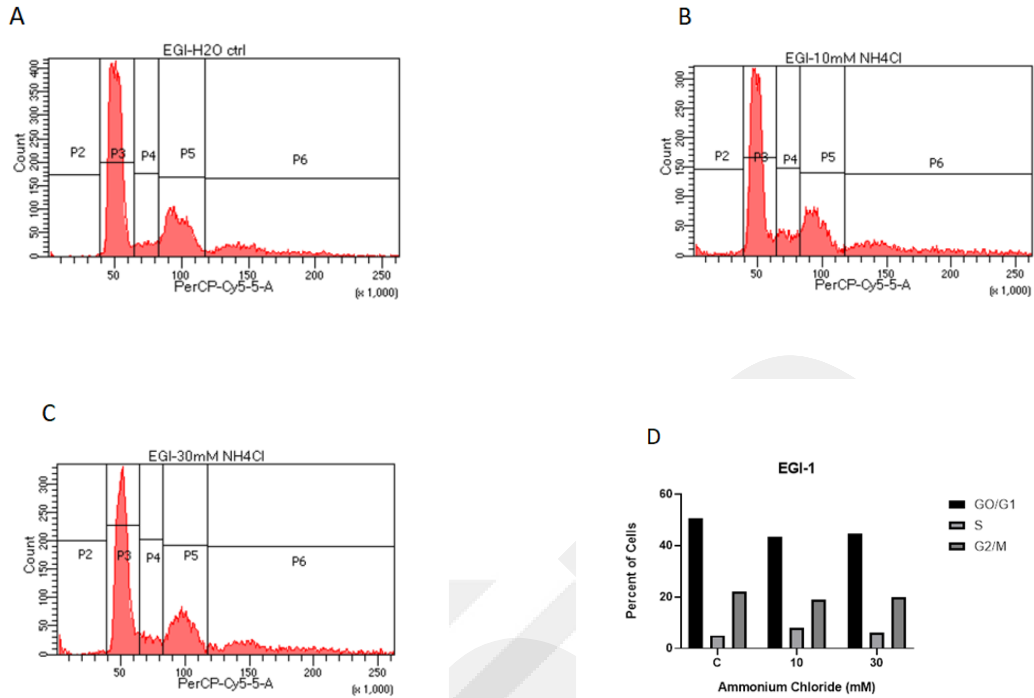


Figure 3.4.6 Cell cycle analysis of EGI-1 cells by flow cytometer. (A) PI staining of water treated EGI-1 cells as control. (B) PI staining of 10 mM ammonium chloride treated EGI-1 cells. (C) PI staining of 30 mM ammonium chloride treated EGI-1 cells. (D) Quantification of the cells in cell cycle after ammonium chloride treatment.

The cell cycle analysis indicates that there is no significant change in the EGI-1 cells after ammonium chloride treatment (Figure 3.4.6).

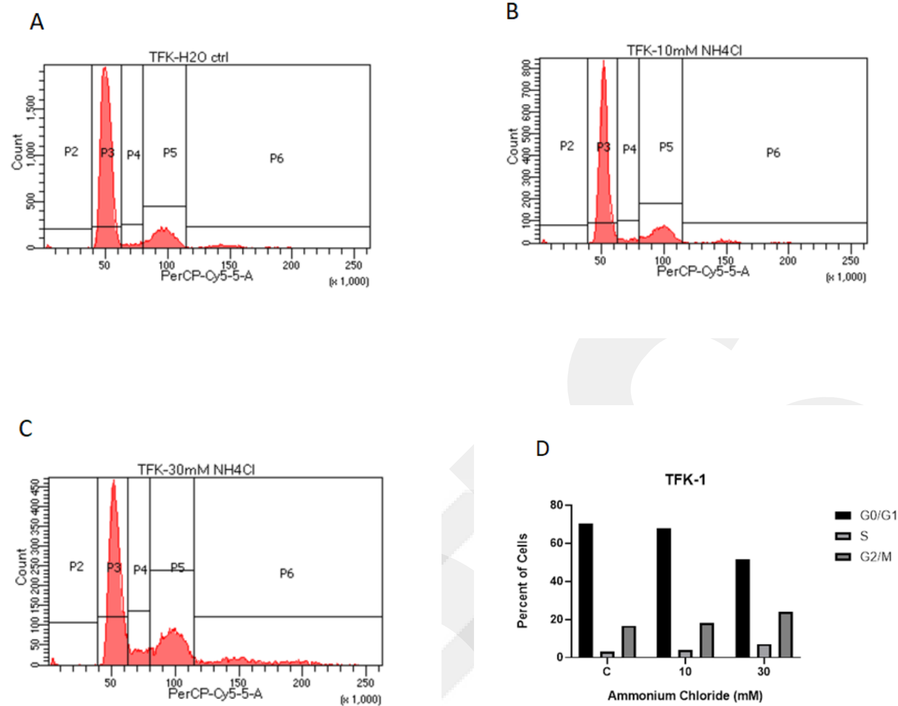


Figure 3.4.7 Cell cycle analysis of TFK-1 cells by flow cytometer. (A) PI staining of water treated TFK-1 cells as control. (B) PI staining of 10 mM ammonium chloride treated TFK-1 cells. (C) PI staining of 30 mM ammonium chloride treated TFK-1 cells. (D) Quantification of the cells in cell cycle after ammonium chloride treatment.

It was observed that ammonium chloride treatment increased the cell number blocked in the G2/M phase, which indicate that treatment with ammonium chloride induces G2/M arrest in TFK-1 cells (Figure 3.4.7). Approximately, ammonium chloride treatment led to around 10% increase in the cell count in the G2/M phase of cell cycle after 30 mM treatment compared to control.

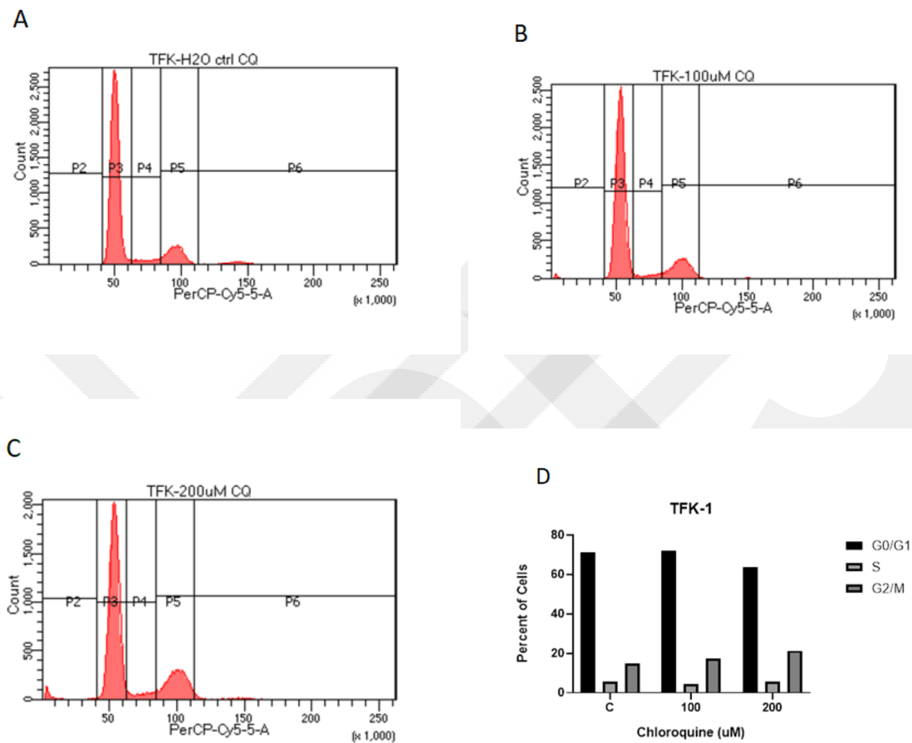


Figure 3.4.8 Cell cycle analysis of TFK-1 cells by flow cytometer. (A) PI staining of water treated TFK-1 cells as control. (B) PI staining of 100 µM chloroquine treated TFK-1 cells. (C) PI staining of 200 µM chloroquine treated TFK-1 cells. (D) Quantification of the cells in cell cycle after chloroquine treatment.

Chloroquine treatment was also shown to cause arrest in G2/M phase at the 200 µM dose after 48h treatment in the TFK-1 cells.

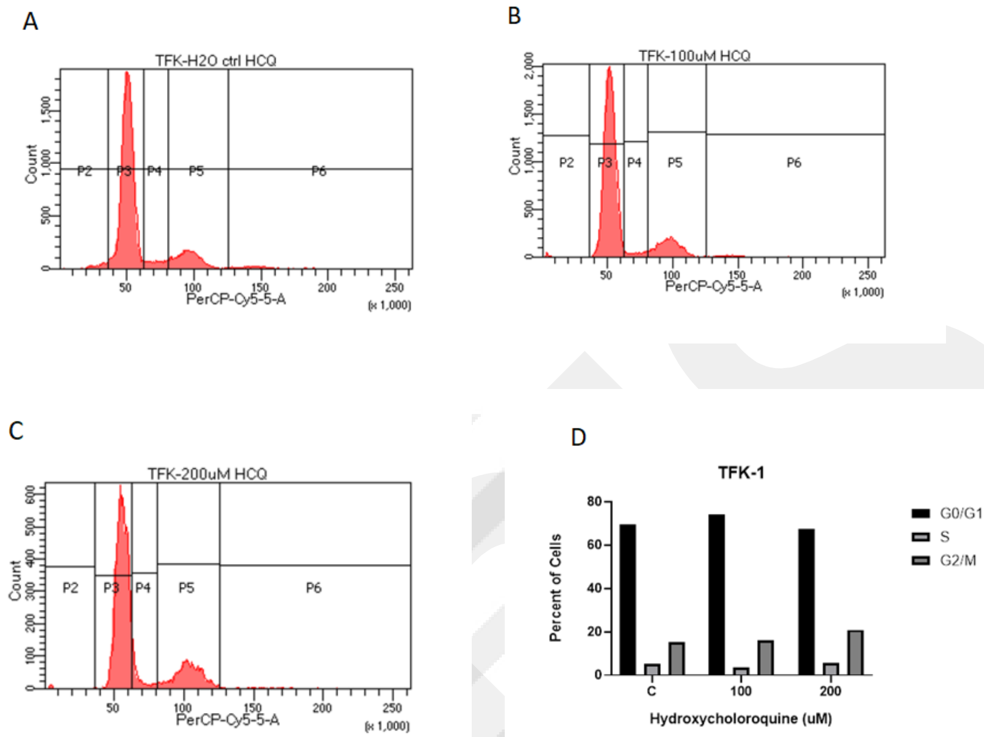
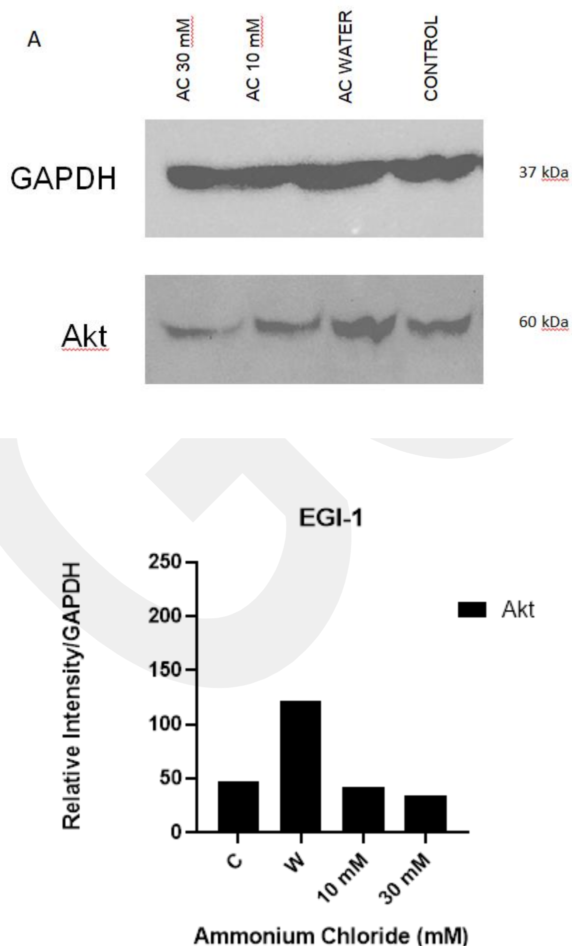


Figure 3.4.9 Cell cycle analysis of TFK-1 cells by flow cytometer. (A) PI staining of water treated TFK-1 cells as control. (B) PI staining of 100 µM hydroxychloroquine treated TFK-1 cells. (C) PI staining of 200 µM hydroxychloroquine treated TFK-1 cells. (D) Quantification of the cells in cell cycle after hydroxychloroquine treatment.

Like ammonium chloride and chloroquine, hydroxychloroquine was shown to induce G2/M arrest in TFK-1 cells (Figure 3.4.8 and 3.4.9). These results indicate that blocking autophagy at autophagosomal degradation leads to G2/M arrest in TFK-1 cells but not in EGI-1 cells.

3.5 The Effect of Autophagy Inhibition or Combination Treatment of Hh and Autophagy inhibition on the markers of Autophagic Pathway

To further understand the mode of action of the modulation of autophagy on the CCA cell lines, we sought to check the underlying molecular mechanisms. In order to analyze protein expression of EGI-1 cells, the cells were first treated with ammonium chloride for 48h. Then, the cell lysates were collected and analyzed by western blotting. Akt is a negative regulator of autophagy by which inhibiting autophagy-promoting proteins. In our study, Akt expression in EGI-1 cells was shown to be decreased after ammonium chloride treatment compared to water control (Figure 3.5.1). Unlike EGI-1 cells, we detected a slight decrease in the expression of Akt in the ammonium chloride treated TFK-1 cells (Figure 3.5.1).



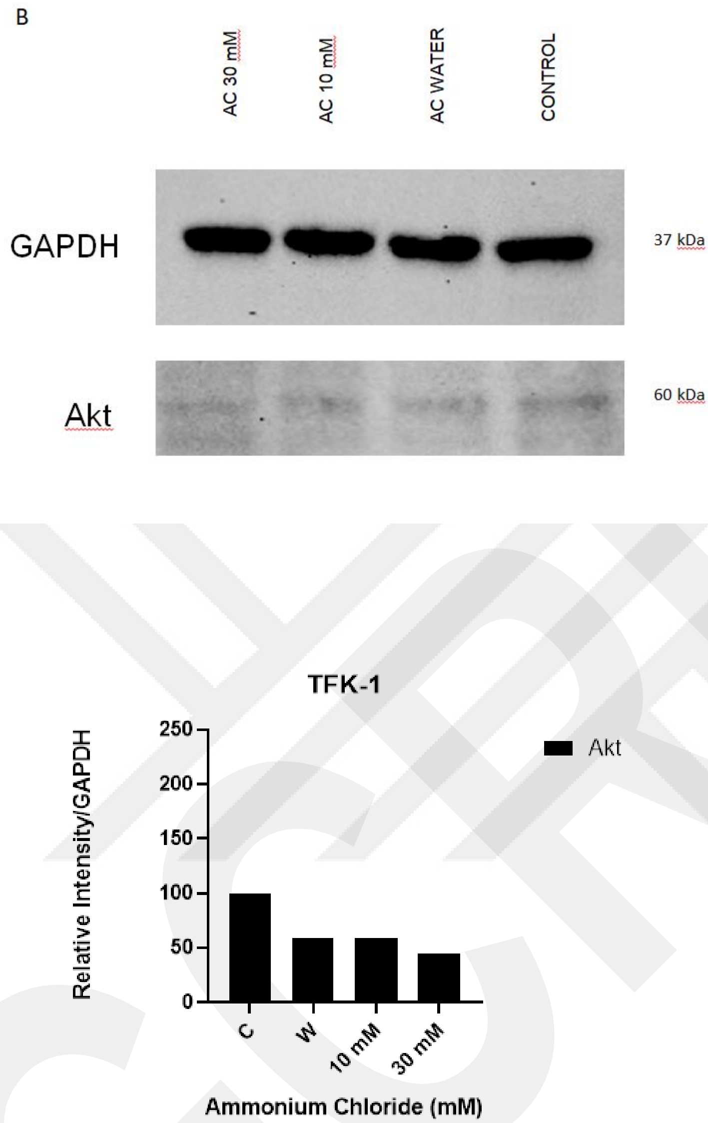


Figure 3.5.1 Western blot analysis of (A) EGI-1 and (B) TFK-1 cells after ammonium treatment with representative western blot analysis of Akt and densitometric graph Akt expression after ammonium chloride. GAPDH was used for protein loading normalization.

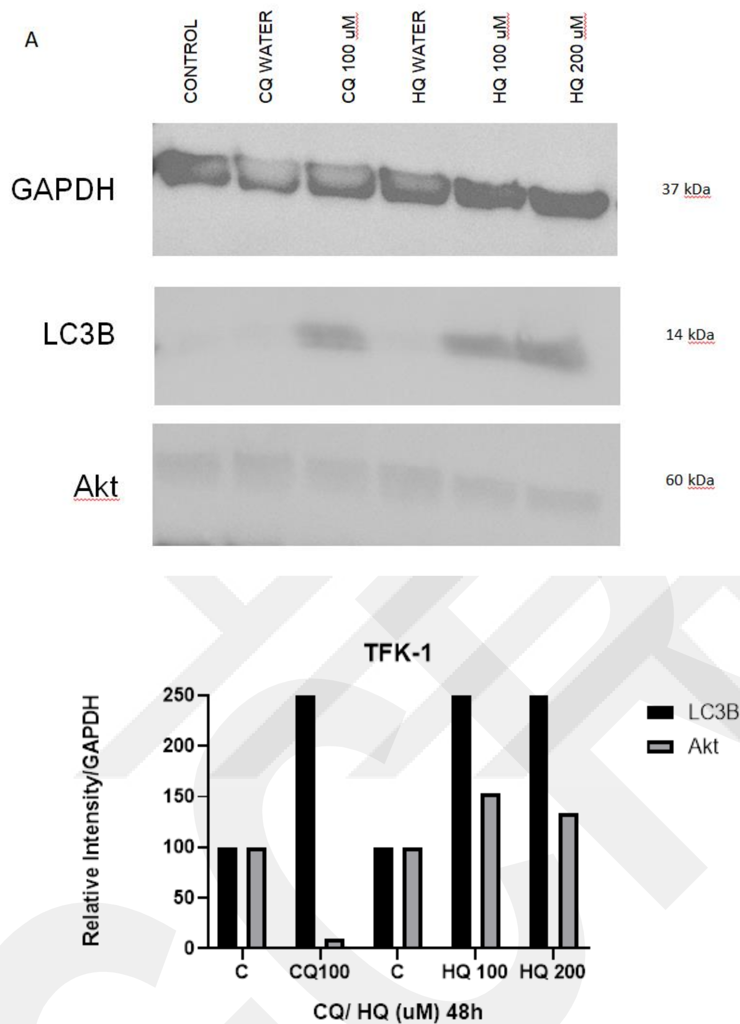
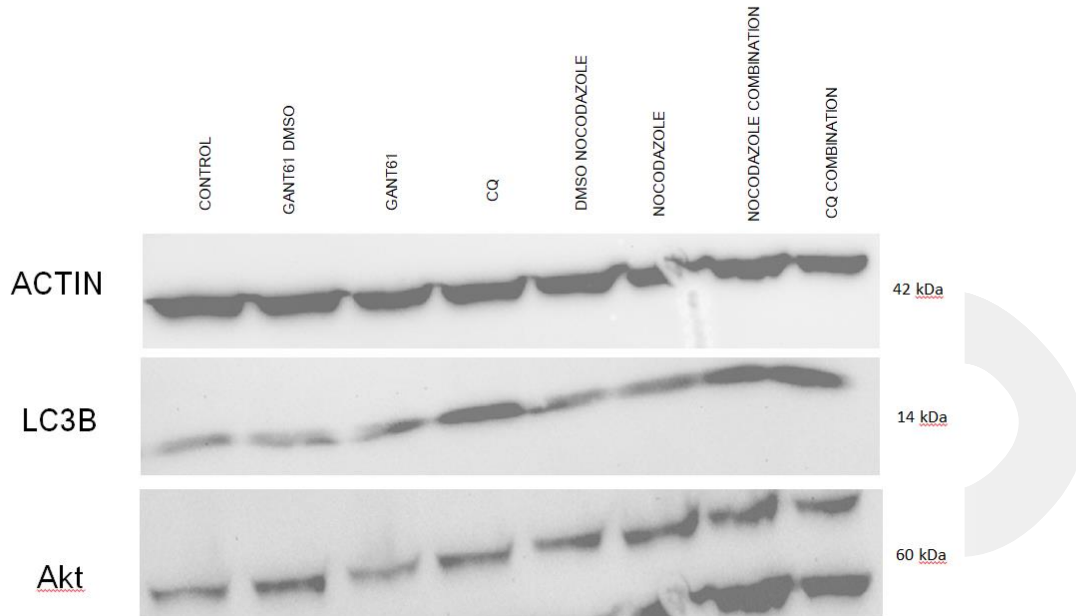
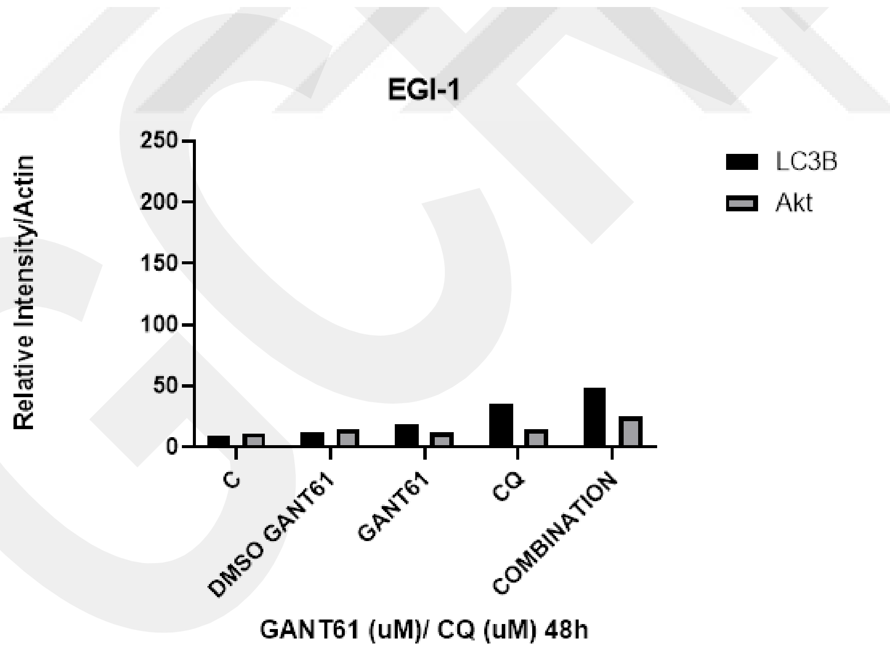


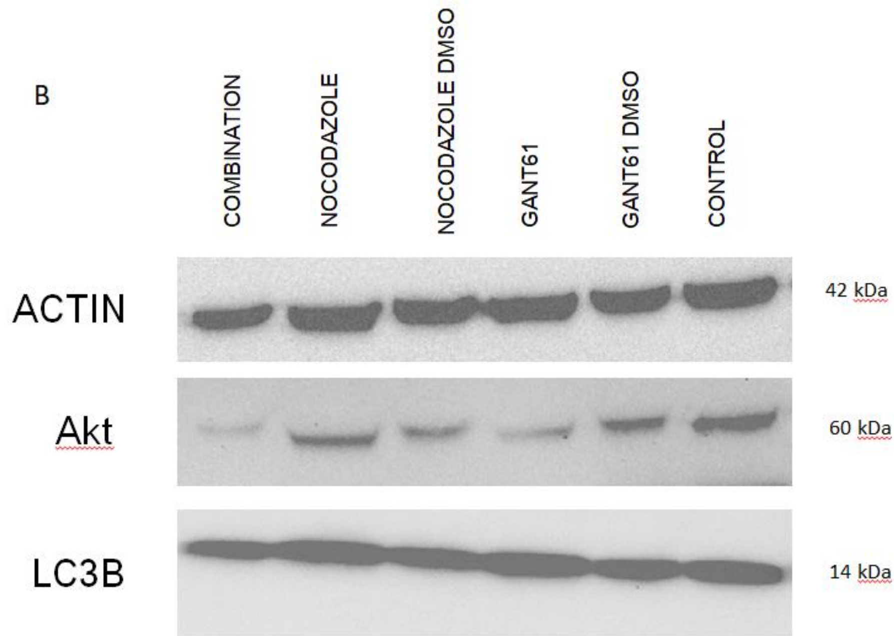
Figure 3.5.3 Western blot analysis of TFK-1 cells after chloroquine and hydroxychloroquine treatments. (A) Representative western blot analysis of LC3B and Akt. GAPDH was used as an internal control and for protein loading normalization. (B) Densitometric comparison of LC3B and Akt expression after chloroquine and hydroxychloroquine treatments.

When the TFK-1 cells were treated with chloroquine and hydroxychloroquine for 48h, we checked expression of LC3B since the presence of LC3 and LC3-II are autophagy indicators [335-337]. It is shown that both chloroquine and hydroxychloroquine increased expression of LC3B compared to water control (Figure 3.5.3). On the other hand, chloroquine treatment is shown to decrease expression of Akt

while, there is not significant change between hydroxychloroquine treatments and water control (Figure 3.5.3)







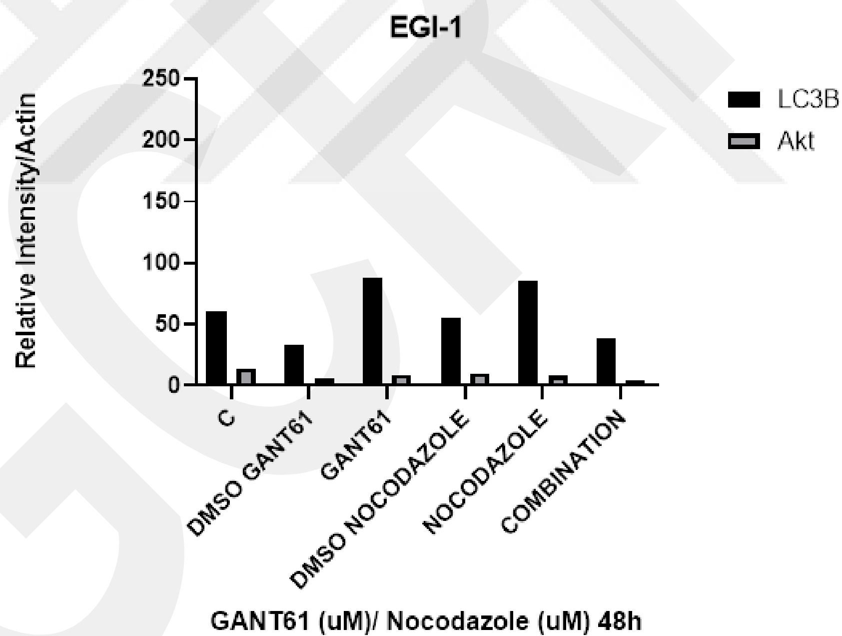
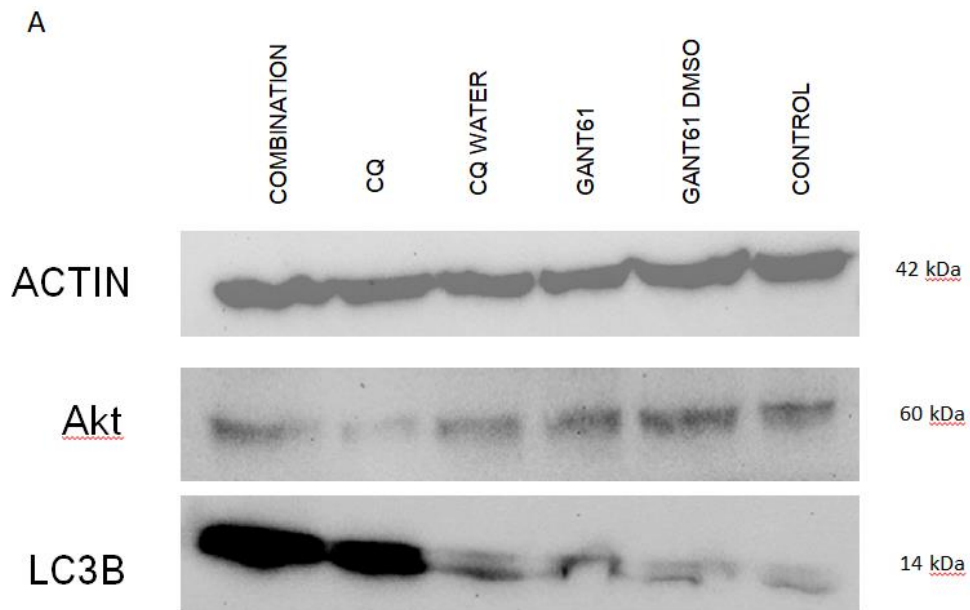
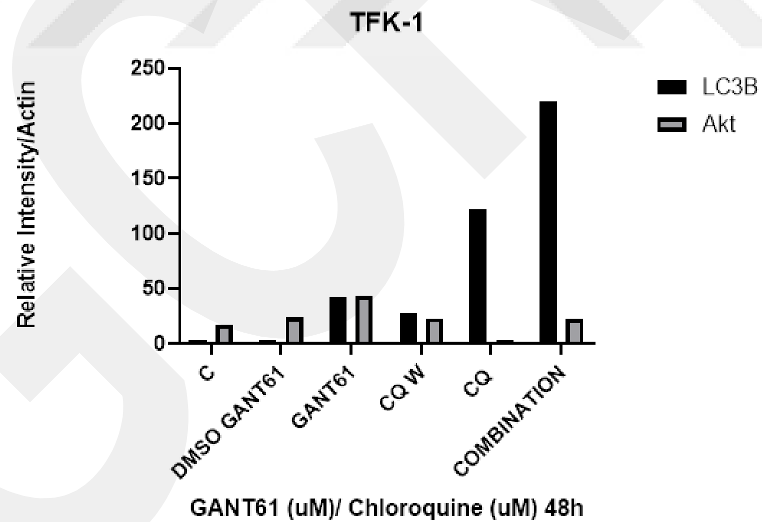
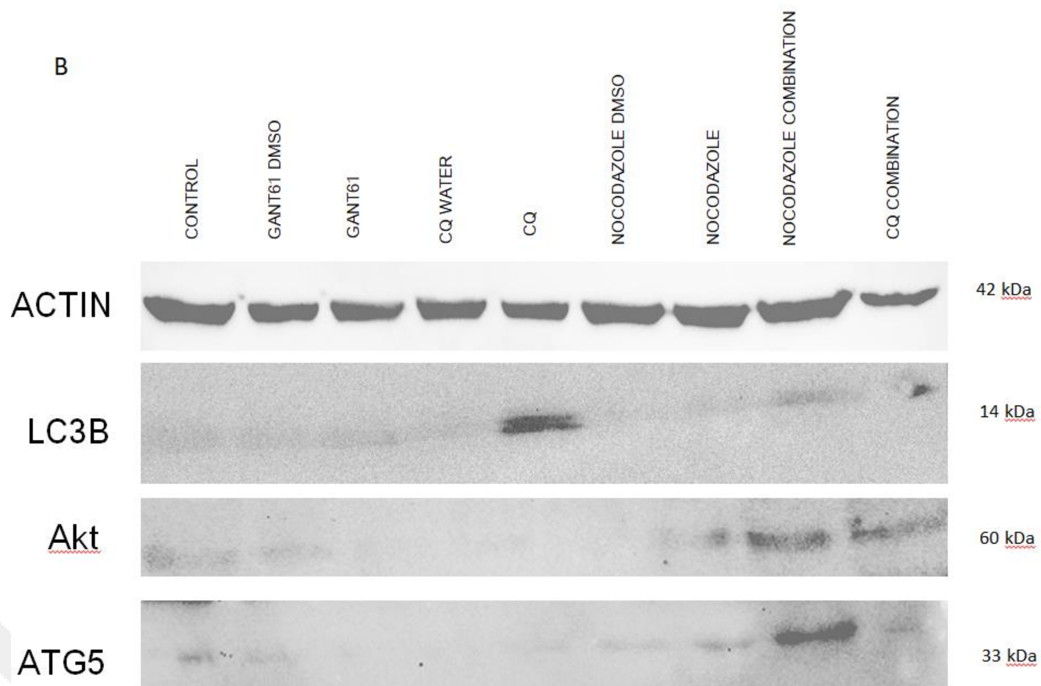


Figure 3.5.4 (A) Western blot analysis of EGI-1 cells after treatment with DMSO as a control, GANT61 alone, chloroquine alone and GANT61/chloroquine combination with representative western blot analysis of LC3B and Akt and densitometric graph LC3B and Akt expression after treatment with DMSO as a control, GANT61 alone, chloroquine alone and GANT61/chloroquine combination. (B) Western blot analysis of EGI-1 cells after treatment with DMSO as a control, GANT61 alone, nocodazole alone and GANT61/nocodazole combination with representative western blot analysis of LC3B and Akt and densitometric graph LC3B and Akt

expression after treatment with DMSO as a control, GANT61 alone, nocodazole alone and GANT61/nocodazole combination. GAPDH was used as an internal control and for protein loading normalization.







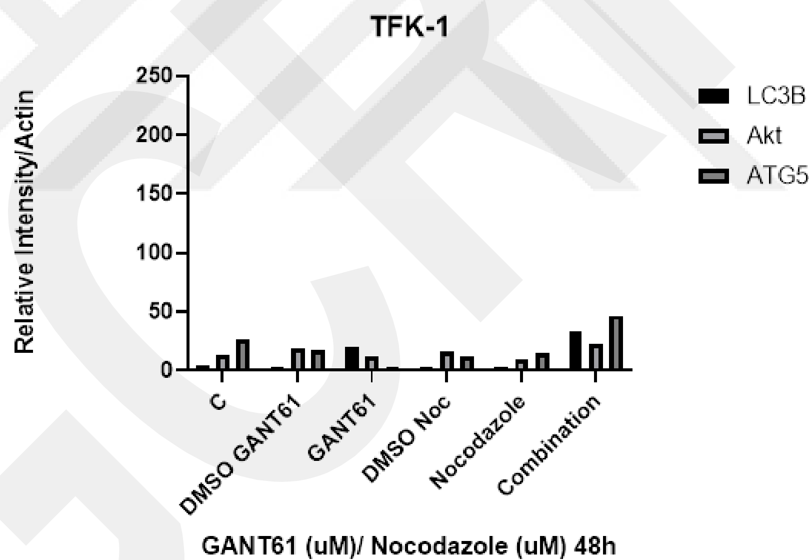


Figure 3.5.5 (A) Western blot analysis of TFK-1 cells after treatment with DMSO as a control, GANT61 alone, water as a control, chloroquine alone and GANT61/chloroquine combination with representative western blot analysis of LC3B and Akt and densitometric graph LC3B and Akt expression after treatment with DMSO as a control, GANT61 alone, chloroquine alone and GANT61/chloroquine combination. (B) Western blot analysis of TFK-1 cells after treatment with DMSO as a control, GANT61 alone, nocodazole alone and GANT61/nocodazole combination with representative western blot analysis of LC3B and Akt and densitometric graph LC3B and Akt expression after treatment with DMSO as a control, GANT61 alone, nocodazole alone and GANT61/nocodazole combination. GAPDH was used for protein loading normalization.

In order to understand if Hh inhibition has an effect on autophagy in the context of CCA, and to see the molecular effect of the combination treatment, we checked the protein expression levels of AKT and LC3B. After determination of IC30 values of GANT61, chloroquine and nocodazole, the EGI-1 cells were treated with DMSO and water as controls, GANT61 alone, chloroquine alone, and combination (GANT61/chloroquine) for 48h. In the GANT61/chloroquine combination, the expression of both LC3B and Akt increased compared to chloroquine alone and GANT61 alone (Figure 3.5.4, Figure 3.5.4).

The same treatment was implemented on TFK-1 cells, too. Like EGI-1 cells, the increase is detected in the LC3B expression. Increase of LC3B expression is an indicator of autophagy [335, 336]. Unlike EGI-1 cells, Akt expression decreased in the chloroquine-treated TFK-1 cells but, GANT61/chloroquine combination treatment increased Akt expression of TFK-1 cells (Figure 3.5.5).

After that, the EGI-1 cells were treated with DMSO as a control, GANT61 alone, nocodazole alone, and combination (GANT61/nocodazole) for 48h. When we compared the expression of LC3B in the combination, we observed decrease in LC3B expression in the combination compared to single treatments (Figure 3.5.4). Like LC3B, the Akt expression is slightly decreased after combination treatment (Figure 3.3.3.6).

The link between ATG5 and ATG12 is essential for modification of LC3 (Ichimura et al., 2000; Mizushima et al., 1998). Unlike EGI-1 cells, we showed that expression of LC3B and ATG5 highly increased in the GANT61/nocodazole combination-treated TFK-1 cells compared to GANT61 and nocodazole alone (Figure 3.5.5). In the autophagy pathway, two ubiquitin-like modification systems are necessary for autophagosome formation. The same increase was observed in the Akt expression of GANT61/nocodazole combination-treated TFK-1 cells (Figure 3.5.5).

As a conclusion, we showed that combination therapy decreased cell proliferation of both EGI-1 and TFK-1 cells. And, inhibition of Hh pathway with either by chloroquine or nocodazole manipulated autophagy.

4. CONCLUSIONS AND FUTURE PROSPECTS

4.1. CONCLUSION

The CCA is the second most common liver cancer. The incidence of CCA is increasing in North America, Europe and Asia day by day. The CCA treatment in both early and advanced stages is really challenging. Surgery is used as an option for CCA treatment; however, percentage of relapses after surgery is high. In addition to surgery, combination of gemcitabine and cisplatin is used as a palliative option for CCA [338]. Therefore, understanding the underlying molecular mechanism of CCA pathogenesis is necessary to combat with CCA. Some pathways are dysregulated in CCA such as, PI3K-AKT-mTOR pathway, Notch signaling pathway, and Hh signaling pathway [339, 290, 184].

In this study, the TFK-1 and EGI-1 cells were treated with GANT61 which is a specific Gli inhibitor in order to check the effects of Hh pathway inhibition on CCA proliferation [340]. Although lower doses of GANT61 did not affect the proliferation of TFK-1 and EGI-1 cells, at the highest doses (15-20 μM), we showed that GANT61 decreased proliferation of TFK-1 and EGI-1 cells 90% compared to DMSO control at the highest concentration; however, the statistical analysis indicate that GANT61 is more effective on the proliferation of TFK-1 cells compared to EGI-1 (Figure 3.2).100 uM GANT 61 was shown to inhibit 50% of SNU1196 pCCA cells [341]. It is also shown that GANT61 reduced cell proliferation of pancreatic cancer stem cells and 20 μM GANT61 was shown to inhibit the growth of ER-positive breast cancer cell [342, 343].

The crosstalk between mTOR/ribosomal S6 Kinase 1 (S6K1) and Hh signaling pathway is established in esophageal adenocarcinoma pathogenesis [344]. mTOR/S6K1 signaling cascade activation induce transcriptional activity of Gli1 by S6K1-mediated Gli1 phosphorylation at Ser84. The dissociation of Gli1 from Sufu activates Gli-target genes, which are involved in carcinogenesis [345]. It is known that mTOR signaling pathway is also involved in autophagy regulation in response to environmental stresses.

mTORC1 stimulation was reported to inhibit autophagy and mTORC2 was also shown to suppress autophagy by mTORC1 activation [191, 346]. Additionally, mTORC2 is activated by PI3K signaling. After that, PI3K phosphorylates AKT, which causes activation of AKT/mTORC1 signaling cascade [346- 348]. In different studies, mTORC1 was shown to modulate autophagy by ULK1, ATG13 and focal adhesion kinase family-interacting protein of 200 kDa (FIP200) regulation. It was reported that for the initiation of autophagy this kinase complex is required to be phosphorylated by mTORC1 [346, 349- 351]. In order to check mTOR inhibition, we treated TFK-1 and EGI-1 cells with PP242, which is mTOR inhibitor and we determined the IC50 concentration of PP242 (Figure 3.1.1 and 6) [315]. We observed that inhibition of mTOR with PP242 was more effective on TFK-1 cells compared to EGI-1 cells. We also detected that inhibition of Hh by GANT61 caused the same results on the proliferation of our cells (Figure 3.2). The combined inhibiton of mTOR and Hh causes autophagy activation, which indicates that activation of autophagy either by mTOR or Hh inhibition might be used as an escape mechanism by EGI-1 cells but not TFK-1 cells. This is supported by the decrease in TFK-1 cell proliferation after PP242 and GANT61 treatment. Xing et al., have determined IC50 of PP242 for gastric cancer cells as 50-500 nmol/L for different cell lines [352]. Moreover, PP242 inhibited mTOR signaling in Ba/F3-ITD/luc/GFP mouse model of leukemia and it showed more anti-leukemia effect compared to another mTOR inhibitor, rapamycin [353]. In order to understand whether mTOR inhibition blocks autophagy in our cells or not, we are planning to check autophagy related proteins which are LC3B and ATG5 in the further experiments.

In addition to mTOR inhibitors and starvation, autophagy could be manipulated with different inhibitors. Nocodazole and vinblastine are microtubule modulators of autophagy pathway [354]. Nocodazole inhibits autophagy by modulating autophagosome fusion [229, 316, 318]. When we treated the EGI-1 and TFK-1 cells with nocodazole, we observed that nocodazole decreased proliferation of EGI-1 and TFK-1 cells around 50% at the highest dose (10 μM), however like PP242 treatment, nocodazole treatment was more effective on the inhibition of the proliferation of TFK-1 cells compared to EGI-1 cells (Figure 3.1.2).We also obtained that vinblastine treatment inhibits cell proliferation of TFK-1 and EGI-1 cell in a time dependent manner (0.25-50 μM for EGI-1 cells, 0.5-50 μM for TFK-1 cells) (Figure 3.1.3). In a study, IC50 of

vinblastine were determined for different cells such as, for MCF-1 human breast and 1/C2 mouse hepatocellular carcinoma as 0.68 nmol/L and 7.69 nmol/L, respectively [355]. The IC50 for these cell lines are low compared to both TFK-1 and EGI-1 cells. In our study, we also used other autophagy inhibitors, chloroquine and hydroxychloroquine, which blocks autophagosomal degradation. Chloroquine and hydroxychloroquine were shown to decrease bladder cancer cell viability in a time-dependent and dose-dependent manner [356]. Jia et al, has determined IC50 concentration of chloroquine as 50 μM in QBC939 cholangiocarcinoma cells [366]. Consistent with this study, we showed that chloroquine decrease cell proliferation of EGI-1 cells 50% after 24h hour incubation (Figure 3.1.9). However, chloroquine is more effective on EGI-1 cell proliferation compared to TFK-1 cells (Figure 3.1.10). When we treated the cells with hydroxychloroquine, we did not observed any significant change in the cell proliferation at the lowest dosess (5-10 μM), however; hydroxychloroquine inhibited cell proliferation of both TFK-1 and EGI-1 cells at the higher doses (50-100 μM) (Figure 3.1.4). Lastly we treated the cells with well-known autophagy inhibitor, ammonium chloride in a dose-dependent and time-dependent manner. Ammonium chloride was shown to induce cell death (70 %) in C6-glioma cells at 15 mM dose at 72h incubation [357]. Consistent with this result, ammonium chloride at the same doses inhibit cell proliferation of our cells more than 60% (Figure 3.1.11 and 3.1.12). Autophagy inhibition in lung, pancreatic and iCCA cells was also shown to inhibit tumor formation and cancer cell survival [358]. Inhibition of autophagy decreased the proliferation of EGI-1 and TFK-1 cells. We can conclude based on our results that autophagy is a survival mechanism for TFK-1 and EGI-1 cells.

Cell cycle checkpoints (the G1/S boundary, the S phase and the G2/M phase) are monitored in order to observe order and timing of cell cycle events. Cell cycle arrest at these checkpoints allows the cell damage repair. In the situations, which the damage cannot be repaired, apoptosis occurs to eliminate the cell [359]. In our study, we checked the apoptotic effects of ammonium chloride. We started with IC50 concentrations; however, we couldn't detect any significant change in the percentage of apoptotic cell. So to rule out that the decrease in cell proliferation due to autophagy inhibitors treatment is a result of apoptotic cell death or any other type of cell death mechanisms we increased the treatment concentration to IC80**.** We showed that there were almost 4 fold apoptotic cells in the ammonium chloride (30 mM) treated-cells

compared to water control (Figure 3.4.1). We also checked apoptotic effects of chloroquine, hydroxychloroquine and ammonium chloride on TFK-1 cells. At the concentration of 100 µM chloroquine and hydroxychloroquine, a 1.5-2 fold increase in the necrotic cell population compared to water control (Figure 3.4.1). Autophagy was shown to activate alternative cell death mechanisms in CCA [387]. Jiang et al., showed that chloroquine both decrease proliferation of Bcap-37 breast cancer cells and induce G2/M cell cycle arrest [360]. In order to understand the underlying mechanism of increase in necrotic cell percentage not in apoptotic cell percentage, we checked cell cycle. We also showed that chloroquine, hydroxychloroquine and ammonium chloride arrested TFK-1 cells at G2/M phase (Figure 3.4.7, 3.4.8 and 3.4.9). This might be an explanation why hydroxychloroquine and chloroquine did not induce apoptosis. We can also further check apoptosis markers caspase-3 and PARP-1 cleavage to confirm these results.

When we treated EGI-1 cells with chloroquine and ammonium chloride, we detected 3 fold necrotic cells compared to water control (Figure 3.4.2). We need to further check the cell cycle checkpoints to understand that which cell death mechanism is induced in the autophagy inhibition by chloroquine and ammonium chloride in EGI-1 cells.

After showing anti-proliferative effects of drug, we started combination experiments. For the combination experiments, we selected a representative of the two different autophagy modulators, which are nocodazole and chloroquine for modulating autophagosome fusion and blocking autophagosomal degradation, respectively. Using MTT cell proliferation assay, we determined IC30 of nocodazole, chloroquine and GANT61 for the further combination experiments. We detected that proliferation of EGI-1 cells decreased in the both GANT61-chloroquine and GANT61-nocodazole combination treated-cells compared to GANT61-alone, chloroquine-alone and nocodazole-alone treated cells (Figure 3.3.1). Like EGI-1 cells, we observed more decrease in TFK-1 cell proliferation in the combination treatment compared to single treatments (Figure 3.3.2). Consistent with our results, Li et al., showed that chloroquine increased GANT61-induced cytotoxicity in human hepatic stellate cell line LX-2 cells [361]. If we compare the effects of combination treatment on our cells, we observed that the combination treatment was more effective on EGI-1 cells compared to TFK-1 cells. The cell proliferation results showed that EGI-1 was more resistant to PP242, GANT61

and nocodazole treatments compared to TFK-1, however, treatment of GANT61 with either nocodazole or CQ sensitized the EGI-1 cells to our single treatments.

Additionally, in order to check basal autophagy level of TFK-1 and EGI-1 cells, we checked the protein level of LC3B. In colon cancer cells, LC3B expression was shown to increase in a dose-dependent manner after chloroquine treatment [362]. Like colon cancer cells, we showed that both chloroquine and hydroxychloroquine increased LC3B expression of TFK-1 cells (Figure 3.5.3). Additionally, we detected highly increased LC3B expression after GANT61/chloroquine-treated TFK-1 and EGI-1 cells compared to GANT61-alone and chloroquine-alone treated cells (Figure 3.5.4 and 3.5.5). Chloroquine was also shown to increase LC3B expression in T lymphocyte Jurkat cells [363]. It has been shown that Hh pathway inhibition by Smo inhibitor, cyclopamine, and increased accumulation of LC3-II in Hela cells [3]. Consistent with this accumulation, we showed that inhibition of Hh pathway activates autophagy. We also shown that after chloroquine treatment, there was slightly cleavage of LC3B. LC3-I is formed after LC3 cleavage and during autophagy, conversion of LC3-I to LC3-II occurs by a ubiquitin-like system so both the presence of LC3 and LC3-II are autophagy indicators [317, 319].

Unlike GANT61/chloroquine treatment, we did not observed any increase in the expression of LC3B of GANT61/nocodazole treated EGI-1 cells. In the autophagy pathway, nocodazole inhibits autophagosome-lysosome fusion that’s why it is not surprising not to see increase in the LC3B expression compared to control after nocodazole treatment and combination treatment. In addition to that, in TFK-1 cells it was also shown that ATG5 expression highly increased in the combination treatment (Figure 3.5.5). Based on the increase in the ATG5 expression, we can conclude that autophagy can also be induced by Hh pathway inhibition at the formation of isolation membrane step of autophagy pathway.

We also checked Akt expression after single treatments and combinational treatments. Akt is a negative regulator of autophagy which inhibits autophagy promoting proteins [205]. When we treated EGI-1 cells with GANT61/nocodazole, we detected that Akt expression decreased in GANT61/nocodazole-treated cells compared to single treatment; however, GANT61/chloroquine increased Akt expression of EGI-1 cells (Figure 3.5.4 and 3.5.5). GANT61’s role in the activation of autophagy was

highlighted in chronic myeloid leukemia (CML). Additionally, in CML GANT61 decreased the level of p-AKT, thus leading to the activation of autophagy.To be able to explain the crosstalk between autophagy and hedgehog, it is essential to check the expression level of the active form of Akt, which is p-Akt without that we cannot come up with any conclusive statement regarding the change of the expression level of Akt due to our treatment in the EGI-1 and TFK-1 cell lines.

We couldn't detect any significant change in Akt expression of after inhibition of both Hh pathway and autophagosomal degradation in TFK-1 cells but GANT61/nocodazole increased Akt expression compared to single treatments unlike EGI-1 cells (Figure 3.5.5). We detected that Akt expression decreased after Hh inhibition with GANT61/nocodazole treated EGI-1 but not in combination-treated TFK-1 cells and GANT61/chloroquine-treated EGI-1 cells. This difference is might be caused by inhibition of autophagy in different steps. Also, combination treatment either increase or decrease expression of the inhibition of different steps in autophagy with Hh pathway inhibition compared to Hh pathway inhibition alone that's why further deciphering of the crosstalk between these two pathways is required to understand which mechanisms control autophagy and what is the role of Akt in the induction or inhibition of autophagy in the context of CCA.

As a conclusion, targeting the different steps of the autophagic pathway with chloroquine and nocodazole and inhibiting Hh pathway by GANT61 decreased cell proliferation of both EGI-1 and TFK-1 cells. Since cholangiocarcinoma cells use autophagy as an escape mechanism, we conducted a combinational therapy targeting both Hh pathway and autophagy pathway. Our results showed that both GANT61/chloroquine and GANT61/nocodazole treatment negatively affected cell proliferation of TFK-1 and EGI-1 cells compared to single treatment with autophagy and Hh pathway inhibitors. We also concluded that, inhibition of different steps of autophagy with Hh pathway induce autophagy in TFK-1 cells; however, inhibition of Hh pathway with autophagy in EGI-1 cells, cause either inhibition or induction of autophagy depends on the treatment.

4.2. FUTURE PERSPECTIVES

The CCA treatment is really challenging and several factors contribute CCA pathogenesis. Dysregulation of signaling pathways is an important factor in CCA

pathogenesis. Autophagy is an example for these pathways and CCA cells use autophagy as an escape mechanism. Akt or ERK 1/2 inhibition was shown to sensitize cisplatin-resistant iCCA cells to cisplatin. Moreover, this inhibition induced apoptosis and inhibited growth of iCCA, which indicates that combination experiment using mTOR and autophagy pathway prevents chemoresistance in iCCA cells [254, 364]. Autophagy pathway crosstalks with Hh signaling pathway, which is dysregulated in CCA.Considering the link between autophagy and Hh pathways, we performed a combinational therapy using autophagy and Hh inhibitors and our results were promising. The combination therapy decrease cell proliferation of CCA cells compared to single treatments. We also checked the expression of autophagy related protein LC3B after combination treatment and we observed that the expression of LC3B increased in GANT61/chloroquine-treated cells but not in GANT61/nocodazole-treated cells. Like our results, nocodazole treatment accumulated LC3II and p62 in Mouse NSC34 (motor neuron-like hybrid cell line) [365]. We also detected either decrease or increase of Akt expression after combination of Hh pathway inhibition with different autophagy modulators. Further experiments are required to confirm the role of Akt. In order to clarify this idea, p-Akt protein levels can be check after combination experiment in the future. Additionally, further experiments need to be done to understand the crosstalk between PI3K/mTOR/ Akt pathway and autophagy in cholangiocarcinoma cells. We are also planning to check expression levels of other PI3K/mTOR/ Akt pathway members in the further experiments.

In our study, we showed that combination experiment has good outcomes with regard to proliferation of cholangiocarcinoma cells but the underlying mechanisms in the inhibition of cell proliferation, which cell death mechanisms are activated by combination, still needs to be clarified. Also, we need to check other apoptosis markers like PARP-1 cleavage and caspases in the future. Understanding and clarifying the proper regulation of autophagy in CCA and the role of hedgehog pathaway in it allow us to develop better treatment options and more personalized targeted therapy for CCA patients. In order to further confirm our *in vitro* data, we need to investigate these two pathways *in vivo* using mouse models for the CCA carcinogenesis.

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