Helin Sağır

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THE EFFECT OF HISTONE DEACETYLASE INHIBITORS ON PTEN/PI3K/AKT/mTOR PATHWAY AND THE CARCINOGENESIS OF CHEMORESISTANT CHOLANGIOCARCINOMA AT A MOLECULAR LEVEL

A THESIS
SUBMITTED TO THE DEPARTMENT OF BIOENGINEERING
AND THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF ABDULLAH GUL UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

By
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M.Sc. thesis titled The Effect of Histone Deacetylase Inhibitors on PTEN/PI3K/AKT/mTOR Pathway and the Carcinogenesis of Chemoresistant Cholangiocarcinoma at a Molecular Level has been prepared in accordance with the Thesis Writing Guidelines of the Abdullah Gül University, Graduate School of Engineering & Science.

Prepared By
Helin Sağır

Advisor
Assist. Prof. Dr. Emel Başak Gencer Akçok

Head of the Bioengineering Program
Prof. Dr. Sevil Dinçer İşoğlu

ACCEPTANCE AND APPROVAL

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JURY:

Advisor : Assist. Prof. Dr. Emel Başak Gencer Akçok……..

Member : Assist. Prof. Dr. Şerife Ayaz Güner……

Member : Assist. Prof. Dr. Melis Kartal Yandım……

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The acceptance of this M.Sc. thesis has been approved by the decision of the Abdullah Gül University, Graduate School of Engineering & Science, Executive Board dated ….. /….. / …….. and numbered ……..……. .

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(Date)

Graduate School Dean
Prof. Dr. Hakan USTA

ABSTRACT

THE EFFECT OF HISTONE DEACETYLASE INHIBITORS ON PTEN/PI3K/AKT/mTOR PATHWAY AND THE CARCINOGENESIS OF CHEMORESISTANT CHOLANGIOCARCINOMA AT A MOLECULAR LEVEL

Helin Sağır
MSc. in Bioengineering
Advisor: Assist. Prof. Emel Başak Gencer Akçok

June 2021

Cholangiocarcinoma (CCA) is an aggressive adenocarcinoma and the second most common primary liver tumor. The precise etiology of the development of CCA is still not clearly defined. Since the current chemotherapeutic treatments are not effective because of the multidrug resistance, chemoresistant CCA is prevalent. The histone deacetylase inhibitors (HDACis) have shown promising anticancer properties and dysregulation of HDAC related to pathways in chemoresistance CCA such as PTEN/PI3K/AKT/mTOR which is important for autophagy. Hence, we generated cisplatin-resistant CCA cell lines and investigated the effect of HDAC inhibition by SAHA, MS-275, and Romidepsin and autophagy inhibition by Nocodazole and Chloroquine. The combination treatment of Romidepsin and Nocodazole has decreased the proliferation of cisplatin-resistant cells. The apoptosis assay has been performed, the result has been proved the increase in early apoptotic and apoptotic cell death. Also, cell cycle analysis results have shown apparent cell cycle arrest. We checked the protein expression level of PTEN, Histone 3 (H3), and Acetylated H3 by western blotting. The results were showing the possible association between the PTEN protein expression and HDAC inhibition. Since the PTEN localization is crucial in the case of resistance, we detected the localization of PTEN in sensitive and resistant cells. Outcomes were showing the PTEN translocation to the cytoplasm in cisplatin-resistant cells. In conclusion, combination therapy of HDAC and autophagy inhibition is may be a promising therapy against chemoresistant cholangiocarcinoma.

Keywords: Chemoresistance, Cholangiocarcinoma, HDAC inhibitors, Autophagy, Combination therapy

ÖZET

HİSTON DEASETİLAZ İNHİBİTÖRLERİNİN PTEN/PI3K/AKT/mTOR YOLAĞI VE KEMOREZISTAN KOLANJİOKARSİNOMA GELİŞİMİNE OLAN ETKİLERİNİN MOLEKÜLER DÜZEYDE BELİRLENMESİ

Helin Sağır
Biyomühendislik Anabilim Dalı Yüksek Lisans
Tez Yöneticisi: Dr. Öğr. Üyesi Emel Başak Gencer Akçok
Haziran 2021

Kolanjiokarsinom (CCA) agresif bir adenokarsinomdur ve ikinci en sık görülen birincil karaciğer tümörüdür. CCA gelişiminin kesin etiyolojisi hala net olarak tanımlanmamıştır. Mevcut kemoterapötik tedaviler, çoklu ilaç direnci nedeniyle etkili olmadığından, kemorezistant CCA yaygındır. Histon deasetilaz inhibitörleri (HDACis); umut verici antikanser karakteri göstermektedir ve HDAC işlevindeki düzensizlikler otofaji için önemli olan ve kemo-dirençli CCA'da bulunan yolaklar ile ilişkilidir, örneğin PTEN/PI3K/AKT/mTOR. Bu nedenle, sisplatine dirençli CCA hücre hatları ürettik ve SAHA, MS-275 ve Romidepsin yoluyla HDAC inhibisyonunun ve Nocodazol ve Klorokin ile otofaji inhibisyonunun etkisini kontrol ettik. Romidepsin ve Nocodazol'ün kombinasyon tedavisi sisplatine dirençli hücrelerin proliferasyonunu azalttı. Apoptotik analiz yapıldı ve sonuçlar erken apoptotik ve apoptotik hücre ölümündeki artışı kanıtladı. Ayrıca, hücre döngüsü analizi sonuçları, hücre döngüsünde durdurulma göstermiştir. Western blotlama ile PTEN, Histon H3 ve Asetillenmiş H3 protein ekspresyonlarını kontrol ettik. Sonuçlar, PTEN ekspresyon seviyesi ile HDAC inhibisyonu arasındaki olası ilişkiyi gösteriyordu. Direnç durumunda PTEN lokalizasyonu çok önemli olduğundan, immünofloresan boyama gerçekleştirdik ve hem hassas hem de sisplatine dirençli hücrelerde PTEN'in yerini tespit ettik. Sonuçlar, sisplatine dirençli hücrelerinde sitoplazmaya PTEN translokasyonunu gösteriyordu. Sonuç olarak, HDAC ve otofaji inhibisyonunun kombinasyon tedavisi, kemorezistan kolanjiokarsinomaya karşı umut verici bir tedavidir.

Anahtar Kelimeler: Kemorezistans, Kolanjiokarsinom, HDAC inhibisyonu, Otofaji, Kombinasyon terapisi

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Helin Sağır

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
AKT	RAC-alpha serine/threonine-protein kinase ALT
AMBRA1	Activating Molecule In BECN1-Regulated Autophagy Protein 1
AMPK	AMP-activated protein kinase
API-2	AKT/PKB signaling inhibitor-2
ASBT	The apical sodium-dependent bile acid transporter
AST	Aspartate aminotransferase
ATF2	Activating Transcription Factor 2
ATG101	Autophagy-related protein 101
ATG12	Autophagy-related protein 12
ATG13	Autophagy-related protein 13
ATG18	Autophagy-related protein 18
ATG2	Autophagy-related protein 2
ATG5	Autophagy-related protein 5
ATG8	Autophagy-related protein 8
ATG9A	Autophagy-related protein 9A
ATGs	Autophagy-related
ATP	Adenozin trifosfat
BCL-2	B-cells lymphoma 2
BSA	Bovine Serum Albumin
BTCs	Biliary tract cancers
CA 19-9	Cancer antigen 19-9
CCI-779	Temsirolimus
CK-19	Cytokeratin 19
CMA	Chaperone-mediated autophagy
CT	Computed tomography
CTCL	Cutaneous T-cell lymphoma
dCCA	Distal extrahepatic CCA
DDR	DNA damage response
DFCP1	Double FYVE-containing protein 1

DMSO	Dimethylsulfoxide
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EGFR	Endothelial Growth Factor receptor
EMT	Epithelial-mesenchymal transition
ERCP	Endoscopic retrograde cholangiopancreatography
ERK	Extracellular signal-regulated kinase
ERK1/2	Extracellularly-Regulated Kinase-1 and -2
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FIP200	Family interacting protein of 200 kD
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBC	Gallbladder carcinoma
GFR	Growth factor receptors
GGT	Gamma-glutamyl transpeptidase
GPCR	G-protein coupled receptor
GPCRs	G protein-coupled receptors
HAT	Histone acetyltransferase
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis A virus
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitors
HH	Hemochromatosis
IGFR1	Insulin Growth factor Receptor1
IL	Interleukin
IMPATH	Interactive Microcomputer Patient Assessment Tool for Health
iCCA	Intrahepatic CCA
JAK	Janus kinase
LC3	Microtubule-associated protein 1A/1B-light chain 3
LOH	Loss of heterozygosity
MDM2	Mouse double minute 2 homolog

MDR	Multidrug resistance SIRT1
MDR1	Multidrug resistance protein 1
MEK1/2	Mitogen-activated protein kinase kinase
MOC	Mechanism of chemoresistance
MRI	Magnetic resonance imagining
MRP1	Multidrug resistance-associated protein 1
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NASH	Non-alcoholic fatty liver disease
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OCT1	Organic cation transporter 1
PBS	Phosphate-buffered saline
pCCA	Perihilar CCA
PDKs	3-phosphoinositide-dependent protein kinases
PFA	Paraformaldehyde
P-gp	P-glycoprotein
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinases
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PPAR γ	Peroxisome proliferator-activated receptor γ
PTEN	Phosphatase and tensin homolog deleted in chromo- some 10
RAD-001	Everolimus
RAF1	V-Raf-1 Murine Leukemia Viral Oncogene Homolog 1
Ras	Rat sarcoma
SIRT1	Silent mating type information regulation 1 homolog
SIRT2	Silent mating type information regulation 2 homolog
STAT	Signal transducer and activator of transcription
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor beta
TKIs	Tyrosine kinase inhibitors
TKR	Tyrosine kinase receptor
TP53	Tumor Protein 53

TRAIL	Telomere homolog oligonucleotides and calpeptin
TSA	Trichostatin A
TSC	Tuberous sclerosis complex
TSC1	Tuberous sclerosis complex 1
TSC2	Tuberous sclerosis complex 2
ULK1	Unc-51-like kinase 1
VEGFR	Vascular Growth Factor receptor
VPS15	Vacuolar protein sorting 15
VPS34	Vacuolar protein sorting 34
Wnt	Wingless/Int-1

To my lovely parents; Zeynep and Devriş SAĞIR

Chapter 1

1. Introduction

1.1 Liver and Biliary Tract Anatomy

The liver is the main organ which is not only crucial for homeostasis but also plays key roles in a range of functions. These functions are including metabolism, bile secretion, drug detoxification, glycogen storage, and production of different serum proteins. Liver diseases which include fibrosis, hepatitis, end with morbidity and mortality because liver functions are fundamental for homeostasis. Most of the functions of the liver are performed by hepatocytes which are almost including 60% of total liver cells and approximately 80% of the volume of the liver. One of the epithelial cells called hepatocytes are extremely polarized and form cords. Mature hepatocytes are secreting bile and exporting the bile sequently through bile canaliculi which are surrounded via the apical membrane of adjacent hepatocytes, intrahepatic bile ducts which is forming a network connected through the canals of Hering to the bile canaliculi, extrahepatic bile ducts which is including gallbladder, the cystic, the hepatic and common bile ducts, unload the bile from the liver to the duodenum, and lastly the duodenum [1,2].

The majority of the pathways in cell signaling such as Notch, Wnt, Transforming growth factor β and sonic hedgehog signaling regulate the development of both liver and biliary tract. These major pathways are also important for both biliary and pancreatic malignancies and their pathogenesis [3]. Tumors of the biliary tract have proven to be ineffective in terms of treatment and control because of their low sensitivity to existing treatments and due to the unsuccessful detection of early tumor formation. Therefore, the etiology of the biliary tract tumors is not fully understood. The tumors of the biliary tract can originate from intrahepatic bile ducts (intrahepatic cholangiocarcinoma), extrahepatic bile ducts (extrahepatic cholangiocarcinoma), and also the gall bladder. In some regions

including Thailand and South Asia, there is a high incidence of intrahepatic cholangiocarcinoma. Since the infection of the parasitic biliary tract is also common in similar areas, the infection of liver fluke and the inflammation of chronic biliary tract has been established as a major risk factor of cholangiocarcinoma [4]. Cholangiocarcinoma comprises approximately 10-15% of all primary hepatobiliary cancers. However, in most cases, no etiological factor can be established. There are a variety of risk factors which are crucial for the development of cholangiocarcinomas, such as long-term inflammation and chronic biliary epithelium damage [5].

1.2 Types of Liver Cancer

Liver cancer is one of the most commonly seen types of cancer around the world. After lung and stomach cancer, liver cancer is the third cancer in the most common cancer types among men in developing countries. Moreover, liver cancer is 2-8 times more common in men than in women. Liver cancer is divided into two types; primary liver cancer which includes Hepatocellular Carcinoma (HCC), Cholangiocarcinoma, Angiosarcoma and Hemangiosarcoma, Hepatoblastoma, and secondary liver cancer (metastatic liver cancer) [6,7].

1.2.1 Hepatocellular carcinoma (HCC)

Hepatocellular carcinoma (HCC) is a worldwide health difficulty that mostly results in morbidity and mortality. HCC is the third type of cancer which is in the list of the deaths associated with cancer and the seventh most seen type of cancer around the world. Since the majority of the secondary tumors which are composing the epidemiology of liver cancer are more complex, histological verification is important to separate primary liver cancers from secondary tumors.

It was reported that the countries with high infections rate of Hepatitis B virus (HBV) and Hepatitis C virus (HCV) mostly have a high HCC incidence. Although there are different incidence rates in different locations of the world, in men, the risk of HCC is 2

to 7 times higher than in women. The main reasons of sex differences are; firstly, men may be exposed to liver carcinogens and hepatitis virus infection. Secondly, interleukin (IL)-6-mediated inflammation in women can be suppressed by estrogen efficacy which is reducing not only the liver damage but also compensatory proliferation. Lastly, in males, the effects of testosterone could increase the signaling of androgen receptor which is important for promoting of liver cell proliferation. HCC is a type of carcinoma that is also related with age that mostly peaks from 75 to 79 years of age [6, 8-10].

Risk Factors of HCC

According to several studies, there are crucial risk factors for liver cancer. The most well-known ones are infections of HBV and HCV, alcohol consumption, tobacco smoking, obesity and diabetes, aflatoxin, oral contraceptives, and iron overload (hemochromatosis).

HBV and HCV infection: The essential risk factors of HCC are HBV and HCV infection**.** Almost 75%-80% of primary liver cancer cases are associated with HBV (50%-55%) or HCV (25%-30%). In chronic HBV and HCV infection, HCC pathogenesis occurs as a result of proliferation, apoptosis of host cells, inflammation, fibrosis, cirrhosis, and lastly dysplasia [6,8,9,11,12].

Alcohol consumption: Extreme alcohol consumption is counted among the main risk factors of primary liver cancer. The formation of liver cirrhosis which is an HCC risk factor is the most possible mechanism of liver cancer that is related with alcohol. In addition to that, alcohol may act as a cofactor in the presence of HBV and/or HCV infection [6,10,11].

Aflatoxins: Aflatoxins are secondary metabolites which are producing by fungi naturally. The most common factor of non-infectious food-borne are aflatoxins. The extremely long-term exposure to the aflatoxins may lead to the development of HCC. The parent molecule of aflatoxin is harmless however, cytochrome p450 superfamily members are converting it to mutagenic and carcinogenic electrophilic intermediates. AFB1, AFB2, AFG1, AFG2 are major aflatoxins. AFB1 is related with HCC development in humans. The cytochrome p450 enzyme metabolizes aflatoxin and turn it into another form which

can react with the p53 protein and cause changes that are increasing the risk of malignant transformation [10,13].

Tobacco Smoking: Several studies are showing the relationship between smoking and primary liver cancer. Approximately 25% of HCC cases may be related with smoking. Since smoking is interacting with other viral and environmental factors such as drinking, a higher risk of cigarette-related HCC production in drinkers compared to non-drinkers has shown potential changes in effect, it increases the HBV and HCV associated carcinogenesis [6,10,11].

Obesity and Diabetes: In the course of the development of many cancer types, obesity is an important risk factor. Diabetes is a condition related with obesity, the main result of diabetes is high blood glucose level. Since there is a strong possibility to observe predisposition of impaired glucose tolerance in patients who have liver diseases, there is a relationship between HCC and diabetes. Fatty liver or non-alcoholic fatty liver disease (NASH) may be the potential precursor for HCC from obesity and diabetes. In addition to that, insulin resistance, hyperinsulinemia and insulin-like growth factors levels may have ability to act as mitogenic factors that induce hepatocyte proliferation [6,10].

Oral Contraceptives: The use of oral contraceptives which are including the combination of estrogen-progestogen hormones are increasing the risk of liver adenomas, therefore, leads to HCC formation. Several studies are showing that women who are long-term oral contraceptives users have benign adenomas and primary liver cancer [10,11].

Iron overload (hemochromatosis): Another possible cause of HCC is the rise in iron storage in the body. Studies are showing that iron metabolism problems, specifically hemochromatosis (HH) are increasing the risk of HCC. Since the studies are not showing the relation between the mechanism and cirrhosis development, the mechanism may be related with HBV-HBC infections and alcohol [6,10].

1.2.2 Cholangiocarcinoma (CCA)

Cholangiocarcinoma (CCA) is a type of cancer which includes a diverse group of malignancies that have features of cholangiocyte differentiation. CCAs are epithelial tumors occurring at any location through the biliary tree and express cholangiocyte differentiation markers. After HCC, CCA is counted as the second most prevalent primary liver cancer around the world however, in certain areas CCA is more common than HCC [14-18]. Classification of the CCAs is based on the location of the tumors that arise from. However there are other types of classifications such as molecular classification, genetic classification according to the mutations and gene functions, epigenetic classification depends on methylation profiles, Integrative genomic classification, emerging non-coding RNA-based classification, microenvironment based classification, proteomics-based classification, radiogenomic-based classification [19].

CCA anatomically classified as intrahepatic, perihilar, and extrahepatic. The first one is Intrahepatic CCA (iCCA) which arises close to bile ducts of the second order, secondly, Perihilar CCA (pCCA) that arises among the bile ducts of second order and the insertion of the cytic duct which is into the common bile duct. The third one is distal extrahepatic CCA (dCCA) which arises among the ampulla of Vater and the insertion of the cystic duct [15,17,20-22].

Several studies show that iCCA, pCCA, dCCA, accounted for approximately 10%, 50% and 40% of cases of CCA, respectively. These different percentages are important not only to identify the tumors which are developing in small - large bile ducts have dissimilar progression patterns and symptoms but also CCA subtypes have associations with various genetic factors [21].

Risk Factors of CCA

In most cases of CCA, there are several common risk factors which increase the possibility of CCA formation [23]

Table 1.1 Risk factors for cholangiocarcinoma [23]

Risk factors for cholangiocarcinoma	
Cholestatic liver diseases	Primary Sclerosing Cholangitis (PSC)
	Fibropolycystic liver diseases
	Congenital hepatic fibrosis
	Caroli disease
	Choledochal cysts
	Biliary hamartomas
Liver cirrhosis (any aetiology)	
Biliary stone disease	Cholecystolithiasis
	Hepatolithiasis
	Choledocholithiasis
Infections	Liver flukes
	Hepatitis B and C
	Chronic typhoid disease
	Recurrent pyogenic cholangitis
	Human Immunodeficiency Virus (HIV)
Inflammatory disorders	Inflammatory bowel disease
	Chronic pancreatitis
	Gout
	Thyrotoxicosis
Toxins	Alcohol
	Tobacco
	Thorotrast (contrast agent)
	Chemical toxins, e.g. dioxins, vinyl chloride, nitrosamines
Metabolic conditions	Diabetes
	Obesity
	Non-Alcoholic Fatty Liver Disease (NAFLD)
Genetic disorders	
	Lynch syndrome (Hereditary Non-Polyposis Colorectal Cancer)
	Bile salt transporter protein gene defects
Other	Intraductal Papillary Neoplasms of the Bile duct (IPNB)

Molecular Pathogenesis of CCA

A majority of oncogenic mutations have been identified in human CCA fragments. There are several factors such as etiology, ethnicity, tumor location and the stage of the tumor which are affecting the frequency of the mutations. Several studies showed that KRAS and TP53 abnormal gene expression, and Interleukin-6 (IL-6) overexpression in CCA patients. In addition, IL-6 production is not only crucial for MCL-1 antiapoptotic protein overexpression which makes CCA cells resistant to treatments but also affecting other signaling pathways via cross-talk. Tumor suppressor genes inactivation is one of the crucial reasons of cancer formation in CCA [15].

There are many different genes which involved in the CCA formation process. In most cases of CCA, MDM2 (Mouse double minute 2 homolog) gene is overexpressed, MDM2 is an oncoprotein that controls tumorigenesis and the key negative regulator of p53, therefore MDM2 can affect the expression and function of p53. During the cancer progression, in most cases, there are p53 accumulation in the cell. p53 is not only a tumor suppressor gene but also a transcription factor for p21. Accumulation of p53 induces the expression of a cyclin-dependent kinase inhibitor called p21, since the cell cycle process occurs and controls via many cyclins and cyclin-dependent kinases. The p53 protein has a critical role both in cancer signaling pathways and in the resistance to apoptosis [15,24]. Since the MDM2 gene expression is related with p53, p21, cell cycle regulation, and arrest which are the key factors to formation of many cancer types, it is important to understand MDM2 interactions with other crucial cellular mechanism members in the case of CCA formation. The protein MDM2 and its subcellular localization are modulated post-translationally by AKT (RAC-alpha serine/threonine-protein kinase) [25].

In addition, MDM2 transcription and isoform selection are modulated via negatively regulation of its promoter by phosphatase and tensin homolog deleted in chromo- some 10 (PTEN) [26]. PTEN is one of the tumors suppressor genes which found as deleted or mutated in many human cancers. The upregulation of PTEN gene perform by several factors to positively regulate the PTEN expression such as PPARγ, p53, ATF2 and on the other hand, TGF-β, NF-κB and Jun regulate the expression of PTEN negatively [27,28]. Several Loss of heterozygosity (LOH) studies have indicated that PTEN may have a role in advanced cancers of specific tissues. Poor prognosis of many tumors are associated with the alterations of PTEN [29,30].

In most of the human cancer types including CCA, PTEN and TP53 tumor suppressor genes are found as mutated or inactivated. Since the PTEN has the ability to regulate p53 stability and p53 can improve PTEN transcription, the association between PTEN and p53 is crucial to progression of cancer [31-34].

Table 1.2 Genetic mutations and polymorphisms associated with cholangiocarcinoma [23]

Gene abbreviation	Gene name	Protein abbreviation	Protein name	Normal function(s)a
Congenital mutations/polymorphisms				
ABCB4	ATP Binding Cassette Subfamily B Member 4	MDR3	Multidrug resistance protein 3	Transport of lipids from hepatocytes to bile
ABCB11	ATP Binding Cassette Subfamily B Member 11	BSEP	Bile Salt Exporter Pump	Transport of cholate conjugates from hepatocytes to bile
ABCC2	ATP Binding Cassette Subfamily C Member 2	MRP2	Multidrug resistance-associated protein 2	Transport of endogenous and xenobiotic compounds from hepatocytes to bile
ATP8B1	ATPase Phospholipid Transporting 8B1	FIC1	Familial Intrahepatic Cholestasis type 1	Transmembrane phospholipid transfer
COX-2	Cyclooxygenase 2	COX-2	Cyclooxygenase 2	Inflammatory cytokine
CYP1A2	Cytochrome P450 1A2	CYP1A2	Cytochrome P450 1A2	Xenobiotic metabolism
GST01	Glutathione S-transferase omega-1	GST01	Glutathione S-transferase omega-1	Detoxification of endogenous and xenobiotic compounds
KLRK1	Killer Cell Lectin Like Receptor K1	NKG2D	NKG2-D type II integral membrane protein	Tumour surveillance
MTHFR	Methylenetetrahydrofolate Reductase	MTHFR	5,10-Methylenetetrahydrofolate reductase	DNA methylation
NAT2	N-Acetyltransferase 2	ARY2	Arylamine N-acetyltransferase 2	Drug and carcinogen metabolism
NR1H4	Nuclear Receptor Subfamily 1 Group H Member 4	BAR (FXR)	Bile acid receptor (Farnesoid X receptor)	Negative feedback inhibitor of bile acid synthesis
TYMS	Thymidylate Synthetase	TYMS	Thymidylate synthase	DNA repair

XRCC1	X-Ray Repair Complementing Defective Repair In Chinese Hamster Cells 1	XRCC1	DNA repair protein XRCC1	DNA repair
Acquired mutations				
APC	Adenomatous polyposis coli	APC	Adenomatous polyposis coli	Tumour suppressor
ARID1A	AT-Rich Interaction Domain 1A	ARID1a	AT-rich interactive domain-containing protein 1A	Transcription factor
AXIN1	AXIN1	Axin-1	Axis inhibitor protein 1	Regulates apoptosis
BAP1	BRCA1 Associated Protein 1	BAP1	Ubiquitin carboxyl-terminal hydrolase BAP1	Regulates cell growth
BCL-2	B cell Lymphoma-2	Bcl-2	B-cell lymphoma 2	Regulates apoptosis
BCL2L1	B Cell Lymphoma Like 1	Bcl-xL [b]	B-cell lymphoma-extra large	Inhibits apoptosis
		Bcl-xS [b]	B-cell lymphoma-extra small	Promotes apoptosis
BRAF	B Rapidly Accelerated Fibrosarcoma	B-Raf	B-Rapidly Accelerated Fibrosarcoma	Proto-oncogene
BRCA1	Breast Cancer 1	BRCA1	Breast cancer type 1 susceptibility protein	Tumour suppressor and DNA repair
BRCA2	Breast Cancer 2	BRCA2	Breast cancer type 2 susceptibility protein	DNA repair
CCND1	Cyclin D1	CCND1	G1/S-specific cyclin-D1	Regulates cell growth
CDH1	Cadherin 1	E-cadherin	Epithelial cadherin	Tumour suppressor, cell adhesion
CDK6	Cyclin-Dependent Kinase 6	CDK6	Cyclin-Dependent Kinase 6	Controls cell cycle and differentiation
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A	p16 [b]	Protein 16	Tumour suppressor
		p14arf [b]	Protein 14 Alternate Reading Frame	Tumour suppressor
CTNNB1	Catenin Beta 1	B-catenin	B-catenin	Proto-oncogene
EGFR (ERBB1)	Epidermal Growth Factor Receptor	EGFR (ErbB-1)	Epidermal Growth Factor Receptor	Proto-oncogene
ERBB2 (HER2)	Avian Erythroblastosis oncogene B2	ErbB-2 (HER2)	Receptor tyrosine-protein kinase erbB-2	Proto-oncogene
FBXW7	F-Box And WD Repeat Domain Containing 7	FBXW7	F-box/WD repeat-containing protein 7	Component of proteasomal protein degradation pathway
FGF19	Fibroblast Growth Factor 19	FGF19	Fibroblast Growth Factor 19	Regulation of bile salt synthesis

FGFR2	Fibroblast Growth Factor Receptor 2	FGFR2	Fibroblast Growth Factor Receptor 2	Cell surface receptor regulating cell proliferation, differentiation, migration and apoptosis
IDH1	Isocitrate dehydrogenase 1	Isocitrate de-hydrogenase 1	Isocitrate dehydrogenase (cytoplasmic)	Glucose metabolism, indirectly mitigates oxidative stress
IDH2	Isocitrate dehydrogenase 2	Isocitrate de-hydrogenase 2	Isocitrate dehydrogenase (mitochondrial)	Glucose metabolism, indirectly mitigates oxidative stress
Keap1	Kelch-like ECH-associated protein 1	KEAP1	Kelch-like ECH-associated protein 1	Prevents Nrf2-driven transcription
KRAS	Kirsten Rat Sarcoma	K-Ras	Kirsten Rat Sarcoma	Proto-oncogene
LTO1	LTO1, ABCE1 maturation factor	LTO1	Protein LTO1 homolog	Ribosome biogenesis
MCL-1	Myeloid Cell Leukaemia 1	Mcl-1 (3 isoforms) [b]	Induced myeloid leukaemia cell differentiation protein Mcl-1	Isoform 1 resists apoptosis, isoforms 2 & 3 promote apoptosis
MDM2	Mouse Double Minute 2	Mdm2	E3 ubiquitin-protein ligase Mdm2	Proto-oncogene, p53 inhibitor
MYC	Avian myelocytomatosis virus oncogene cellular homolog	Myc	Myc proto-oncogene protein	Proto-oncogene
NF1	Neurofibromin 1	NF1	Neurofibromin	Stimulates Ras activity
PBRM1	Polybromo 1	PBRM1	Protein polybromo-1	Negative regulator of cell proliferation
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha	PIK3CA	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform	Generates PIP3 that activates signalling cascades for cell growth, survival and motility
PRSS1	Protease, Serine 1	TRY1	Trypsin-1	Serine protease
PRSS2	Protease, Serine 2	TRY2	Trypsin-2	Serine protease
PTEN	Phosphatase And Tensin Homolog	PTEN	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN	Tumour suppressor

RAD51AP 1	RAD51 Associated Protein 1	RAD51AP1	RAD51 Associated Protein 1	DNA damage repair
RASSF1A	Ras association domain family 1 isoform A	RASSF1A	Ras association domain-containing protein 1 isoform A	Tumour suppressor
ROS1	Reactive Oxygen Species Proto-Oncogene 1, Receptor Tyrosine Kinase	ROS1	Proto-oncogene tyrosine-protein kinase ROS	Epithelial cell differentiation, activation of signal pathways of cell differentiation, proliferation, growth and survival
SMAD4	Small Mothers Against Decapentaplegic 4	SMAD4	Small Mothers Against Decapentaplegic 4	Tumour suppressor, transcription factor
SOCS3	Suppressor Of Cytokine Signaling 3	SOCS3	Suppressor Of Cytokine Signaling 3	Signal transduction inhibitor
TP53	Tumour Protein 53	p53	Protein 53	Tumour suppressor

1.3 Chemoresistance in CCA

1.3.1 Chemoresistance mechanisms in CCA

Drug resistance is one of the most well-known situations which occurs after the diseases have become resistant to pharmaceutical treatments. The resistance mechanism was firstly observed when bacteria gained resistance to specific antibiotics, later on, similar resistance mechanism have been observed in other diseases including cancer. There are various resistance mechanisms, some of them are specific for each disease while others are evolutionary conserved. Drug resistance mechanisms do not always occur from the onset of the treatment, it is also possible to observe the resistance developing after the pharmaceutical treatment.

Several factors such as epigenetic changes, cell death inhibition, DNA damage repair, drug inactivation, drug efflux, epithelial-mesenchymal transition, drug target alteration are directly or indirectly promote the drug resistance mechanisms in human cancer cells [35].

Drug Inactivation

Drugs have the ability to initiate complex mechanisms to interact with different proteins and perform its activation. These interactions are crucial for modification of proteins, partially degradation, formation of the complexes which include drug and proteins or drug and other molecules. All these processes are the key factors that lead to activation [35,36].

Alteration of drug targets

The molecular target of the drug and a variety of alterations of this target including mutations, expression level modifications are crucially important for the drug efficacy. In the case of cancers, target alterations are an important cause of drug resistance [35].

Drug Efflux

One of the well-known mechanisms of drug resistance in cancer is the drug efflux mechanism which reduces the accumulation of drug within the cell by enhancement of efflux process. In healthy cells, there are specific types of regulators at the plasma membrane called ATP-binding cassette (ABC) transporter family proteins which are capable of performing the efflux process. ABC transporters are important transmembrane proteins, these transporters are not only for human cells but also found in entire existing phyla. The efflux mechanism depends on two main domain's of transporters, the first one is nucleotide binding domain which s highly conserved and second one is transmembrane domain which is more variable [35]. The efflux mechanism is a key mechanism to prevent the accumulation of toxins in the cell. The process starts with substrate binding to the transmembrane domain and continues with the conformational change via ATP hydrolysis at the nucleotide binding site. The change which occurs in the conformation pumps the substrate out of the cell [35,37].

Since the function of ABC transporters is protecting the body against harmful molecules and drugs by pumping them into the intestinal lumen and bile duct, the expression level of ABC transporters is high in the intestine and liver epithelium [35,38]. ABC transporter associated drug efflux mechanism is important to protect cells against toxic molecules but also it is an important mechanism to gain resistance against drugs in

the case overexpression in cancer. In many drug resistance cancer types, there are three important transporters which play key roles in resistance mechanism. These are MDR1, MRP1, and BCRP. The overexpression of transporters prevent the effects of the first line chemotherapies and protects cancer cells from pharmacological inhibition [35,39]. The first of these to be defined and extensively studied was MDR1, which produced P-glycoprotein (P-gp) [40]. Since some of the oncogenic kinases are activated in many type of cancers, it would be beneficial to reduce P-gp expression via targeting of these oncogenic kinases and sensitize cancer cells to drugs [35].

DNA Damage Repair

DNA damage repair mechanism is one of the most well-known mechanisms in anticancer drug resistance. The mechanism of DNA damage response (DDR) has a capacity to reverse drug-induced damage in response to chemotherapy drugs which target DNA damage in a direct or indirect way. Targeting DNA damage repair pathways in combination with chemotherapy can induce DNA damage and might sensitize cancer cells and such a way could increase the effectiveness of the treatment [35].

Cell Death Inhibition

Two major regulatory processes of cell death are apoptosis and autophagy. Although these processes are antagonists to each other, both lead to the death of cells. Apoptosis is a process of programmed cell death which is crucial for various types of processes that include embryonic development, cell turnover, immune system, and cell death [41]. Apoptosis consists of two main pathways; an intrinsic pathway which includes BCL-2 family proteins, Akt, and caspase-9 and an extrinsic pathway which is associated with cell death receptors of cell surface. Both of these pathways activate the caspase-3 enzyme which is a downstream caspase and lead to apoptosis process. In various types of cancer, overexpression of the proteins have an antiapoptotic character such as BCL-2 family proteins and Akt is one of the common conditions.

Another well-known condition is highly active downstream transcription modulators such as NF-κB and STAT (Signal transducer and activator of transcription). Since the targets are important in drug development against cancer cells for successful therapy, targeting these overactive proteins and modulators might be beneficial for drug development [35].

Several studies with inhibitors such as kinase inhibitors, histone deacetylase inhibitors (HDACi), BCL-2 family inhibitors, and protease inhibitors have shown promising results in drug trials [35,42]. Another major cell death process is autophagy which involves lysosomal degradation. During the autophagy process, cellular organelle and protein degradation occurs in order to protect cellular biosynthesis and viability under compelling circumstances such as nutrient deprivation or stress. Autophagy has two important roles in cancer; the first one is the inhibition of tumor initiation due to its tumor suppressor activity, the second one is to facilitate the survival of cancer cells under the metabolic stresses due to anticancer agents and caused drug resistance mechanism [42,43].

Epithelial-Mesenchymal Transition (EMT) and Metastasis

Epithelial cells can turn into mesenchymal cells via the epithelial-mesenchymal transition (EMT) process where solid tumors turn into metastatic cells. Mesenchymal cells have the capacity to increase motility and invade the body which are the main steps of metastasis. Epithelial and mesenchymal cells have their own specific surface markers. During the EMT, the expression of some cell adhesion receptors of epithelial cells such as integrins and cadherins, which are important for cell-cell attachment, decrease, and at the same time other cell adhesion receptors, which help cell motility increase. Nowadays, several studies are demonstrating the link between EMT and resistance of therapies [42]. During EMT, several signaling processes of differentiation occur which may play important roles in the process of development of drug resistance. However, the association between EMT and drug resistance depends on the degree of EMT and the differentiation level [35].

Cancer Cell Heterogeneity

Drug resistance is not a process that develops only in progenitor or mature cancer cells. Several studies have shown that heterogeneous populations of cancer cells which include stem cell properties are mostly drug resistant. Tumors which contain different types of clonal subpopulations may display different characteristics, such as different levels of resistance or sensitivity against different drugs [35,44].

Epigenetics in Cancer Drug Resistance

Resistance to chemotherapy is one of the common phenomena in human tumor cells. Chemoresistance mechanisms of tumor cells are mainly; defense mechanisms, drug-target interactions, and cellular response, as mentioned above [45]. Recent studies have shown different effects of epigenetic mechanisms on the development of cancer and drug resistance. DNA methylation and histone modification either by acetylation or methylation are two main forms of epigenetic changes. These two processes are crucial for the regulation process of gene expression throughout chromosomes. In cancer formation and development, this regulation is disrupted [35]. Several recent studies, which suggest the association between epigenetic alterations and the development of drug resistance, have shown the possible crucial role of the epigenetic alterations in the drug resistance development process and possible target site. Studies have shown the importance of either methylation or demethylation of MDR1 for multidrug resistance phenotype. [35,46]. According to [47] Trichostatin A (TSA, histone deactylase inhibitor) and 5-aza-2'-deoxycytidine (5AC, a DNA methyltransferase inhibitor) increased the expression of MDR1 mRNA in different levels in both gastric cells and colon cancer cells. Combination of TSA and 5AC has shown the potential to increase MDR1 mRNA levels. Consequently, these results demonstrate the effect of different epigenetic regulations includes DNA methyltransferase inhibitors (DNMTis) and histone deacetylase inhibitors (HDACis) on combination therapy [47]

Epigenetic alterations also have the capacity to influence the mechanism of DNA damage repair which has an effect in the process of the development of drug resistance. Since epigenetic changes plays a role not only in progression of drug resistance but also in cancer and cancer stem cell formation in many types of cancer, epigenetic therapy may have the capacity to sensitize cancer cells which have drug resistance [35,48]. According

to several studies, HDACis treatment sensitizes both ovarian and breast cancer cell lines to the TRAIL, telomere homolog oligonucleotides, and calpeptin [49-51]. According to the study that classified the efficacy of the mechanism of chemoresistance (MOC), there are seven major groups of MOC. Classification depends on the limitation that demonstrates the success in the treatment of CCA with chemotherapy [52,53]. Since the complex structure of MOC have the capacity to work with cancer cells and help them to escape from the effects of drugs, in chemoresistance CCA, MOC has a crucial role. Because of these resistance mechanisms, CCA shows a poor response to anticancer agents. In addition to that, MOC has a crucial role in the characterization of the multidrug resistance (MDR) phenotype [53].

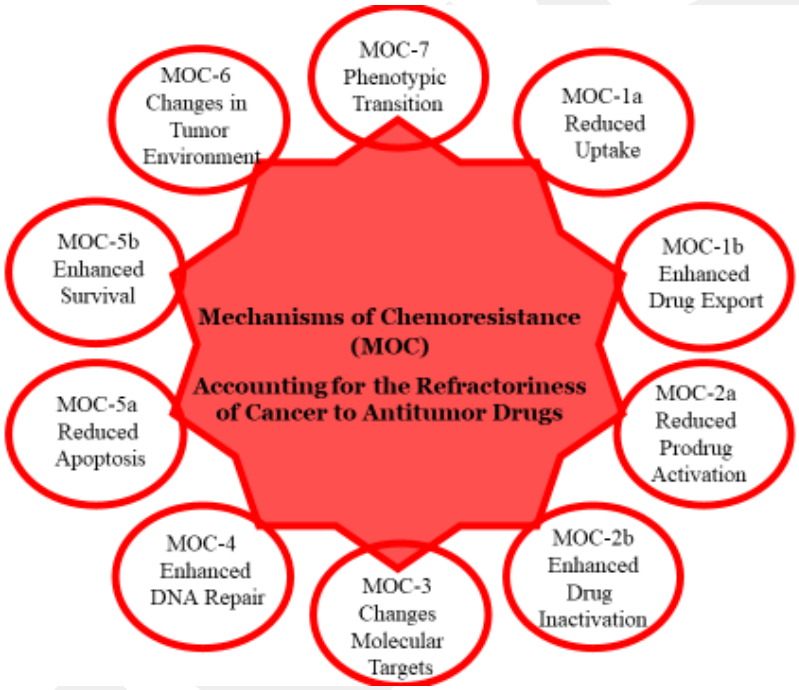


Figure 1.1 Classification of the mechanisms of chemoresistance (MOC) involved in the poor response of cholangiocarcinoma (CCA) to available chemotherapy. (Adapted from [53])

1.3.2 Diagnosis of CCA and chemoresistance CCA

Tests for diagnosis of cholangiocarcinoma

Liver Function Test: Blood tests which show the function of the liver and gallbladder with specific substances in the blood. Checking the amount of bilirubin, albumin and liver enzymes such as AST, GGT, ALT, and alkaline phosphatase in the blood. [54]

Tumor marker Test: CA 19-9 is one of the cancer antigens for CCA diagnosis. In bile duct cancer cells. CA 19-9 protein expression is high however this level in blood does not shown certain bile duct cancer, other diseases that are related to the bile duct also increase the expression of the protein [54].

Test with a small camera: Observation of the area where bile ducts and small intestine connect with each other via small camera. The process called endoscopic retrograde cholangiopancreatography (ERCP) [54].

Imaging tests: Imaging tests can be beneficial to check abnormalities. There are a variety of the image tests that use magnetic fields, x-rays, waves of sound to create image of inside body. Imagining tests include ultrasound, computed tomography scan, magnetic resonance imagining, Cholangiography, Angiography, Biopsy, Laparoscopy [54,55].
In chemoresistance CCA diagnosis, firstly diagnosis of CCA should perform with the tests which mentioned above. Since it is not possible to determine the possibility of developing any chemoresistance clearly prior to chemotherapy and estimate the failure the therapy, it is important to examine molecular markers and evaluate the possible resistance mechanisms.
Additionally, a commercial company, IMPATH (Interactive Microcomputer Patient Assessment Tool for Health), is providing a cell culture-based drug resistance assay. This strategy measures the uptake of 3 H-thymidine into tumor cells that were cultured and obtained by biopsy. An algorithm determines the probability of the patient's response to a variety of treatments with several drugs [56]. After diagnosis, the second step is to choose the appropriate treatment method. Current treatment methods of cholangiocarcinoma include; surgery, liver transplant, chemotherapy, radiation therapy, photodynamic, and biliary drainage [57].

1.3.3 Current methods to overcome chemoresistance

In chemoresistant CCA, the approaches to overcome drug resistance are crucial to complete the chemotherapy treatment.

Targeted therapies: Therapies that target TKR (tyrosine kinase receptor) to inactivate. One of the promising methods for several cancer types is, development of a wide family

of TKIs (tyrosine kinase inhibitors) with monoclonal antibodies that are capable of inhibiting TKR activity. In clinical oncology, the TKI which is called imatinib was firstly used. In the treatment of advanced HCC, sorafenib is one of the TKIs which has shown some efficacious effect. In the CCA treatment, sorafenib decreased the proliferation of CCA cells and improved apoptosis in vitro. Since the biological variability in CCA is a limitation for targeted therapy, results of the sorafenib treatments with different patients might show distinct effects. Therefore the therapeutic efficacy of sorafenib in patients with biliary cancers is still unknown in clinical practice. Another well known combination targeted therapy for CCA includes gemcitabine and oxaliplatin. Additionally, the combination of gemcitabine, oxaliplatin with erlotinib has shown certain antitumor benefit. Several targeting therapy drugs are under evolution for better results [53].

Enhancing intracellular drug concentration: Increasing the number of inhibitors that can interact with the action site is a beneficial method to enhance the efficacy of anticancer drugs. Several antitumor drugs enter the cell via diffusion through the cell membrane, changing the membrane fluidity of cancer cells make them more sensitive to anticancer drug influx, therefore, help to overcome MDR. Nanoparticles containing anticancer drugs can enter the cell via endocytosis, this lipid-polymer nanoparticles give rise to increased intracellular concentrations of anticancer drugs and cell killing rates [53]. One of the possible limitations in the nanoparticle drug complex method is leak and deterioration in systemic circulation however targeted systems of nanocarriers with antibodies to cancer specific targets have shown promising results [53,58]. Intracellular pH related strategy is another way to overcome chemoresistance. Since lysosome has inner ph value than cytoplasm when nanoparticles fuse with lysosome nanoparticles release the drug. This strategy may help to treat chemoresistance tumor cells with nanoparticles contain anticancer drugs encapsulated in ph-sensitive formulations [53,59].

CCA chemotherapy with using bile acids as Trojan horses: In liver cancer, several studies have shown promising results in the use of derivatives of bile acid as conjugated molecules for the treatment. Three main conditions in this method; i) a certain level of expression of bile acid transporter must be performed by liver tumors; ii) these transporters must also be capable of absorbing the synthetic conjugate of bile acid; iii) some of the pharmacological activity of the parent drug must be preserved by the new

complex. Bamet-UD2 is an acid derivative contains cisplatin-ursodeoxycholic and it provides all these requirements [53,60].

Enhance the expression level of drug uptake transporters via gene therapy: Bamet-UD2 is the most well known bile acid derivative. In many CCA cases, the expression of the ASBT is observed, it is possible to use other bile acid derivatives. There are several other available inhibitors such as sorafenib that are mostly taken up by OCT1 however they are not giving stable results. Studies have shown that the expression of this carrier in HCC and CCA is significantly reduced [53,61].

According to the studies, it may be possible to overcome the chemoresistance in CCA with viral or non-viral vectors [53]. Since the chemotherapy efficacy is dependent on the up-regulation of ABC export pumps, promoters have a considerable function in the regulation of expression. With specific vectors, that have an ability to express proteins, such as drug transporter or a tumor suppressor, controlled via a specific promoter which is found in the tumor cell of interest and upregulated, and by this way gene therapy may be possible [62]. TERT and CK-19 are tumor specific promoters that are found in CCA, also they have the potential to be beneficial in adenoviral gene therapy in CCA [63].

Inhibition of drug export pumps via chemosensitizing agents development: Inhibition of ABC transporters is a popular strategy in the last few decades. The combination therapy of chemosensitizers includes inhibitors and anticancer drugs with lower doses. Classification of chemosensitizers are; ı) First generation drugs such as quinidine, verapamil, have shown the blocking of ABC pumps in vitro and in vivo however since they were not specific, their side effects in normal tissues and organs caused them to fail in clinical trials. ıı) Inhibitors of the second generation such as PSC-833 and valspodar were designed to enhance drug efflux inhibitory properties but unexpected interactions such as pharmacokinetic interactions were observed. ııı) Third generation inhibitors such as laniquidar, and zosuquidar have shown promising results but in clinical trials they have not been proven to be successful yet [53,64].

Inhibition of ABC-transport-mediated drug efflux with nanotechnological strategies: Nanotechnology increases the chance to fight cancer with specific nanotechnological substances to improve the efficacy of treatment. Natural polymers, such as alginates xanthan gum, dextran, gellan gum, and fucoidan, are anionic

polysaccharides which enhance oral bioavailability and help to overcome MDR in tumor cells [65]. Additionally, studies have demonstrated the inhibitory effects of thiolated derivatives of chitosan against MDR1 and MRPs [66,67]. Studies have shown that polymers such as poly(alkyl cyanoacrylate) which do not have amphiphilic features can overcome MDR [68]. Another strategy is using the surfactants polyethoxylated castor oil and polysorbate 80 with non-cytotoxic doses to reverse MDR protein function [69]. There are several studies that have shown the success of nanotechnology substances to overcome chemoresistance mechanisms [53].

Inhibition of ABC-mediated drug efflux via TKIs: Since various TKIs are substrates of ABC pumps, combination therapy with anticancer drugs that are the substrate for the same ABC pumps can improve chemotherapy treatment in cancer. The combination of TKIs and cytotoxic anticancer agents may be an option to overcome chemoresistance [53].

miRNA treatment: One of the non-translated RNA types is miRNA that has a capacity to regulate cellular functions such as apoptosis, senescence, and drug resistance. Studies demonstrated that miRNA have a role in the regulation of MDR in CCA. Since miRNA has a role in the generation of anti-apoptotic and chemoresistant phenotype, miRNAs might be biomarkers to target for the treatment of chemoresistance CCA [62].

1.4 Histone deacetylase (HDAC)

The genomic DNA of the eukaryotic cell is packed around extremely conserved histone proteins H2A, H2B, H3, and H4 into nucleosomes. The complex structure of nucleosomes forms the key and main units of chromatin. The control of gene expression has crucial parts, one of the main parts is chromatin organization [70]. Gene expression is not only changed by altering the DNA sequence but also is changed via epigenetic modifications. Epigenetic modifications have the ability to regulate gene expression both heritable or reversible without changing the DNA sequence. The epigenetic modifications can regulate chromatin organization via modifying the architecture and accessibility of chromatin [71,72].

Epigenetic regulation of gene expression has two main mechanisms. DNA methylation and acetylation of histones post-translationally [73]. Acetylation of histones reduces the affinity of DNA to histones and releases the interaction between histone tails and the linker DNA, therefore, opens the structure of condensed chromatin. The open structure allows access of transcription factors, RNA polymerase II, and co-factors complexes to the DNA. There are two main enzymes that regulate the levels of acetylation, histone acetyltransferase (HAT) and histone deacetylase (HDAC). HATs targets the lysine-rich amino-terminal tails of histone proteins and acetylate lysines. HATs neutralize the charge and form open, relax, and transcriptionally available, active chromatin structures. On the other hand, HDACs target acetyl groups from 1-N-acetyl lysine amino acids on histone proteins. HDACs remove the acetyl group and return the effect of HATs on the histone proteins. HDACs reform histone proteins with basal state and prevent transcriptional availability. The close structure of chromatin mostly suppresses gene expression [70]. HDACs are mostly found in the complexes of co-repressor multiprotein. In these complexes, they can mediate the removal of an acetyl group of histone tails, specifically from lysine residues. In mammals, there are 18 HDACs [74].

HDACs are classified depending on their homology to their yeast counterparts, thereby HDACs can be classified into four main classes [75]. Class I consist of HDAC1, 2, 3, and 8. These HDACs have shown homology with Rpd3 in yeast, additionally, they are mostly found in the nucleus. Class I HDACs have shown their expression in a variety of mammalian cell lines and tissues [76]. Class II includes HDAC4, 5, 6, 7, 9, and 10. Class II members have shown homology with Hda1 protein. Class II is classified into two main subclasses; Class IIa (HDAC4, 5, 7, and 9) and class IIb (HDAC6, and 10). Class II has shown specific expression depends on the tissue. Moreover, Class II HDACs can change their location among the nucleus and cytoplasm via a phosphorylation-regulated manner. The change in the location of class II HDACs, suggests their functions in not only acetylation of histone proteins, but also for non-histone proteins [70]. Class III includes Sirt1, 2, 3, 4, -5, 6, and 7. These HDACs have shown a homology with Sir2 and SIRT1-7 [77]. HDAC11 is the only member of class IV. There are some common features that classes I, II, and IV share. These features include these classes' enzymatic activity and inhibition by HDAC inhibitors (HDACis), such as trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), and LB589, related with dependence on

zinc^{2+}. However, class III HDACs, depend on NAD+, this feature makes them insensitive to TSA [78].

1.4.1 Histone Deacetylase Inhibitors (HDACis)

HDAC enzymes inhibition is performed by both natural or chemical compounds called HDAC inhibitors (HDACis). Several HDAC inhibitors have been formed to target HDACs' catalytic sites. Inhibition of HDACs can affect the balance between HATs and HDACs. This change in balance results in acetylated histones accumulation. Since the acetylation and deacetylation of histones affect chromatin organization, inhibitors can change the molecular effects related to the expression [70]. Four major ways that can be affected by balance change are; signaling pathways, such as ERK and Wnt, proteasomal degradation, protein kinase C activity, and status of DNA methylation [79]. The classification of HDACs is dependent on their structure and specificity. There are several classes include; hydroxamates, short chain fatty acids, cyclic peptides, benzamides, and sirtuins inhibitors [70,79].

a. Hydroxamates: Trichostatin A (TSA) is the first natural hydroxamate that has been found to inhibit HDACs [80]. However, because of the toxicity of TSA, it has been using only in laboratory experiments [79]. Vorinostat (SAHA) is the first HDAC inhibitor approved by the FDA to treat patients with cutaneous T-cell lymphoma (CTCL) [81]. There are other HDACis that are approved for different therapies such as, Belinostat which is approved for therapy of peripheral T cell lymphoma, panobinostat which is approved for therapy of multiple myeloma. Several other compounds such as, givinostat, abexinostat ,resminostat, and quisinostat, are the pan-HDAC inhibitors which are tested in clinical studies lately. In addition to these inhibitors mentioned above, there are several selective inhibitors for HDAC classes that are under clinical trials. Rocilinostat which inhibits HDAC class II, practinostat (SB939) which inhibits I, II, and IV classes, and CHR-3996 is a selective inhibitor of class I [70,79].

b. The short chain fatty acids: Valproic acid (VPA) is an inhibitor of HDAC class I and IIa, butyric acid and phenylbutyric acid inhibits HDAC class I and II [79].

c. Cyclic peptides: Romidepsin is a prodrug approved by the FDA and EMA for the treatment of CTCL, FK228 inhibits class I HDACs [82].

d. Benzamides: Entinostat (MS-275-SNDX-275), tacedinaline, and 4SC202 are inhibitors of class I HDACs. classes I and IV HDACs inhibit by Mocetinostat (MGCD0103) selectively [79].

e. Sirtuins inhibitors: Sirtuin inhibitors consist of specific SIRT1 and SIRT2 inhibitors; sirtinol, EX-527, cambinol, and pan-inhibitor nicotinamide [83]. Pan-HDAC inhibitors are inhibiting the activity of HDACs' in a broad spectrum, most of the currently available HDAC inhibitors are pan-inhibitors [70].

1.4.2 HDACis as an anticancer drug

HDAC inhibitors can affect and induce different mechanisms such as cell cycle arrest, differentiation, and cell death. Additionally, it is possible to see the effect of HDACis on immune response modulation and decreasing angiogenesis. Important mechanisms that HDAC inhibitors can affect are not only the ways it was mentioned above but also apoptosis induction, autophagy induction, altering non-coding RNA expression, effects on cellular signaling pathways are the ways might be affected. [79]. Since the various types of HDACs' effects on cellular mechanisms, and the relation of these mechanisms with cancer formation there is a hypothesis about HDACs and cancer. The hypothesis of Dawson and Kouzarides [84] has been shown the possible specific function of the HDACs in cancer cells. This hypothesis might show the importance of HDACs in cancer formation and therefore HDACis have crucial role in cancer treatment [84]. HDACis' anticancer effects mechanisms are not common in all cancer types. The features of inhibitors, and different mechanims of action could be dependent on the cancer type, the dose of the inhibitor, and other factors [85]. There are specific overexpression of different HDACs in specific cancer types, these have shown the potential of HDACis on various cancer types include prostate, ovarian, bladder, colon cancer, and hepatocellular carcinoma [70].

In addition to the single anticancer effect of HDACis, there are several examples of combination therapy of cancer with HDACis. These combinations mostly consist of chemotherapy or radiotherapy with HDACis and it is possible to see clinical trials of these combinations [86].

1.4.3 HDACis in Chemoresistance CCA

As mentioned above, CCA is a kind of fatal malignancy formation in the epithelium of the biliary. One of the most well-known treatment methods is surgical resection. The diagnosis of CCA patients is not easily found and early detection is not very common because of complexity. This main reason leads to diagnose the CCA at a late stage and makes poor survival rate, therefore the majority of CCA patients are not suitable for surgical resection and the same reasons make them not suitable for therapeutic modalities [87-89]. Limitations of therapeutic modalities make these two main therapy methods which are chemotherapy and radiotherapy, ineffective in many CCA patients. Clinical studies have shown that this resistance mechanism against chemotherapeutic drugs, such as gemcitabine, cisplatin, 5-fluorouracil, either single or combination therapy, which have been used in CCA cases, leads to a decrease in the successful response to chemotherapy in CCA recently [90-92]. The decrease in the effectiveness of chemotherapy leads to combinations of chemotherapy and other agents. As we mentioned above HDACs overexpression is specific for different cancer types which makes HDACis a potential combination therapy method [93,94]. Nowadays there are several studies to figure out which HDAC is crucial for CCA and chemoresistance CCA treatment. These studies are also demonstrated the possible combinations for therapy.

Chemoresistance mechanism of CCA and HDAC interaction is crucial to decrease the resistance level and make chemotherapy more effective [95-98]. Mastoraki and his colleagues mentioned the overexpressed HDACs in CCA. This study has shown that 21 of 35 patients with iCCA have HDAC1 expression, which means 60% of patients have positive HDAC1 expression. The prognosis of HDAC1 positive patients is worse than HDAC1 negative patients. This study demonstrated the relation between HDAC1 expression and an increase in lymph node metastasis, microscopic vascular tumor infiltration, and tumor staging [99]. Another study has shown the relation between HDACs Class I and II expression levels and CCA. Class I HDACs which are 2, 3, and 8 have shown remarkably high expression levels in the nucleus, however, these HDACs' expression levels are low in non-tumor tissues. HDAC2 and HDAC3 overexpression is correlated with the increase in lymph node metastasis. As a treatment method, HDAC2 and HDAC3 inhibitors are effective to treat these overexpressions and induce apoptosis via modulation of the cell cycle [97].

A combination of HDACis and chemotherapy agents in CCA perform by scientists recently. Iwahashi et al. have shown the potential treatment method for CCA with VPA and gemcitabine combination [100], Sriraksa and Limpaiboon have shown the successful combination effect of VPA or SAHA with 5-FU (5-fluorouracil) [95]. Depends on the specific HDACs for specific cancer types, combination therapy has the potential to treat CCA and chemoresistance CCA [99].

1.5 Autophagy

The degradation process within the lysosome which degrades the cytoplasmic components is called autophagy [101-103]. Autophagy is a different mechanism compared to the degradation of extracellular and plasma membrane proteins via lysosomal degradation which is mediated with endocytosis. Autophagy induction is mainly triggered by the starvation of nutrients, and essential nutrient lacking could initiate the autophagy process [104]. The autophagy process is not only the response to nutrient stress but also has roles in removing proteins that are misfolded or aggregated, elimination of intracellular pathogens, and clearing organelles that are damaged. The autophagy process is crucial for the promotion of cellular senescence, presentation of cell surface antigen, protection to the instability of genome, and prevention of necrosis. All of these multiple functions are giving autophagy one of the main roles in the prevention of several diseases such as autoimmune diseases, liver disease, cardiomyopathy, neurodegeneration, and cancer [105].

Autophagy is classified into three fundamental types; macroautophagy, microautophagy, and chaperone-mediated autophagy. Macro-autophagy depends on the autolysosome formation which is a unique fusion of autophagosome that is a double membrane-bound vesicle and lysosome. Micro-autophagy directly happens via the lysosome, cytoplasmic components taken inside the lysosome by the lysosomal membranes' invagination and degradation process performed by the lysosome [105]. The chaperone-mediated autophagy (CMA) with the help of chaperone-proteins. Chaperone-proteins help the translocation of the targetted proteins through the lysosomal membrane. Specific chaperone-proteins recognize by the lysosomal membrane receptor, after

recognition and crossing to the lysosome, targetted proteins are unfolded or degraded in the lysosome [106].

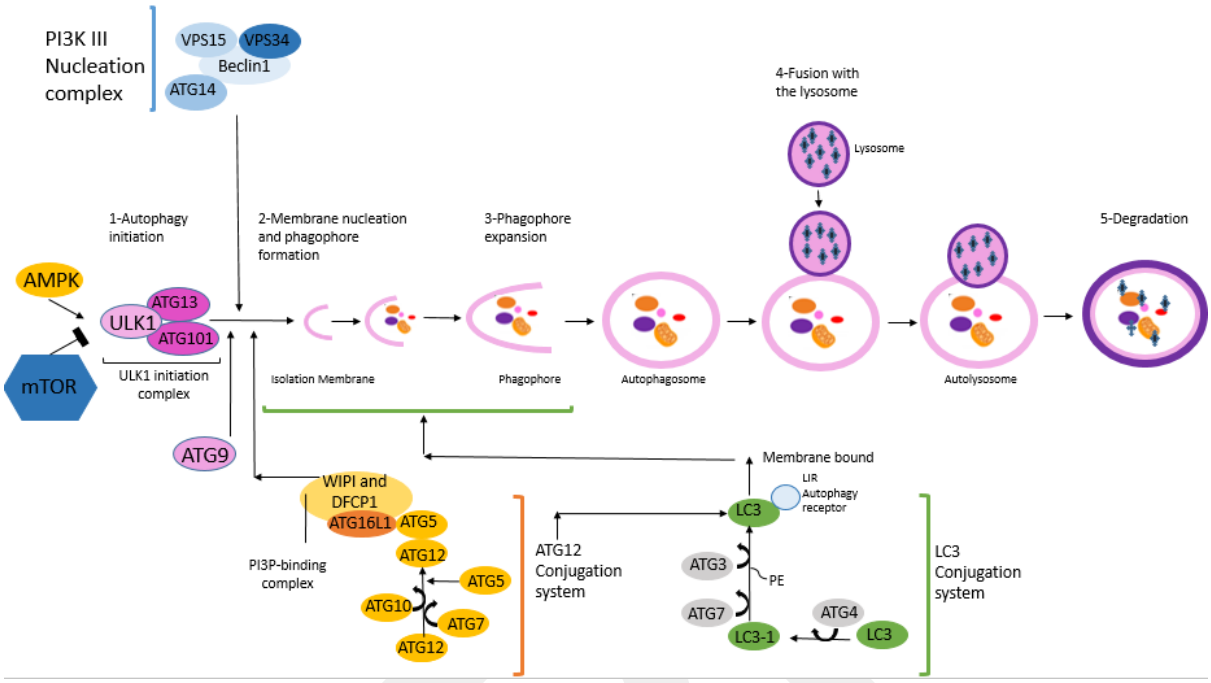


Figure 1.2 Molecular mechanisms of autophagy (Adapted from [107])

The regulation and control of the mechanism of the autophagic process are performed by a variety of proteins. The most well-known one is mTOR (mammalian target of rapamycin) which has an association with crucial mechanisms for cancer formation including cell proliferation, stress, and cancer progression. mTORC1 and mTORC2 are two complexes of mTOR, each of these two complexes have shown distinct in their functions and localizations [108-110]. The mTOR1 has an essential role in the inhibition of autophagy via phosphorylation of ATG [111]. The mTORC2 has been shown an indirect suppress on autophagy via activation of mTORC2. The activation of mTORC2 by PI3K signaling and after this interaction, AKT phosphorylation at two different sites leads to AKT/mTORC1 signaling activation [112,113]. In addition to the proteins that is mentioned above, several different non-ATG proteins such as mTOR, AMPK, AKT, AMBRA1, BCL2, DFCP1, and VPS34 have a role in the regulation and process of autophagy [114]. ATGs proteins are the main regulators and members of the autophagy process, there are six clusters depending on their function; (1) the protein kinase complex of ULK1–ATG13–FIP200–ATG101, (2) the PtdIns3K class III complex which include VPS34, VPS15, and Beclin 1 core proteins, (3) the complex of PtdIns3P-binding WIPI/ATG18 –ATG2, (4) ATG9A, the multi-spanning transmembrane protein,

(5) the ubiquitin-like system of ATG5/ATG12, (6) the ubiquitin-like conjugation system of ATG8-LC3 [115]. The six different clusters of ATGs are responsible for different steps of autophagosome formation. Moreover, these six clusters have the ability to crosstalk [116].

1.5.1 Role of Autophagy pathway in cancer

As it was mentioned above, the autophagy process has many functions which make it a key regulator in maintaining metabolism and homeostasis. Since the process can affect many different signal mechanisms and processes, the autophagy process may have the capacity to be related to cell death and survival [117-119].

In normal cells, basal levels of autophagy are necessary to maintain the biological functions of the cells, control the quality of the cell contents, the elimination of damaged organelles and old proteins for recycling, and most importantly homeostasis [104,120]. Moreover, since the autophagy process is related to the maintenance of differentiation and self-renewal which are unique and the most well-known properties of stem cells, the autophagy process is also important for cancer cells also [121,122].

In cancer cells, autophagy has two different effects. The first one is the suppression of tumorigenesis via inhibition of survival of cancer cells and induction cell death. In contrast to the first one, the second one is, facilitation of tumorigenesis via promotion of the cancer cell proliferation and thereby tumor growth [123,124]. In addition to a variety of different effects of the autophagy process on signaling pathways and cellular regulations, there is an association between autophagy and the expression of oncogenes or tumor suppressor proteins. The negative regulation of mTOR and AMPK by tumor suppressor factors leads to the induction of autophagy and thereby induce cancer suppression [125]. On the other hand, mTOR, class I PI3K and AKT may activate oncogenes, this activation leads to autophagy suppression and finally induces an increase the level of cancer formation [126]. Regulation of autophagy is important for cancer formation, this regulation may happen via the PI3K/AKT/mTOR pathway, p53 (tumor suppressor protein), crosstalk with apoptosis, calcium signaling, ER (endoplasmic reticulum) stress, Ras/RAF1/MEK1/2/ERK1/2 pathway [127].

The PI3K/AKT/mTOR pathway is one of the most well-known survival mechanisms in a variety of human cancer types. Abnormal activation of members of these pathways and cellular events related to the members often lead to cancer formation. The loss of the PTEN (tumor suppressors phosphatase and tensin-homolog deleted on chromosome 10) and TSC1 and TSC2, class I PI3K mutation, AKT overexpression, exposure to different carcinogens, tyrosine kinase growth factor receptors' activation are some of the abnormalities which may result in unexpected activation of related pathways and suppression of autophagy [128-132]. On the other hand, there are a variety of inhibitors that provide autophagy induction and modulation to treat cancer. These inhibitors include; GPCR antagonists to GFR, inhibitors such as carbamazepine, and lithium to inhibit class I PI3K, inhibitors such as API-2, and perifostine to inhibit AKT, CCI-779, rapamycin, and everolimus inhibitors to inhibit mTOR [127]. In addition to the crucial effects of autophagy on cancer formation, autophagy has also a role in chemoresistant cancer formations due to the effects on drug resistance mechanism in cancer. Nowadays several studies have shown promising results proving the effect of autophagy against the formation of drug resistance as chemotherapeutic drugs [133-136]
.

1.5.2 Autophagy in Chemoresistance CCA

Chemotherapy is one of the most well-known treatment methods for cancer patients. However, cancer cells have the potential to gain resistance via different mechanisms against a variety of chemotherapeutic drugs. This resistance mechanism is a common problem for the clinical issue for the treatment of patients. Drug resistance may be related to intrinsic reasons or acquired abilities after the treatment, mechanism of resistance is mostly about genetic or epigenetic alterations such as heterogeneity of tumor cells, alterations in drug efflux mechanism, metabolism, and tumor microenvironment. These alterations are general cellular responses to drug exposure [137]. Cholangiocarcinoma is one of the cancer type which have a chemoresistance mechanism in most of the cases as it was mentioned above. Depending on the late diagnosis of the CCA, surgery is not recommended for approximately 70% of the patients , thereby chemotherapy is the first option for treatment in most of the cases [138]. As indicated previously, CCA has a complex mechanism which called as MOC and this complex mechanism is related to MDR phenotype, this interaction makes CCA cells more prone to gain chemoresistance against anticancer drugs which results in poor prognosis [53]. The function of autophagy as a regulatory mechanism in cancer is still unclear. This uncertainty makes it hard to understand the results of autophagy either as cell death or cell survival. When considered the different roles of autophagy in cancer such as tumor suppressor, an ability to promote cancer, and drug resistance, autophagy is a contradiction in the case of cancer [139-141].

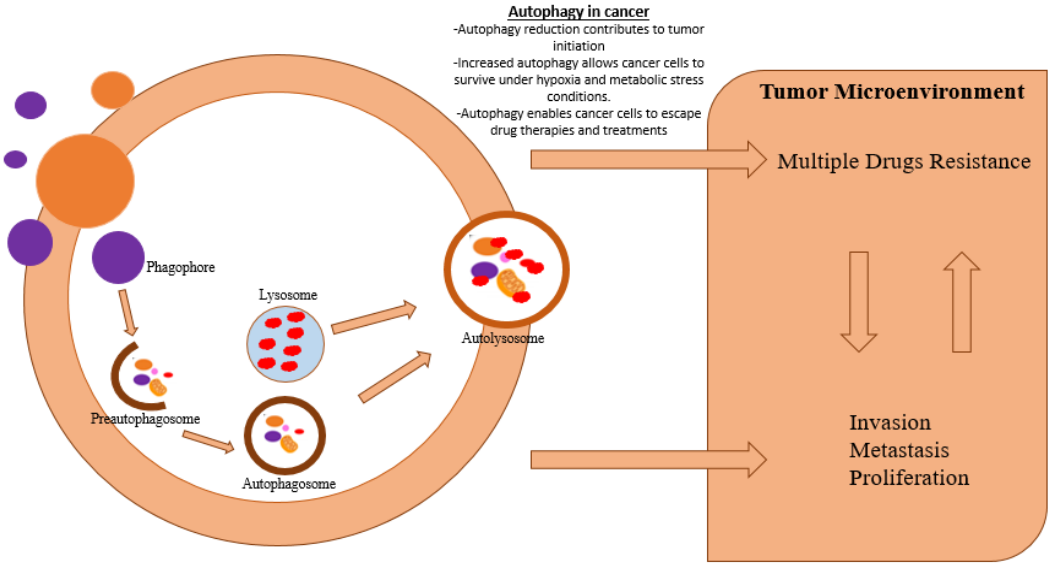


Figure 1.3 Autophagy: A novel mechanism of chemoresistance in cancers Adapted from [141]

The targetting of autophagy is one of the treatment methods for chemoresistance cancer. Inhibition of autophagy with a variety of autophagy inhibitors which are approved by the FDA such as chloroquine, hydroxychloroquine, could be a potential therapeutic method [142]. In CCA like some other cancer types, EMT (epithelial to mesenchymal transition) has a link with autophagy. This link is demonstrated with several pathways which have a regulatory role in autophagy and has impact on EMT dramatically. These pathways include Beclin1, PI3K/AKT/mTOR, JAK/STAT, and p53 signaling pathways. Moreover, a variety of signaling pathways such as WNTs, NF-kB, TGF-β, and integrins are crucially found in EMT and have roles in the autophagy process [143]. Since the EMT has a role in the development of resistance mechanism against chemotherapeutic drugs, this interaction between EMT and autophagy is important for the chemoresistance mechanism [144]. Recently, depends on the studies about resistance, researchers believe the effect of the PI3K-Akt activation in gaining resistance mechanism. One of these studies indicated the modulation effect of PI3K pathways in the mechanism of one of the chemotherapy drugs called oxaliplatin resistance in CCA cells [145]. One of the other studies to prove the effect of AKT activation in chemoresistance in CCA is performed by H.Yoon and his colleagues. This study demonstrated the association between AKT inhibition and sensitizing cisplatin resistance cells [146]. As it was mentioned above, the effects of autophagic pathways specifically the PI3K/Akt/mTOR pathway on the gaining resistance mechanism in chemoresistance CCA is proved with several studies.

Considering these studies the crucial role of activation/inactivation of this pathway makes it an excellent target point in the chemoresistance CCA [145,146].

1.5.3 PTEN/PI3K/AKT/mTOR autophagic pathway

The PI3K/AKT/mTOR pathway has a regulatory function in majority of cellular mechanisms such as cell growth, metabolism, survival, cell cycle, angiogenesis, intracellular metabolism, response to intracellular and extracellular stimuli. In addition to these roles, the PI3K/AKT/mTOR pathway has roles in carcinogenesis and progression of BTCs (Biliary tract cancers), BTCs include GBC (gallbladder carcinoma) and CCA [147,148]. In several BTCs cases, PI3K, AKT,mTOR, and the autophagy regulator called PTEN have shown alterations in their copy number, phosphorylation of proteins aberrantly, these abnormalities show the relation with poor survival [149]. The PI3K/AKT/mTOR pathway phosphorylation starts via RTKs such as IGFR1, VEGFR and EGFR, GPCRs, and the RAS familys' oncogenic proteins to trigger the activation of PI3K [150]. Activation of PI3K converts PIP2 to PIP3, this conversion provides a membrane docking site that PDKs requires. Association of PIP3 and PDKs induces the phosphorylation of AKT and thereby the activation of AKT serine/threonine kinase. Active AKT inhibits the mTOR inhibitors called TSC proteins, thereafter induces mTOR activation. In addition to all these steps, there is also a negative regulator of the cascade which is PTEN, PTEN is a phosphatase that has the ability to convert active PIP3 to PIP2 which is inactive, therefore, prevent the AKT activation [150,151].

In BTCs patients PI3K/AKT/mTOR pathway has deregulation. Alterations, mutations, and aberrant phosphorylations of PI3K, AKT,mTOR, and PTEN have an association with BTCs carcinogenesis. The alteration of the pathway is also possible with the over-activation of membrane receptors that are found upstream of the pathway and their ligands such as inflammatory cytokines, stromal-derived peptides, growth factors, and pro-angiogenic molecules [140,150-152].

In CCA patients, loss of PTEN is related with tumor differentiation, survival, and invasion depth [153-155]. PI3K/Akt/mTOR pathway is one of the crucial aberrant pathways in chemoresistance CCA. This pathway is activated in nearly 60% of iCCA patients and almost 80% of eCCA patients. These percentages clearly have shown the importance of this pathway in the case of chemoresistance CCA [156,157].

All of these studies were mentioned previously and the proves that have shown the effects, suggest the important targeting point which is PI3K/Akt/mTOR for the sensitize resistance CCA cells and the treatment of chemoresistance CCA. As it was mentioned above there are combinational therapies that depend on cancer treatment. The combination of mTOR and autophagy inhibition is one of these combinational therapies that help to overcome chemoresistance in CCA [145,146].

1.6 Aim of The Study

The autophagy pathway is a seriously complex pathway that crosstalks with a variety of other pathways. However sometimes these associations are not useful for the treatment of cancer, since the pathway has many crosstalks, single therapy via targetting of autophagy is not the best option for the treatment of chemoresistance CCA. To make the treatment method more specific, combination therapies are giving better results. For this combination, HDAC inhibitors are really good anticancer agents and have shown the potential for single and combination therapies.

Autophagy and HDACis combination is a novel combination treatment method to facilitate the sensitization of chemoresistance CCA cells and treatment of CCA formation. Considering their single treatment achievements, the potential of combining with another inhibitor, and promising results, this dual inhibition could be a potential treatment method for chemoresistance CCA.

We propose a combinational targeting therapy consist of inhibition of both autophagy and HDAC to overcome chemoresistance CCA treatment.

Chapter 2

2. Material and Methods

2.1 Maintenance of Parental Cell Lines

Two different parental extrahepatic CCA cell lines, TFK-1 and EGI-1 were selected to form resistance cell lines and perform the experiments. As a control cell line HCC cell line HepG2 was chosen.

TFK-1, EGI-1, and HepG2 cell lines were procured from the German National Resource Center for Biological Material (DSMZ), three parental cell lines were cultured under the conditions which are recommended. The culturing condition as a medium was the same for all of the cell lines, RPMI medium (Sigma, cat. no. R8758) supplemented with 10% FBS and 100 U/mL penicillin/streptomycin, incubation of the cells performed in a 5% CO2 incubator at 37°C.

TFK-1, EGI-1 and HepG2 cells were seeded out as $5x10^6$ cells /10 mL/96mm x 21mm tissue culture dish. The subculture of the cells after 60-70% confluency was performed every 2-3 days, cells were split into 1:2, using trypsin/EDTA. The trypsinization of the cells were performed after washing the cells with 5ml, 1X PBS. After removal of the washing solution, 1X trypsin was applied to the cells for 10min, 30min, 10min respectively for TFK-1, EGI-1, and HepG2. Subsequently, the cells were collected with the help of a culture medium, to inactivate trypsin enzyme activity on the cells, medium was used with an amount that is 3 times more than trypsin. After collection, cells were centrifuged at 900 rpm for 5 min. Following the centrifugation step, the supernatant was discarded and the pellet was dissolved with a new culture medium. The cells were plated into TPP 96mm x 21mm tissue culture dishes (TPP, product no: 93100).

2.2 Generation of Resistance Cell Lines

2.2.1 Drug Preparation and In Vitro Cell Viability Assay for Generation of The Resistance Cell Lines

The TFK-1, EGI-1, and HepG2 cells were separately plated 10000 cells/ 100 µL into a 96 well plate for cell viability assays, later on, incubated overnight.

The incubated cells were treated with NaCI, cisplatin (Sigma-Aldrich, Cat. No: 232120-50MG) and gemcitabine (Sigma-Aldrich, Cat. No: G6423-10MG) to calculate the IC50 value of the cells to cisplatin and gemcitabine. Cisplatin and Gemcitabine were dissolved in NaCl and the main stocks of the drugs were prepared and stored at +4 ^{o}C and -20 ^{o}C, respectively.

Subsequently, the TFK-1 and EGI-1 cells were treated with NaCI, cisplatin (5µM-200µM), and gemcitabine with different dose range (50µM-2000µM and 1000µM-15000µM) for 24, 48, and 72 hours. HepG2 cells were treated with NaCl, cisplatin (1µM-2,25µM), and gemcitabine (0,5µM-2µM) for 24, 48, and 72 hours. In order to check their IC50 values after drug treatment, Graphpad Prism 7 program was used to calculate the concentrations via proliferation curve [136].

The proliferation of the cells after drug treatment was calculated via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide Cell Viability Assay (MTT). MTT assay is a colorimetric assay that has shown promising results around the 1980s and since that time MTT assay has been used for calculating and determination cellular viability (growth and survival).

The process of MTT assay has been started with cell seeding in 96-well plates, each well has contained $10x10^3$ cells in triplicates for each dose. The cells were incubated 24, 48, and 72 hours, after incubation, 10 µl of MTT solution (cat. no. M2128; Sigma Aldrich) per well was added and subsequently, the cells were incubated between 2-4h at 37°C in a 5% CO2 incubator.

Later on the incubation, the formazan crystals, which formed during incubation with MTT solution and precipitated at the bottom of the wells, were dissolved with 100 µl of DMSO.

For homogenization of formazan crystals and DSMO mixture, plates were incubated on the shaker for 15 min and finally, the absorbance was measured and calculated via Varioskan™ LUX multimode microplate reader (Thermo Scientific™) at 570 nm.

2.2.2 Generation and Maintenance of Resistance Cell Lines

The TFK-1, EGI-1, and HepG2 cells were plated ($5x10^6$ cells/1,5mL for each well) into a 6 well plate and they were incubated overnight at 37°C in a 5% CO2 incubator. The next day, the attached cells were treated with gemcitabine (0,1 uM /1,5mL) and cisplatin (0,1 uM /1,5mL) with low concentrations to start resistance cell lines formation and incubated for 48h.

After 48h incubation, the media was removed and the cells were rinsed with, 2 ml, 1X PBS. TFK-1 and HepG2 cells were trypsinized with 400 µM 1X trypsin for 10 minutes, EGI-1 cells were trypsinized with 750 µM 1X trypsin for 20-30 minutes. After trypsinization, the cells were collected with 3 mL fresh culture media and centrifuged at 900rpm for 5 minutes. Later on the centrifugation, the supernatant was removed and the pellet was dissolved with 1,5 mL media.

The TFK-1 and EGI-1 cells were plated and incubated overnight, later on , the cells were cultured with the same concentrations of each drugs 3 passaging times. After that, the concentration was duplicated as follows; TFK-1 and EGI-1 cells were treated with cisplatin (0,2µM-0,4µM-0,8µM-1,6µM-3,2µM-6,4µM-12,8µM-17µM respectively) and gemcitabine(0,2µM-0,4µM-0,8µM-1,6µM-3,2µM-6,4µM-12,8µM-25,6µM-51,2µM-102,4µM respectively). The cells were cultured with the same concentrations of drugs 3 passaging times. The concentration of drugs increased until the IC50 value of TFK-1 and EGI-1 (IC50 of cisplatın: 17µM) achieved. The cells treated with IC50 of cisplatin for 3 weeks, during this 3 weeks, drug treatment performed almost 10 times, after each passage of the cells. Subsequently, cells were cultured without cisplatin treatment for 3 weeks. Following these culturing with and without drug treatment respectively, cells were cultured with drug treatment for 3 weeks.

2.3 Colony Formation Assay

The TFK-1 and EGI-1 parental and cisplatin-resistant cells were trypsinized at 70-80% confluent plate, collected with fresh culture medium, and centrifuged at 1500 rpm for 3mins. Later on the cell counting, the TFK-1 and EGI-1 parental and cisplatin-resistant cells were plated (1,000 cells/1mL for each well) into a 6 well plate and they were incubated overnight at 37°C in a 5% CO2 incubator. After 24 hours, the incubated cells were treated with, 17 μM and 50 μM cisplatin doses (Sigma-Aldrich, Cat. No: 232120-50MG).

Table 2.1 Concentrations of drugs for colony formation experiments

TFK Cisplatin Control + Medium	TFK Cisplatin Control + 17 μM Cisplatin	TFK Cisplatin Control + 50 μM Cisplatin
TFK Cisplatin Resistant + Medium	TFK Cisplatin Resistant + 17 μM Cisplatin	TFK Cisplatin Resistant + 50 μM Cisplatin

EGI Cisplatin Control + Medium	EGI Cisplatin Control + 17 μM Cisplatin	EGI Cisplatin Control + 50 μM Cisplatin
EGI Cisplatin Resistant + Medium	EGI Cisplatin Resistant + 17 μM Cisplatin	EGI Cisplatin Resistant + 50 μM Cisplatin

After 9-14 days, the old media of the cells were discarded and the cells were rinsed with 1X PBS. Cells were fixed with 4% PFA (1ml per well) and incubated for 15min at room temperature in a rotating shaker.

Following the removal of the PFA, cells were rinsed with 1X PBS twice.

The crystal violet (Serva, Product Code: SERV.2733501) (0.1%,1ml per well) was added to the cells and incubated for 30min at room temperature in a rotating shaker. After removal of the crystal violet, the cells were washed with dH2O to discard the excess color. The plates were allowed to air dry and the cells were visualized under the microscope.

2.4 Preparation of Drug and In Vitro Cell Viability Assay

The TFK-1 and EGI-1 parental and resistance cells were seeded 10000 cells/ 100 µL into a 96 well plate for cell viability assays and incubated overnight. The incubated cells were treated with DMSO, an autophagy inhibitor, HDACs inhibitors, and the combination of autophagy inhibitor and HDACs inhibitors, to figure out the IC50 value of the cells.

In order to manipulate the autophagy pathway, the inhibition of autophagy achieved with autophagosome-lysosome fusion agent Nocodazole [158-159-160-161-162-163] was dissolved in DMSO and the main stocks of the drug were prepared and stored at -20. Subsequently, the TFK-1 and EGI-1 cells were treated with DMSO, nocodazole (0,1µM-10µM) for 48 hours.

In order to inhibit HDACs, SAHA (suberoylanilide hydroxamic acid) , MS-275, and FK-228 (romidepsin) [95,99-100,164-165-166-167] were dissolved in DMSO, and the stock of each inhibitor prepared and stored at -20. After treatment of TFK-1 parental and cisplatin resistance cells with DMSO, Nocodazole (2,89µM), SAHA (2,25 µM), MS-275 (0,0035µM), and Romidepsin (0,0037µM) for 48 and EGI-1 parental and cisplatin resistance cells with DMSO, Nocodazole (2,15µM), SAHA (0,43 µM), MS-275 (0,53µM), and Romidepsin (0,74µM) for 48 hours. The cells proliferation were checked by MTT assay as described above.

In order to calculate their IC50 values after drug treatment, the Graphpad prism 7 program was used to calculate the concentrations via proliferation curve.

2.4.1 Combination Experiments

The combination experiments were performed with Nocodazole, SAHA, MS-275, and Romidepsin. For combination HDACis and Nocodazole, IC30 drug concentrations have been chosen.

Table 2.2 Concentrations of drugs for combination experiments

DRUGS	TFK-1 RESISTANT	EGI-1 RESISTANT
NOCODAZOLE	2,89 μM	2,15 μM
SAHA	2,25 μM	0,43 μM
MS-275	0,0035 μM	0,53 μM
ROMIDEPSIN	0,0037 μM	0,74 μM
NOCODAZOLE+SAHA	2,89 μM+2,25 μM	2,15 μM+0,43 μM
NOCODAZOLE+MS-275	2,89 μM+0,0035 μM	2,15 μM+0,53 μM
NOCODAZOLE+ROMIDEPSIN	2,89 μM+0,0037 μM	2,15 μM+0,74 μM

2.5 Protein Expression Detection via Western Blotting

TFK-1 cisplatin resistance and EGI-1 cisplatin resistance cells were seeded into the 6-well plates ($3X10^5$ cells/mL). Later on the overnight incubation, the cells were treated with drugs contain HDACis and Nocadozole for 48h. Following the incubation step, the cells were rinsed with cold PBS. After removal of the wash solution, the cells were lysed with RIPA lysis buffer (50Mm Tris-HCI, Ph 8.0, with150Mm Sodium Chloride, 1.0% Igepal CA-630 (NP-40), 0.5% Sodium deoxycholate, and 0.1% Sodium dodecyl sulfate) after addition of Pierce™ Phosphatase Inhibitor Mini Tablets (Thermo Fisher Scientific, Cat. No: A32957) and Pierce™ Protease Inhibitor Tablets, EDTA-free (Thermo Fisher Scientific, Cat. No:A32965), and incubated with RIPA on ice for 5 min.

After collecting cells with a scraper, cells were transposed to a falcon tube, then, centrifuged at +4 ºC, 8000g for 10 min. DC protein assay kit (Biorad/USA cat. no. 500-0113, cat.no. 500-0114, cat. no. 500-0115) has been used to calculate the extracted protein concentration. Varioskan™ LUX multimode microplate reader (Thermo Scientific™) has been used to measure the absorption of proteins at 750 nm. The cell lysates were loaded as 50-80 μg per well.

Gel electrophoresis (12% acrylamide gels) were performed (Biorad, München). The PVDF membranes (Serva, Product Code: SERV.4251401) were activated using 99.8 % methanol (Isolab, Product Code: 947.046) and then in 1X TNT containing 5M NaCI, 2M Tris pH 7,5 and 10 % Tween20, following the transfer, membranes were blocked with

5% dried milk (Serva, Cat. No: 42590.01) in 1X TNT containing 5M NaCI, 2M Tris pH 7,5 and 10% Tween20, 1 hour on the shaker at room temperature and they were incubated overnight at 4°C with the primary antibodies which are GAPDH (1:5,000-1:40,000; Cat. No: 10494-1-AP; Proteintech), PTEN (1: 1,000; Cat. No: 138G6; Cell signaling), H3 (1:1,000; Cat. No. 9715S; Cell Signaling), Acetyl-H3 (1:1000; Cat. No: 9677S; Cell Signaling) antibodies.

Following the overnight incubation with primary antibodies that were mentioned above, membranes were rinsed with 1X TNT buffer for 10min on shaker at room temperature and the washing step was repeated thrice. Later on the washing, the membranes were incubated with the secondary antibody for 1 hour 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) (obtained from Jackson immunoresearch europe ltd). Subsequently, the washing steps with 1X TNT buffer were repeated for 3 times and Pierce™ ECL Western Blotting Substrate (cat. no: 32106; UK) with ChemiDoc™ Imaging Systems (Biorad) were used for the signals detection.

2.6 Modulation of Autophagy via Autophagy Blocker

The modulation of the autophagy pathway was performed by an autophagy blocker called Nocodazole. With the purpose of inhibition of the autophagic pathway, autophagosome-lysosome fusion using nocodazole in DMSO (from 0,1μM to 10 μM). The activity of autophagy was determined based on the guidelines [168]. The expression level of protein was checked with the following autophagy markers: PTEN, and GAPDH. The experimental procedure that was mentioned above for western blot was performed for the detection of the expression of autophagic markers that was mentioned.

2.7 siRNA Silencing

siRNA transfection reagents were provided from Santa Cruz Biotechnology, TFK-1 cisplatin resistance cells were plated into the 6-well plates ($2x10^5$ cells/mL) with 2ml normal growth medium supplemented only with FBS, without antibiotic.

Cells were incubated overnight in a 5% CO2 incubator at 37°C. Depending on the protocol (Santa Cruz siRNA Transfection Protocol), three different Solution A and Solution B were prepared to depend on the different siRNAs, contents of the solutions are described in the table below.

Table 2.3 Concentrations of drugs for Solution A and Solution B of siRNA experiment

Solution A	Solution B
5 µl control siRNA (Santa Cruz Cat. No: sc-370007) + 100 siRNA Transfection Medium (Santa Cruz Cat. No: sc-36868)	4 µl siRNA Transfection Reagent (Santa Cruz Cat. No: sc-29528) + 100 µl siRNA Transfection Medium (Santa Cruz Cat. No: sc-36868)
2 µl siPTEN (Santa Cruz Cat. No: sc-29459) + 100 siRNA Transfection Medium (Santa Cruz Cat. No: sc-36868)	4 µl siRNA Transfection Reagent (Santa Cruz Cat. No: sc-29528)+ 100 µl siRNA Transfection Medium (Santa Cruz Cat. No: sc-36868)
5 µl siPTEN (Santa Cruz Cat. No: sc-29459) + 100 siRNA Transfection Medium (Santa Cruz Cat. No: sc-36868)	4 µl siRNA Transfection Reagent (Santa Cruz Cat. No: sc-29528)+ 100 µl siRNA Transfection Medium (Santa Cruz Cat. No: sc-36868)

Solution A and Solution B were mixed and this mixture was incubated for 30 minutes at room temperature. The cells were rinsed with 2 ml of siRNA Transfection Medium (Santa Cruz, Product Code: sc-36868), the medium was removed. For transfection of each sample, 0.8 ml siRNA Transfection Medium was added to each tube that contain the mixture of Solution A and Solution B. The mixed solution overlayed onto the cells that washed before. The cells were incubated 5-7 hours at 37° C in a CO_2 incubator.

Following the incubation, 1 ml normal growth medium which contains 2 times the normal serum and antibiotics concentration (20% FBS and 2% Pen-Strep) was added on transfection mixture, without removing anything. The cells were incubated for 20 hours, after this, the medium was replaced with a fresh normal growth medium (1X, 10%FBS, 1%Pen-Strep). After 48 hours incubation, cells were ready to analyze by Western Blotting. Later on, in the incubation step, the cells were rinsed with phosphate-buffered saline (PBS) (cold). After removal of the wash solution, the cells were lysed with RIPA lysis buffer and incubated with 250µL RIPA lysis buffer on ice for 5 min. After collecting cells with a scraper, cells were transposed to a falcon tube and centrifuged at +4, 8000g for 10 min. DC protein assay kit has been used to calculate the extracted protein

concentration, as described previously. The experimental procedure that was mentioned above for western blot was performed for the detection of the PTEN expression.

2.8. Immunofluorescence Staining

For the immunofluorescence staining of the resistant and parental cells, $5x10^5$ cells/1mL were seeded onto coverslips in a 6 well tissue culture plate, with a normal growth medium which is supplemented with 10% FBS. Cells were incubated overnight in a 5% CO2 incubator at 37°C.

After 24h incubation, the cells were rinsed with 1ml, 1X phosphate-buffered saline (PBS). Followed the aspiration of the PBS, fixation, permeabilization, and blocking steps were performed. The cells were fixed with 4% methanol-free formaldehyde (pH: 7,4), the incubation time of the fixation was 10 minutes, and the process was performed on the shaker at room temperature. Before the permeabilization, the cells were rinsed with 1ml, 1X PBS thrice, after the addition of Triton X–100 (Sigma, Cat. No: T8787) in 1X PBS, permeabilization was performed for 15 minutes at room temperature on the shaker. Later on the permeabilization, the cells were washed with 1ml, 1X PBS 3 times. Lastly blocking was performed with the mixture of 10% Bovine Serum Albumin (BSA) (Sigma, Cat. No: A9647) and 0,5% Tween20 mixture in 1X phosphate-buffered saline (PBS). Followed the 1-hour incubation of the cells with blocking mixture at room temperature on a shaker, primary PTEN antibody (1: 1,000; Cat. No: 138G6; Cell signaling) was added and incubated on the shaker at +4 for a night.

After the overnight incubation, the cells were washed with 1ml, 1X PBS 3 times, and, Anti-Rabbit IgG –FITC antibody (1:200; Cat. No: F0382; Sigma) was added on the coverslips and stayed for incubation on the shaker at room temperature for 1 hour. Subsequently, the cells were washed with 1ml, 1X PBS thrice, DAPI (4′,6-diamidino-2-phenylindole) was added, and incubated for 3 minutes in a dark place. Lastly, the cells were washed with 1ml, 1X PBS, and the coverslips were closed with slides, with the help of a mounting reagent. Analysis was performed with ZEISS LSM 900 with Airyscan 2 confocal microscope.

2.9 Cell Death Assay

With the purpose of apoptosis assay of cisplatin-resistant cells, $1x10^6$ cells/mL were plated in 6 well plates and treated with the combination of autophagy and HDAC inhibitors, later on, incubated for 48h. Following the 48h incubation, the cells were collected via trypsinization. After collection, centrifugated at 1700 rpm for 5 minutes at +4 ^oC. The cells were washed with PBS two times and later on the washing, the cells were centrifugated and the pellet dissolved in 200 µl 1X binding buffer (diluted with ultra-pure water, 1:10).

The control cells which did not exposed any treatment were dissolved in 400 µl 1X binding buffer and divided as PI+ /FITC+, PI- /FITC-. With the aim of the detection of apoptosis, 5 µL of fluorochrome-conjugated Annexin V (eBioscience™ Annexin V Apoptosis Detection Kit APC, cat. no. 88-8007-72) and 5 µL of Propidium Iodide staining solution (eBioscience™ Annexin V Apoptosis Detection Kit APC, cat. no. 88-8007-72) were added samples that contain 200 µL, but the double negative control did not exposed any PI or FITC. The samples were incubated for 15 minutes at room temperature. Analysis was performed with BD FACSAria III Cell Analyzer flow cytometer.

2.10 Analysis of Cell Cycle

For cell cycle analysis of cisplatin-resistant cells, $1x10^6$ cells/well were seeded and incubated for 24h. The resistant cells were treated with a combination of autophagy and HDAC inhibitors and incubated for 48h. The cells were detached via trypsinization and centrifuged at 260 G for 10 min at 4 °C. After centrifuge, supernatant was discarded and the cells were dissolved with 1 mL cold PBS. Following the centrifugation at 260 G for 10 min at 4 °C, the supernatant was discarded and the pellet were rinsed with 1ml PBS step was repeated two times. Following the centrifugation, the cells were dissolved with 1 mL cold PBS, and 4 mL cold ethanol (70%) was added into the falcon. The mixture contain the cells, PBS, and ethanol was homogenize via vortex. The mixture incubated overnight at -20 °C for the fixation of the cell. Followed the 24h incubation, the cells were centrifugated at 260 G for 10 min at 4 °C and the supernatant was removed completely.

The cells were dissolved with 1 mL cold PBS, and centrifuged at 260 G for 10 min at 4 °C, after centrifugation, the supernatant was discarded. The pellet was homogenized with the mixture of 1 mL 0.1% Triton-X (Merck Millipore 1.08603.1000) in PBS and 100 µL RNase (200µg/mL) (Sigma R5503) and incubated at 37 °C for 30 min. The control (untreated) was divided into two samples and the controls were propidium iodide (PI) (Sigma P4170) stained (PI+) as a positive control and PI unstained (PI-) as a negative control. 100 µL PI (1 mg/mL) was added to the samples, one by one and the untreated control was stained with PI. Following 15 minutes of room temperature incubation, the samples were analyzed via BD FACSAria III Cell Analyzer flow cytometer.

Chapter 3

3. Results

Cholangiocarcinoma is a highly aggressive adenocarcinoma. Since specific symptoms or laboratory abnormalities are absent during the early stages of the disease and the histological diagnosis is made at the time of surgical resection, where peritoneal metastasis is found in 83% of the cases, intrahepatic and extrahepatic CCA are usually detected at the later stages of the disease [8,169-172].

For patients without the advanced disease, radical surgery is the only effective treatment. However, most of the CCA patients have advanced disease with high tumor metastasis [172,173]. Besides, the current chemotherapeutic regimens are inefficient due to multidrug resistance and they are based on phase I/II clinical studies [174,175]. Cisplatin, rifampicin, mitomycin C, paclitaxel, gemcitabine, docetaxel, and 5-fluorouracil have been used as chemotherapeutic drugs for CCA but with disappointing results [169,170].

In order to understand the crosstalk between autophagy manipulation and HDAC inhibition effect on chemoresistance CCA cells, specific autophagy modulator, and HDAC inhibitors have been used on the chemoresistant cells which were generated from parental cells. According to this aim, two cholangiocarcinoma cell lines, which, EGI-1 and TFK-1 have been chosen to generate resistance cells against cisplatin and gemcitabine. All experiments of the study were performed on resistant cells in vitro.

3.1 Generation of cisplatin-resistant CCA cell lines

In order to generate the cisplatin-resistant cells from parental TFK-1 and EGI-1 cell lines, we treated TFK-1 and EGI-1 parental cells with increased doses of cisplatin. Doses have been chosen according to the previous studies that include cisplatin treatment

[146,176-183]. With the purpose of finding the most effective range of doses, several cytotoxic assays were performed for 24, 48, and 72 hours.

Various drug dosa ranges have been tried for treatment. Initially, treatments were continued for 24 hours and 48 hours until the IC50 value was found. Doses started with a low concentration and were followed by increasing concentrations (2,5-500 μM).

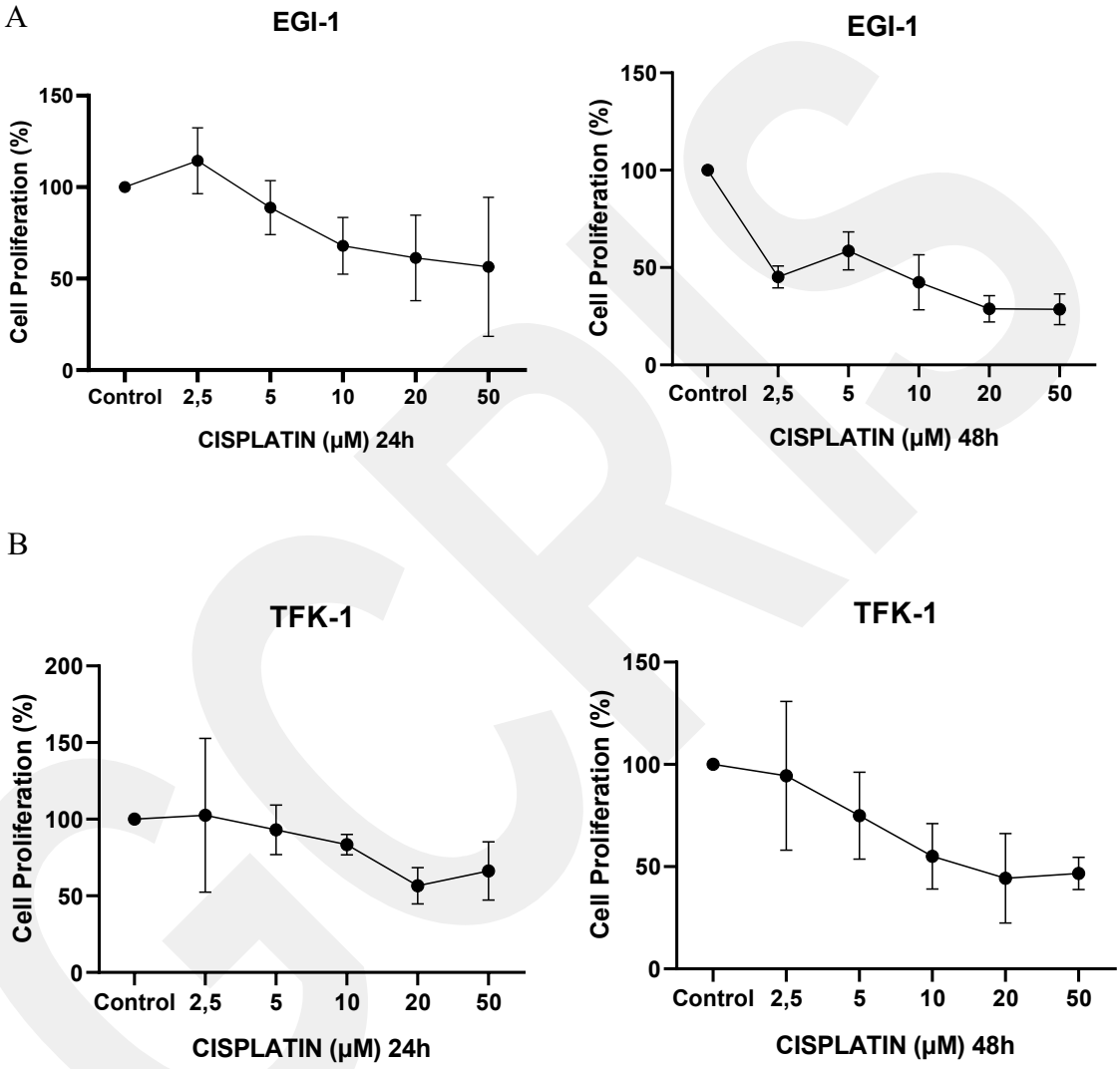


Figure 3.1 The effect of cisplatin treatment (2,5 μM to 50 μM) on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 24h, and 48h. The average of the 3 replicates performed during each experiment were imported to GraphPad.

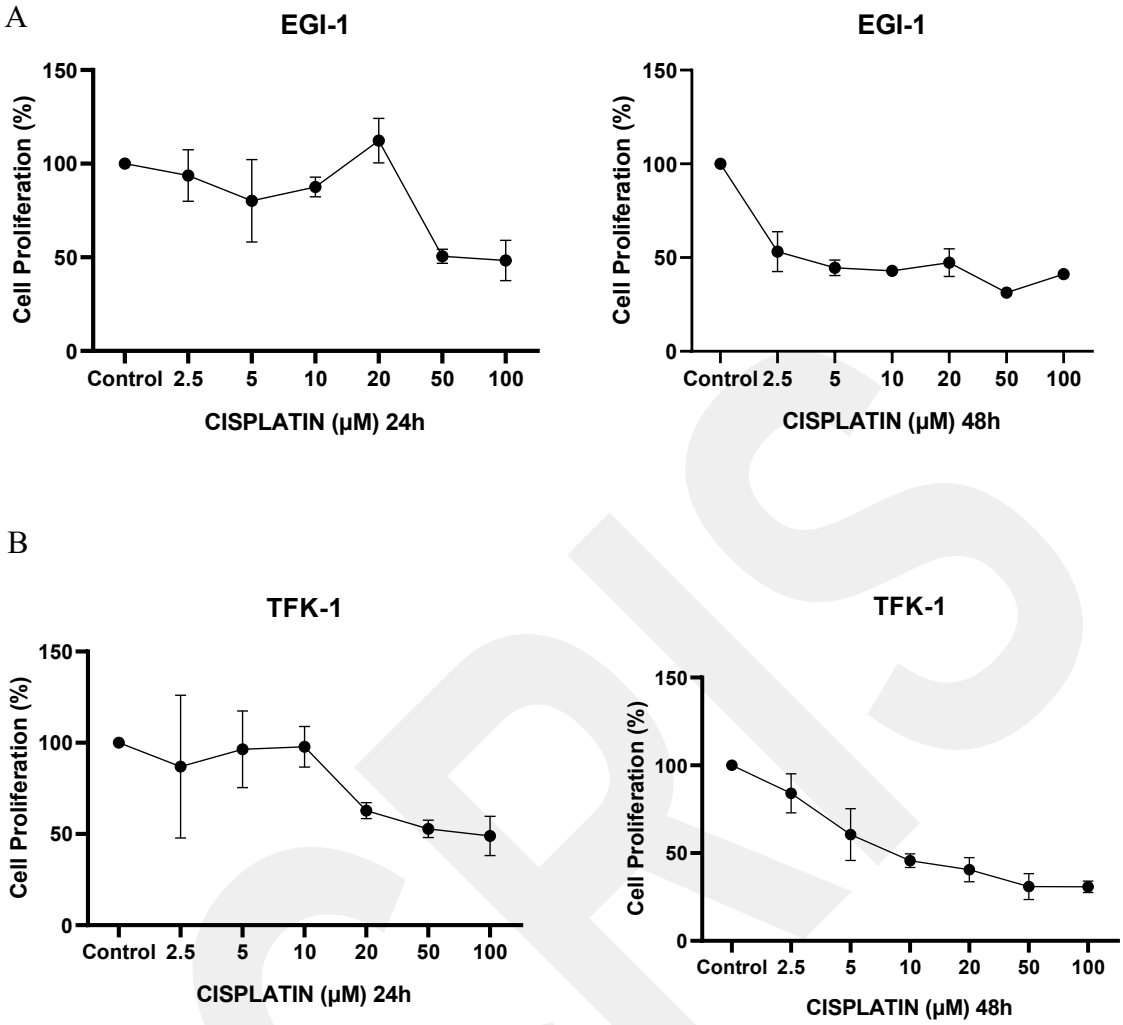


Figure 3.2 The effect of cisplatin treatment (2,5 µM to 100 µM) on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 24h, and 48h. The average of the 3 replicates performed during each experiment were imported to GraphPad.

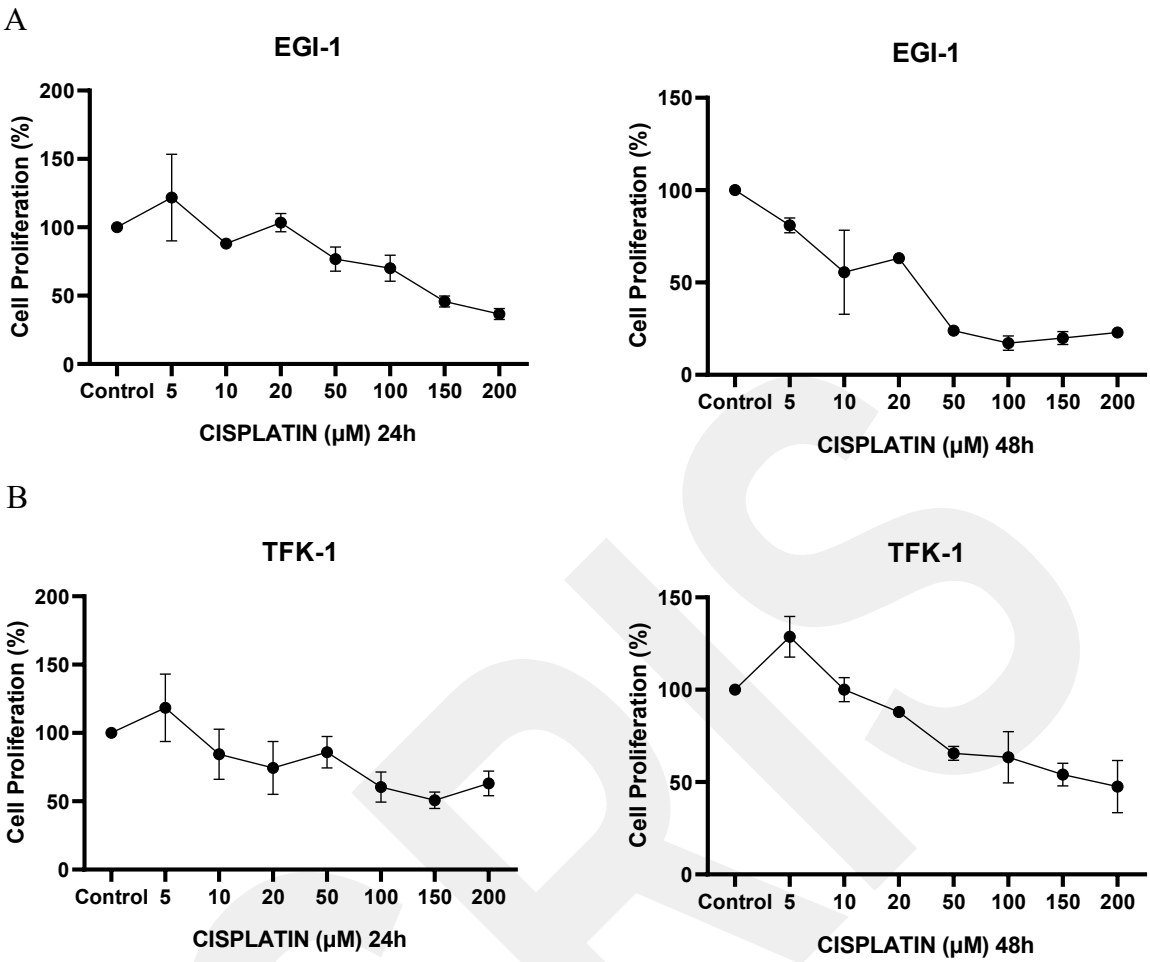
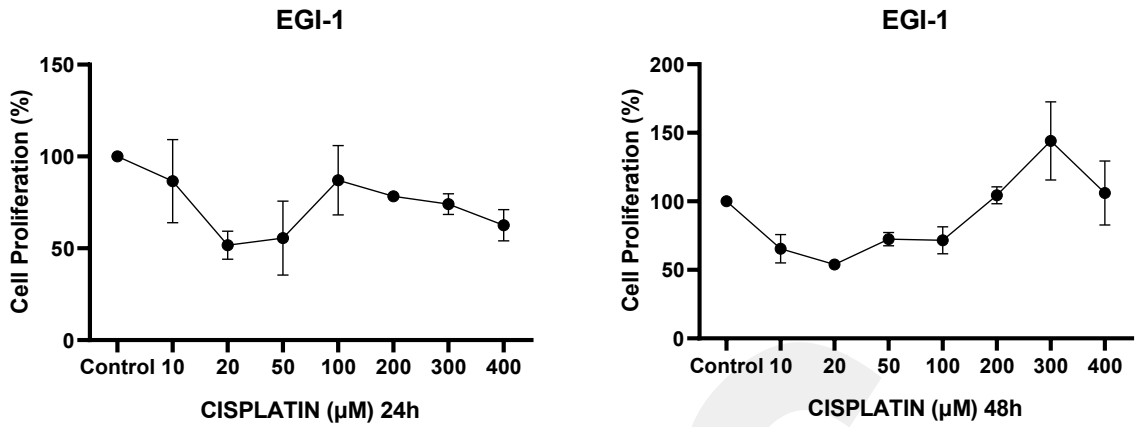


Figure 3.3 The effect of cisplatin treatment (5 µM to 200 µM) on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 24h, and 48h. The average of the 3 replicates performed during each experiment were imported to GraphPad.

A



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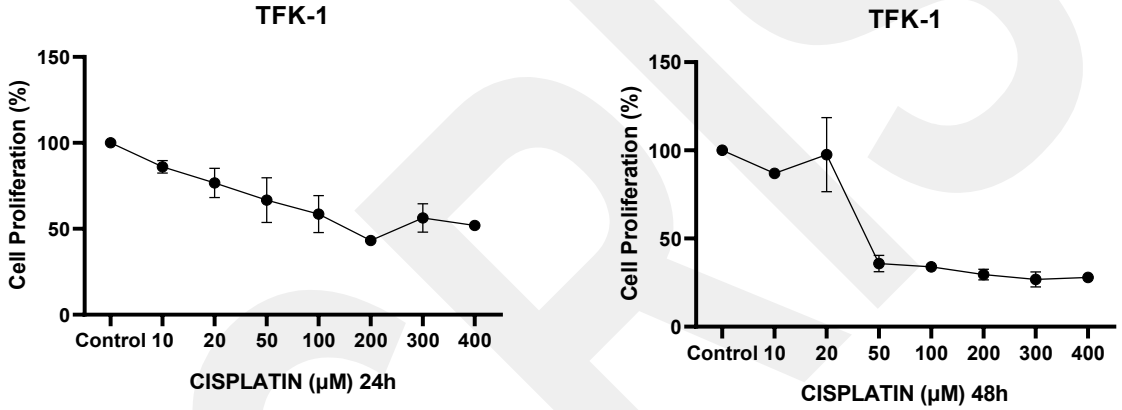
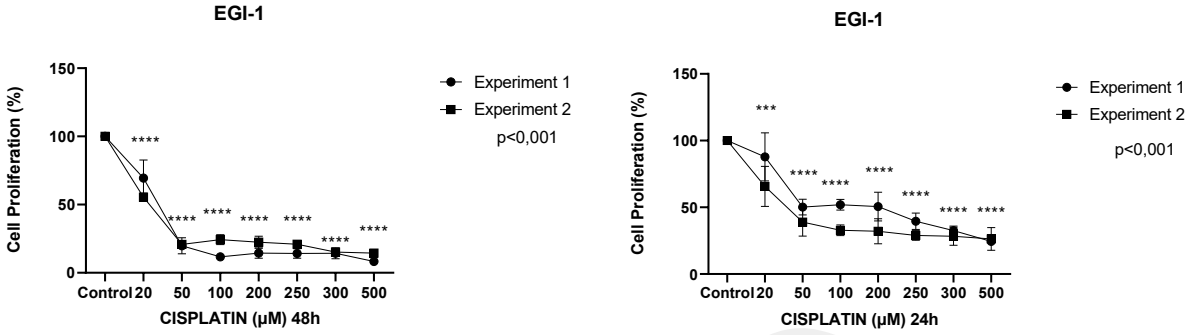


Figure 3.4 The effect of cisplatin treatment (10 µM to 400 µM) on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 24h, and 48h. The average of the 3 replicates performed during each experiment were imported to GraphPad.

A



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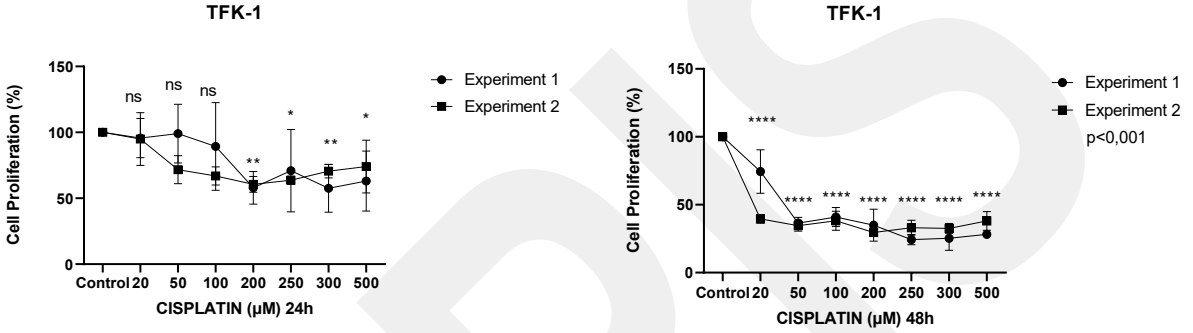


Figure 3.5 The effect of cisplatin treatment (20 µM to 500 µM) on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 24h, and 48h. In each experiment the number of replicates was 2, and the average of the replicates performed during each experiment were imported to GraphPad, where the statistical 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).

As a result of the MTT assays performed with a various dose range of cisplatin. The best dose range was decided between 5 µM and 200 µM for 48h.

After all of the MTT assays to decide the IC50 value for cisplatin, it was observed that 17µM concentrations of cisplatin treatment for 48h, as an IC50 value for both EGI-1 and TFK-1 cells. The cytotoxic assays indicates that treatment of EGI-1 and TFK-1 cells with 17 µM cisplatin for 48h have been decreased the cell number by almost 50% which have shown in the Figure 3.6.

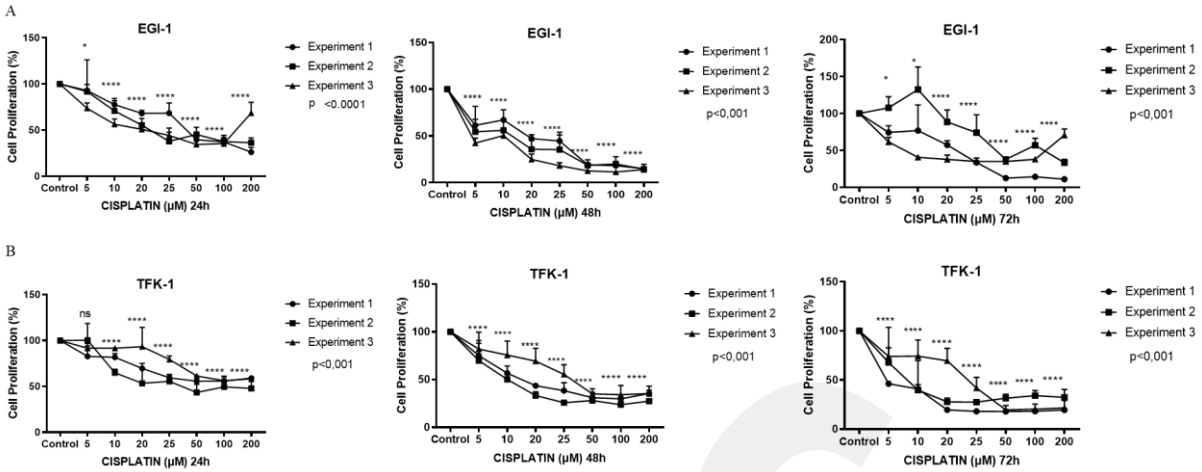


Figure 3.6 The effect of cisplatin treatment (5 μM to 200 μM) on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 24h, 48h, and 72h. In each experiment the number of replicates was 3, and the average of the replicates performed during each experiment were imported to GraphPad, where the statistical 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).

In this set of experiments, we observed the results we expected in general. Drug dose has been determined for IC50 and the doses were almost close to the doses used in the studies that we mentioned previously. With the aim of generating resistance cells, we started to treat EGI-1 and TFK-1 parental cells with the lowest dose of cisplatin (0,1 μM) and incubated them for proliferation, after 24h cells were treated with 0,1 μM cisplatin. The TFK-1 and EGI-1 cells were cultured with the same concentrations of cisplatin 3 passaging times. After each triplet of the treatment, the concentration value was duplicated and TFK-1 and EGI-1 cells were treated with increased doses of the cisplatin until IC50 value [184-186].

In order to determine how the resistant cells responded to the chemotherapeutic drugs compared to the parent cells, different dose amounts of the drugs have been applied to the resistant and parent cells, during drug treatment the cell was observed via microscopy.

In this experiment, we aimed to observe how previously unaffected parental cells would change when the resistant cells were programmed to be tolerated.

The treatment of cisplatin was performed for EGI-1 and TFK-1 parental and 1,6 μM cisplatin-resistant cells. When 1,6 μM cisplatin was applied to control (parental) and cisplatin-resistant cells of both TFK-1 and EGI-1 cell lines, almost all of the parental

(never exposed to cisplatin) cells died, while 1,6 μM cisplatin-resistant lines proliferation was increased and cells were healthy.

In Figure 3.7, cell confluency and number of cells were clearly have shown that generating resistance against cisplatin is achieved for 1,6 μM cisplatin for both EGI-1 and TFK-1 cells.

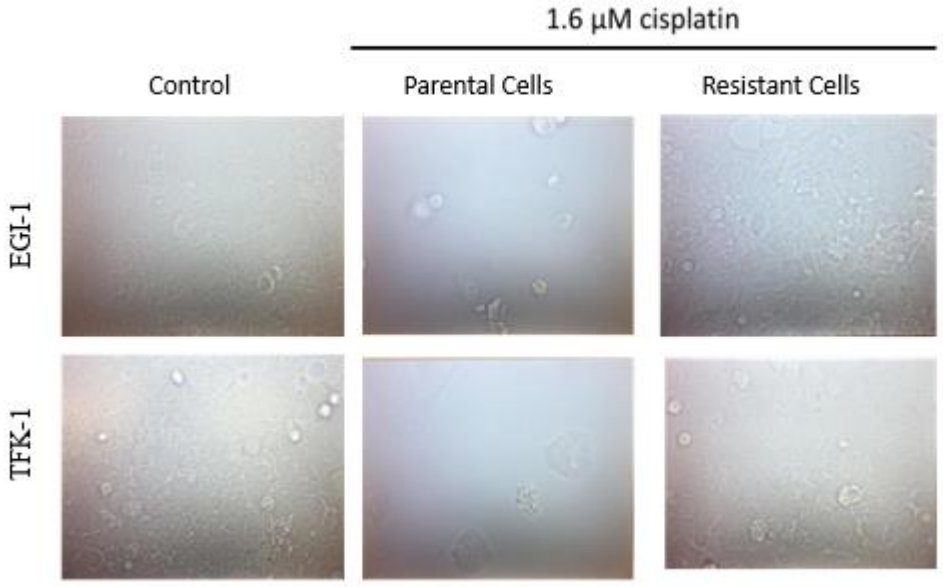


Figure 3.7 The effect of cisplatin treatment (1,6 μM) on the cell proliferation of EGI-1 and TFK-1 parental cells and EGI-1 and TFK-1 1,6 μM cisplatin resistance cells for 48h.

In this experiment, it was observed that the results were the same as we expected in general. Drug doses that did not adversely affect the resistant cells were effective in the parental cells. For the resistant cells; it was observed that the cell proliferation without being affected by the drugs in terms of confluency, attachment, and proliferation. However, when the parent cells were exposed to the same drug doses showed a important decrease in the cell number, an increase in the number dead cells, and a negative effect on the confluency formation when compared to the parental cells. As a result, it was observed that resistant cells gained resistance to different doses of cisplatin.

In order to continue the comparison, since the time passed and the drug dose increased further, the same application was applied to the cells and the cell numbers were recorded after treatment, at the same time. When looking at the TFK-1 cells treated with

cisplatin, a high mortality rate was observed as a result of 17 μM cisplatin, which was very close to this value, although the resistant cells were at a dose of 12.8 μM. We think that the reason for this is the adaptation process of these cells to the high dose and the state that their final passages were performed without cisplatin treatment (Figure 3.8).

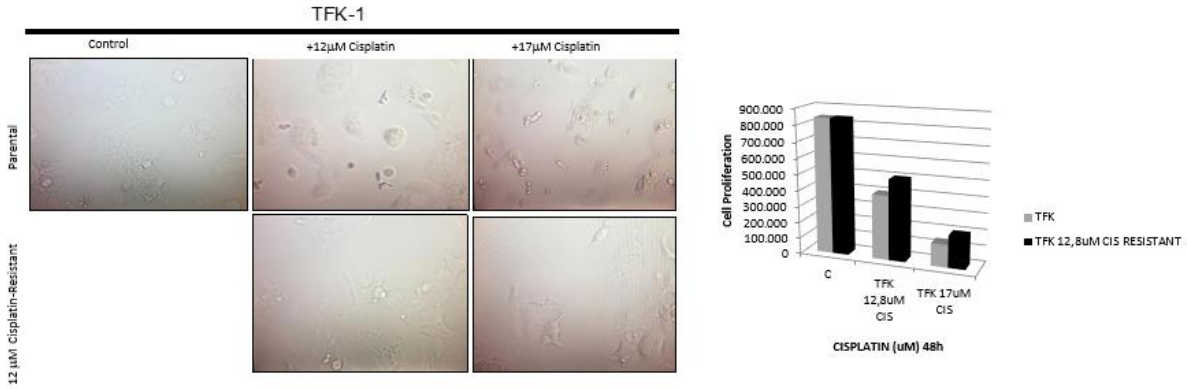
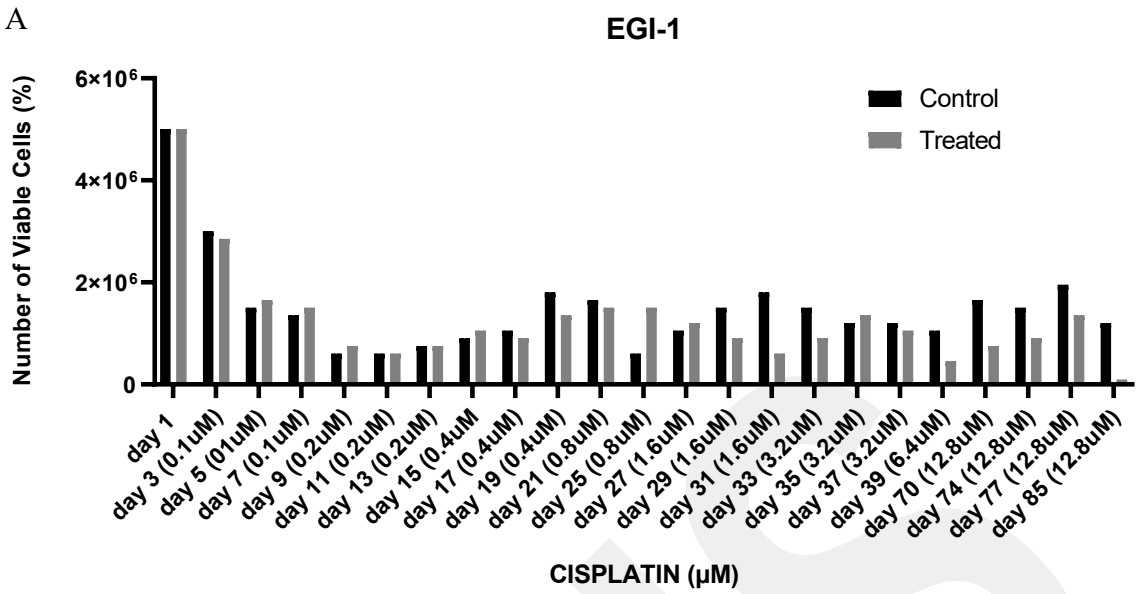


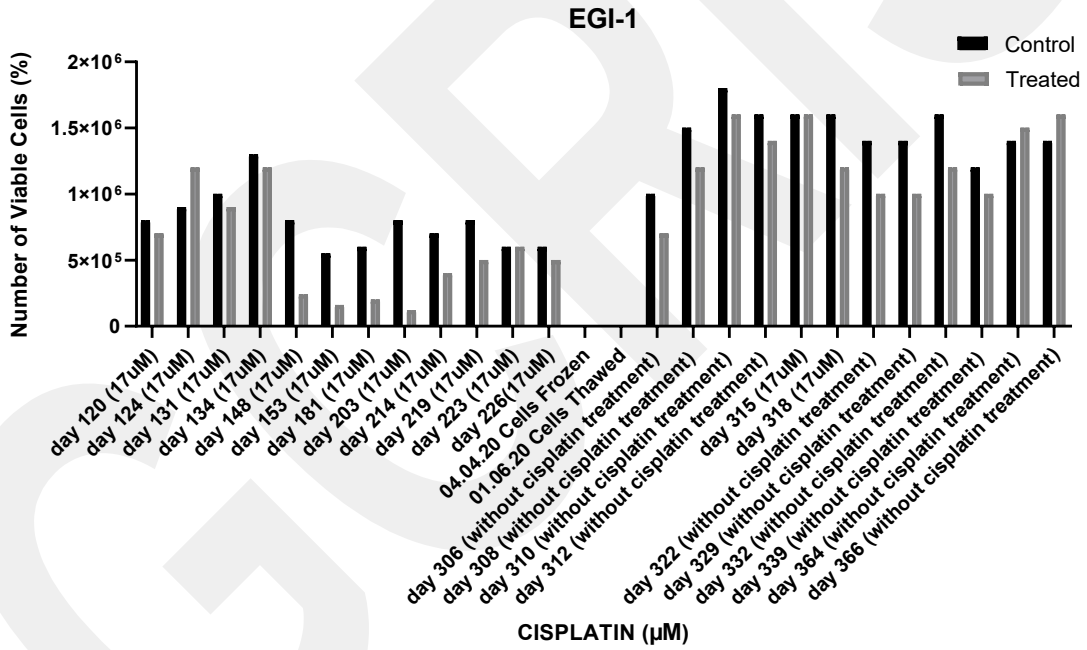
Figure 3.8 The effect of cisplatin treatment (12 μM and 17 μM) on the cell proliferation of TFK-1 parental cells and TFK-1 12 μM cisplatin resistance cells cells for 48h.

With the exact purpose of checking the proliferation of the parental EGI-1 and TFK-1 cells during the generation of cisplatin-resistant cells, the cell number of each cell line has been counted during each passage of the cells and imported via GraphPad.

As described in the Figure 3.9, the concentration of the cisplatin has been duplicated after every 3 passages and after cisplatin treatment, parental cells were needed time to adapted and increased their number. After parental cells have been achieving the IC50 value (17 μM) and become resistant to cisplatin, the passages of resistant EGI-1 and TFK-1 cells has been continued without cisplatin treatment for 3 weeks. After 3 weeks, resistant cells were treated with cisplatin after each passage, with this way we aimed to prove the resistance was not temporary, and even under the situation, the cisplatin treatment was not performed, cells were protected their resistance against cisplatin.

A


EGI-1
Number of Viable Cells (%)
6×10⁶
4×10⁶
2×10⁶
0
Control
Treated
day 1
day 3 (0.1uM)
day 5 (01uM)
day 7 (0.1uM)
day 9 (0.2uM)
day 11 (0.2uM)
day 13 (0.2uM)
day 15 (0.4uM
day 17 (0.4uM)
day 19 (0.4uM)
day 21 (0.8uM)
day 25 (0.8uM)
day 27 (1.6uM)
day 29 (1.6uM)
day 31 (1.6uM)
day 33 (3.2uM)
day 35 (3.2uM)
day 37 (3.2uM)
day 39 (6.4uM)
day 70 (12.8uM)
day 74 (12.8uM)
day 77 (12.8uM)
day 85 (12.8uM)
CISPLATIN (µM)


EGI-1
Number of Viable Cells (%)
2×10⁶
1.5×10⁶
1×10⁶
5×10⁵
0
Control
Treated
day 120 (17uM)
day 124 (17uM)
day 131 (17uM)
day 134 (17uM)
day 148 (17uM)
day 153 (17uM)
day 181 (17uM)
day 203 (17uM)
day 214 (17uM)
day 219 (17uM)
day 223 (17uM)
day 226(17uM)
04.04.20 Cells Frozen
01.06.20 Cells Thawed
day 306 (without cisplatin treatment)
day 308 (without cisplatin treatment)
day 310 (without cisplatin treatment)
day 312 (without cisplatin treatment)
day 315 (17uM)
day 318 (17uM)
day 322 (without cisplatin treatment)
day 329 (without cisplatin treatment)
day 332 (without cisplatin treatment)
day 339 (without cisplatin treatment)
day 364 (without cisplatin treatment)
day 366 (without cisplatin treatment)
CISPLATIN (µM)

B

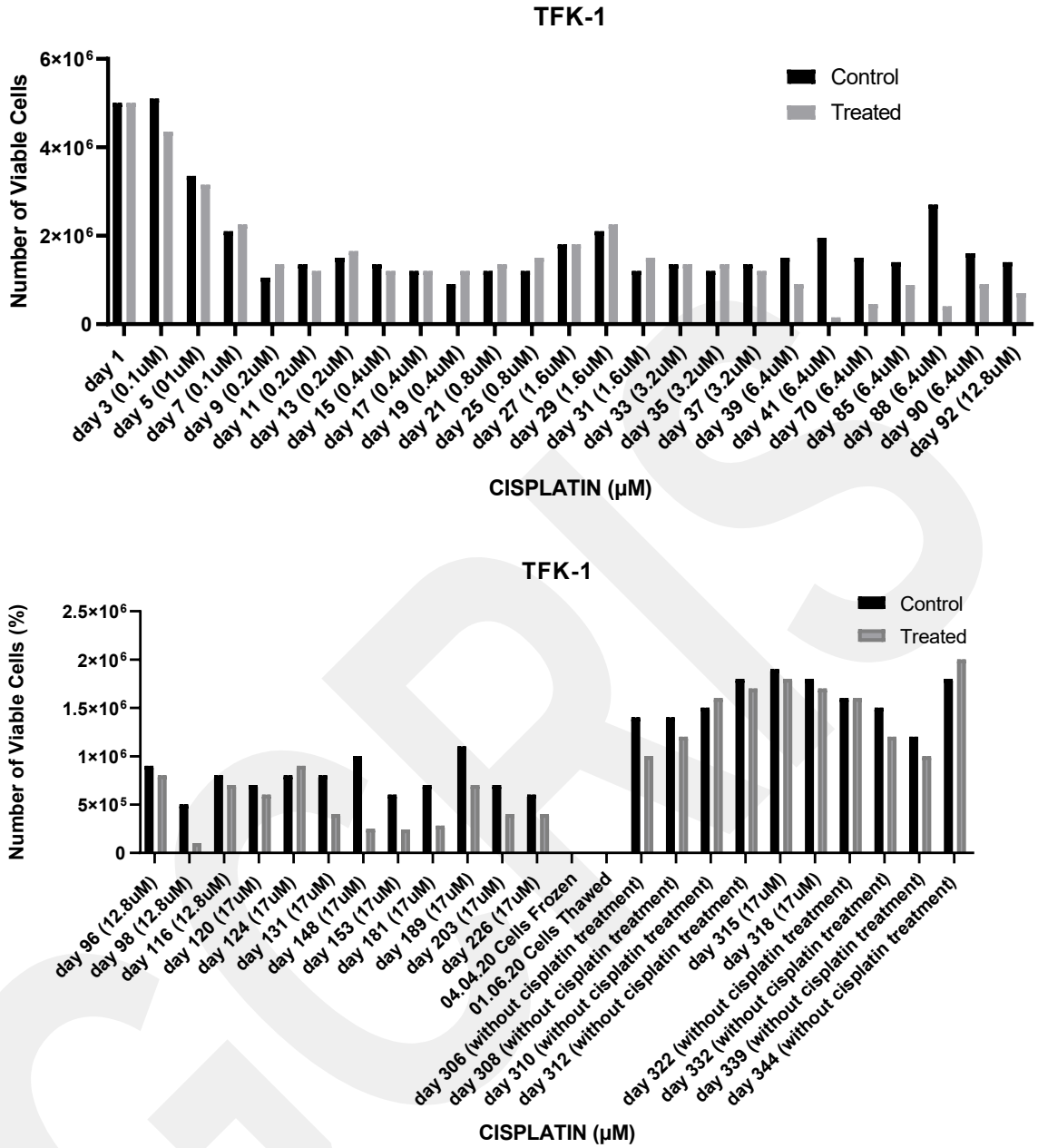
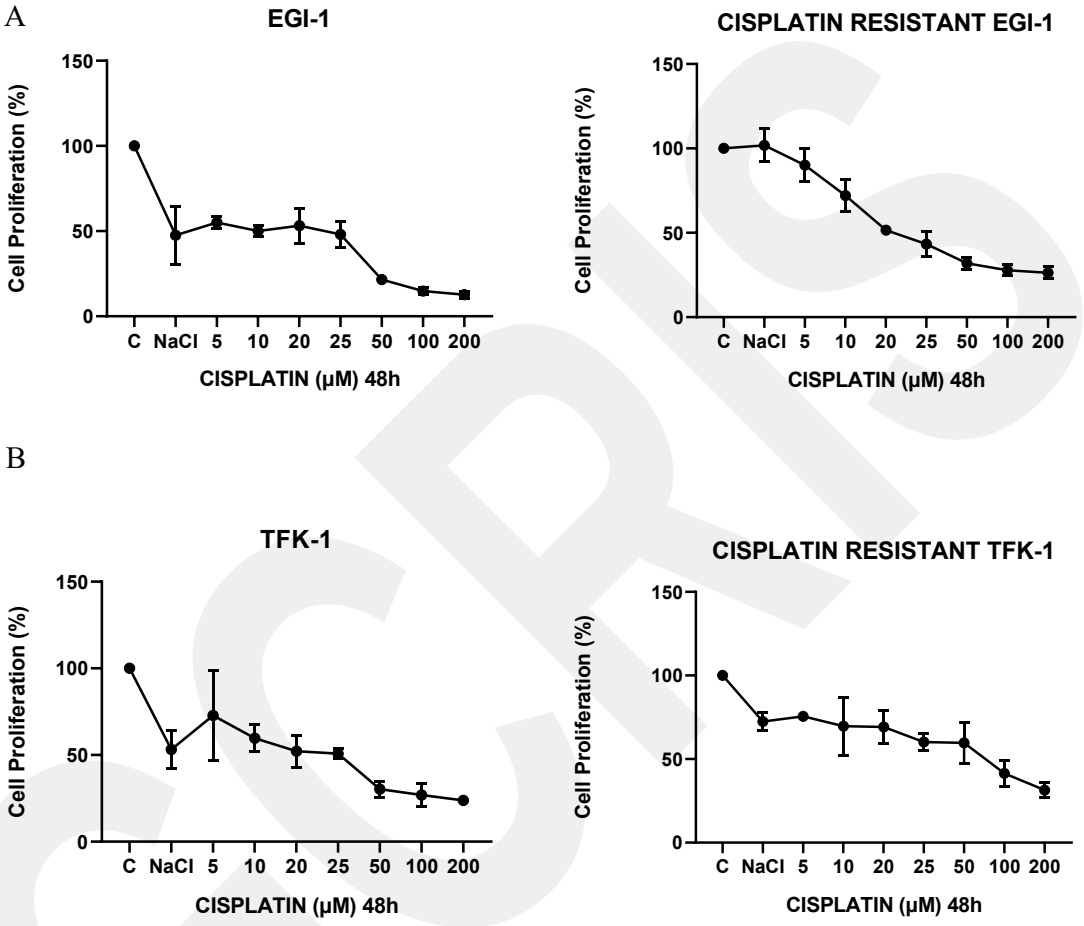


Figure 3.9 The effect of cisplatin treatment on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells during the generation of resistant cells. The number of viable cells during each passage was imported to GraphPad.

To determine the extent of resistance in cisplatin-resistant EGI-1 and TFK-1 cell lines, experiments with resistant cells were performed after cisplatin is removed from the medium.

With the aim of determining the difference in the IC50 for cisplatin in EGI-1 and TFK-1 parental cells and the resistant cells, we performed cytotoxic assays. The results

were showing the increase in IC50 for resistant cells. It has been shown the resistance gaining against cisplatin and for EGI-1 resistant cells IC50 concentration is 1,8 fold more than parental cells, for TFK-1-resistant cells IC50 concentration is 4 fold more than parental cells (Figure 3.10).



IC50 Value (Cisplatin)	Parental Cells	Cisplatin-Resistant Cells
TFK-1	20.3 µM	80.92 µM
EGI-1	15.3 µM	28.36 µM

Figure 3.10 The effect of cisplatin treatment (5 µM to 200 µM) on the cell proliferation of (A) EGI-1 and cisplatin-resistant EGI-1 and (B) TFK-1 and cisplatin resistant TFK-1 cells for 48h. The average of the 3 replicates performed during each experiment were imported to GraphPad.

After achieved to the IC50 value and generated the resistance in both for EGI-1 and TFK-1 against cisplatin, the colony formation assays have been performed to see the

difference between parental and cisplatin resistance EGI-1 and TFK-1 cells. TFK-1 parental and cisplatin resistance cells were treated with 17 μM cisplatin which is IC50 value and 50 μM cisplatin to observe the resistance cells proliferation under a dose that is more than two times higher than the IC50 value.

Table 3.1 Concentrations of drugs for colony formation experiments

TFK Cisplatin Control + Medium	TFK Cisplatin Control + 17 μM Cisplatin	TFK Cisplatin Control + 50 μM Cisplatin
TFK Cisplatin Resistant + Medium	TFK Cisplatin Resistant + 17 μM Cisplatin	TFK Cisplatin Resistant + 50 μM Cisplatin

As a result of the colony formation assays, it was observed that the resistance cells were continued their proliferation under the treatment with IC50 value. Moreover, resistant cells had a capacity to continue their proliferation under 50 μM cisplatin treatment while parental cells have almost died totally. The number of colonies after treatment for each well were shown in Figure 3.11.

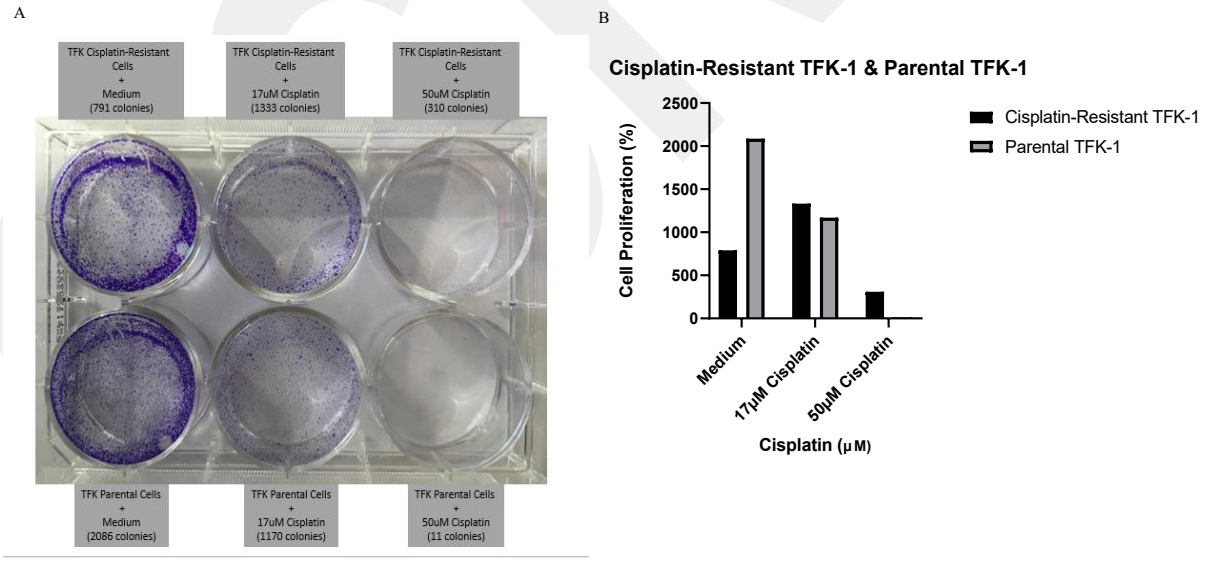


Figure 3.11 (A) The effect of cisplatin treatment (17 μM and 50 μM) on the cell proliferation of EGI-1 and TFK-1 parental cells and EGI-1 and TFK-1 17 cisplatin resistance cells for 10 days. (B) The number of the colony that formed after cisplatin treatment for each well has been stated. The cell proliferation after each treatment was imported to GraphPad.

3.2 Generation of gemcitabine-resistant CCA cell lines

In order to generate the gemcitabine-resistant cells from parental TFK-1 and EGI-1 cell lines, we treated TFK-1 and EGI-1 parental cells with increased doses of gemcitabine. Doses have been chosen according to the previous studies that include gemcitabine treatment [184,187-191]. With the purpose of finding the most effective range of doses, several cytotoxic assays were performed for 24, 48, and 72 hours.

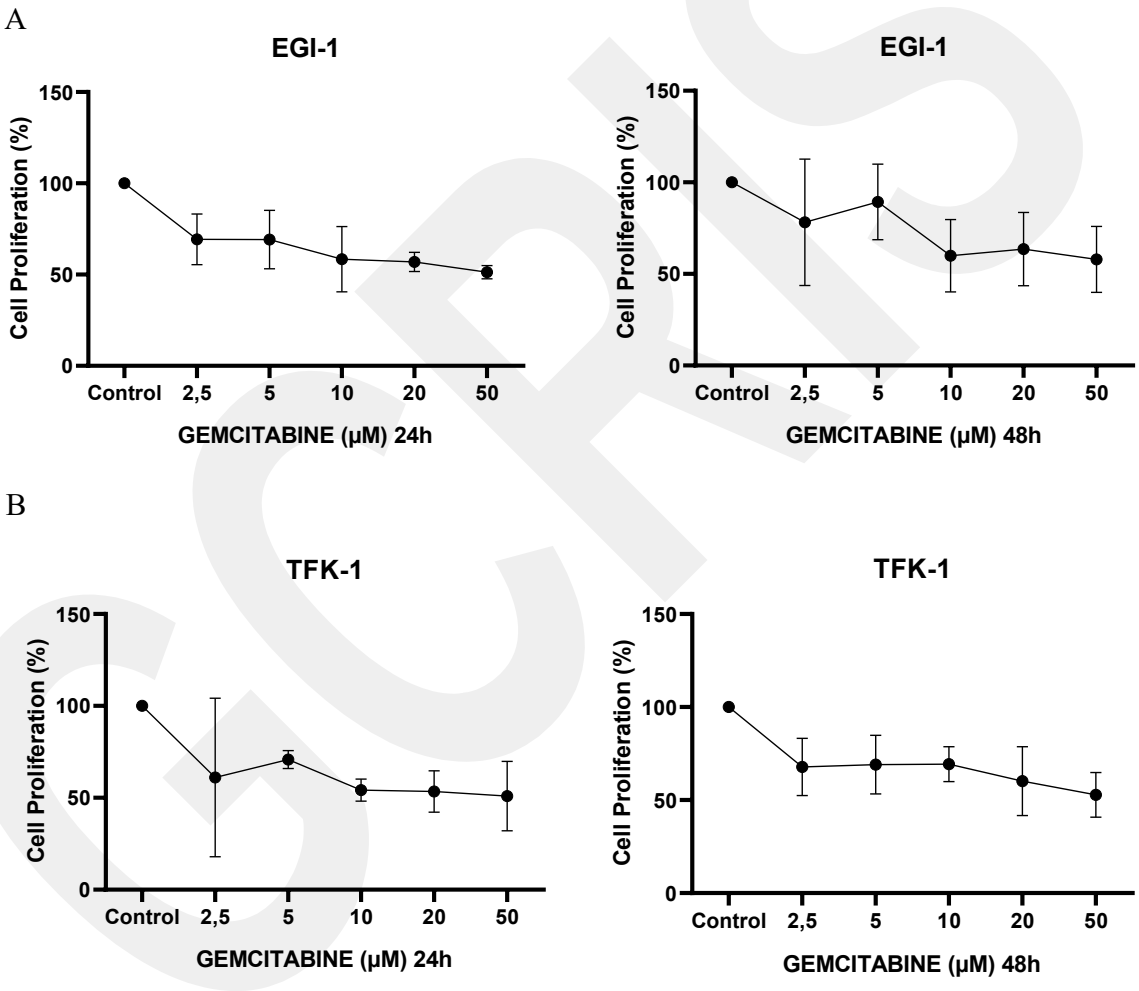


Figure 3.12 The effect of gemcitabine treatment (2,5 μM to 50 μM) on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 24h, and 48h. The average of the 3 replicates performed during each experiment were imported to GraphPad.

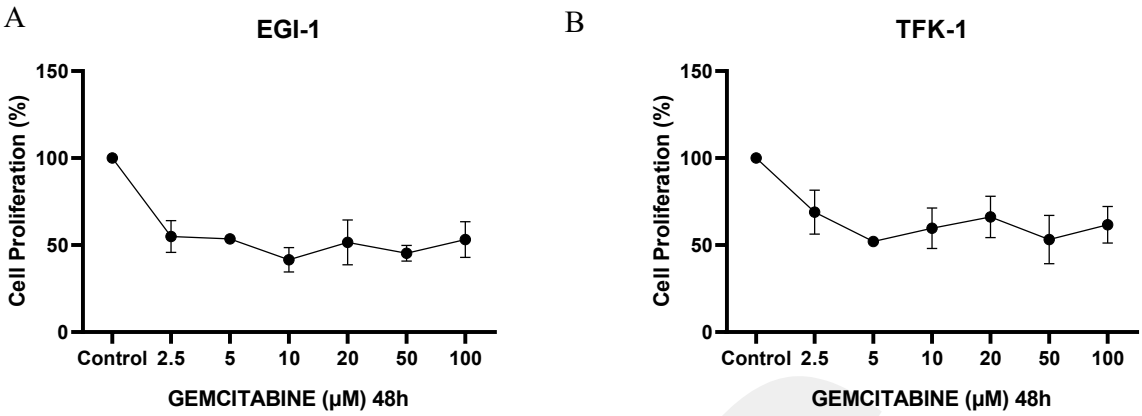


Figure 3.13 The effect of gemcitabine treatment (2,5 μM to 100 μM) on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 48h. The average of the 3 replicates performed during each experiment were imported to GraphPad.

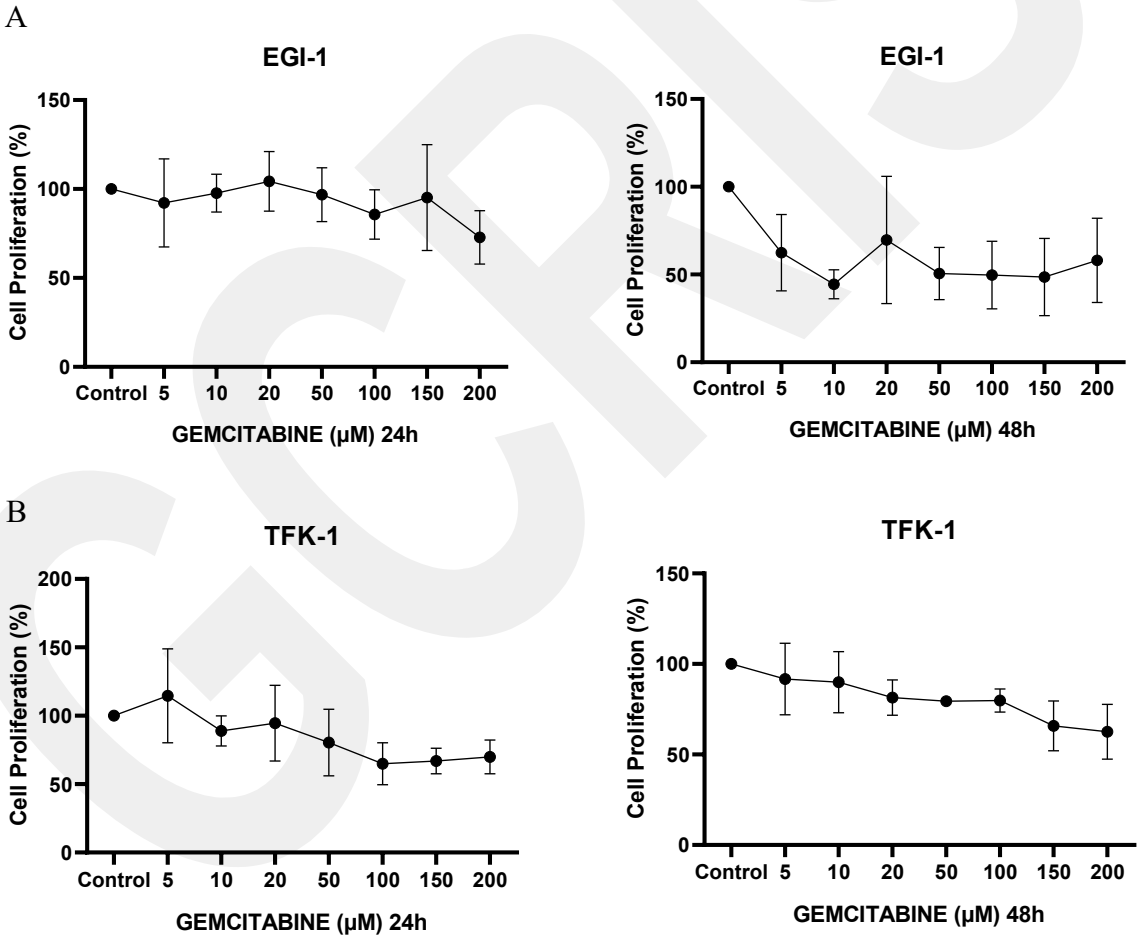


Figure 3.14 The effect of gemcitabine treatment (5 μM to 200 μM) on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 24h, and 48h. The average of the 3 replicates performed during each experiment were imported to GraphPad.

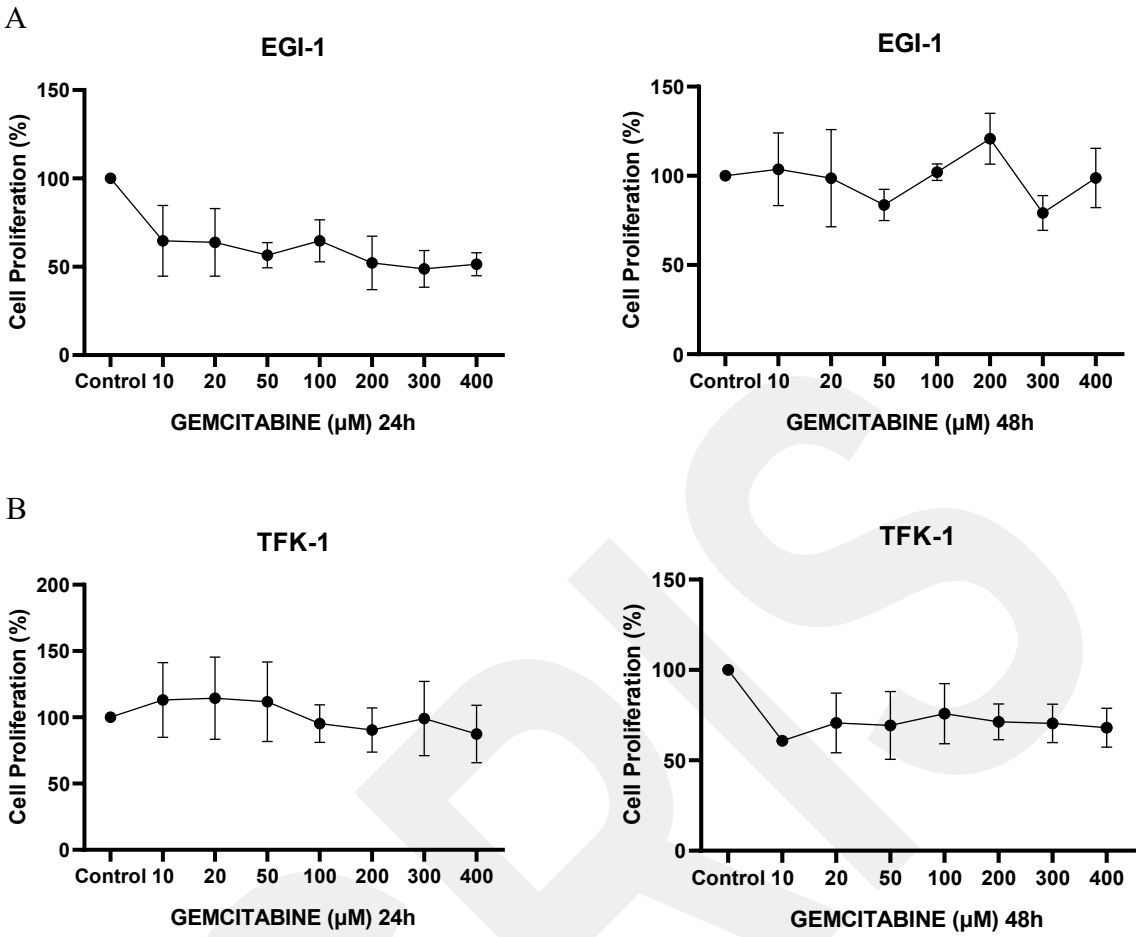


Figure 3.15 The effect of gemcitabine treatment (10 µM to 400 µM) on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 24h, and 48h. The average of the 3 replicates performed during each experiment were imported to GraphPad.

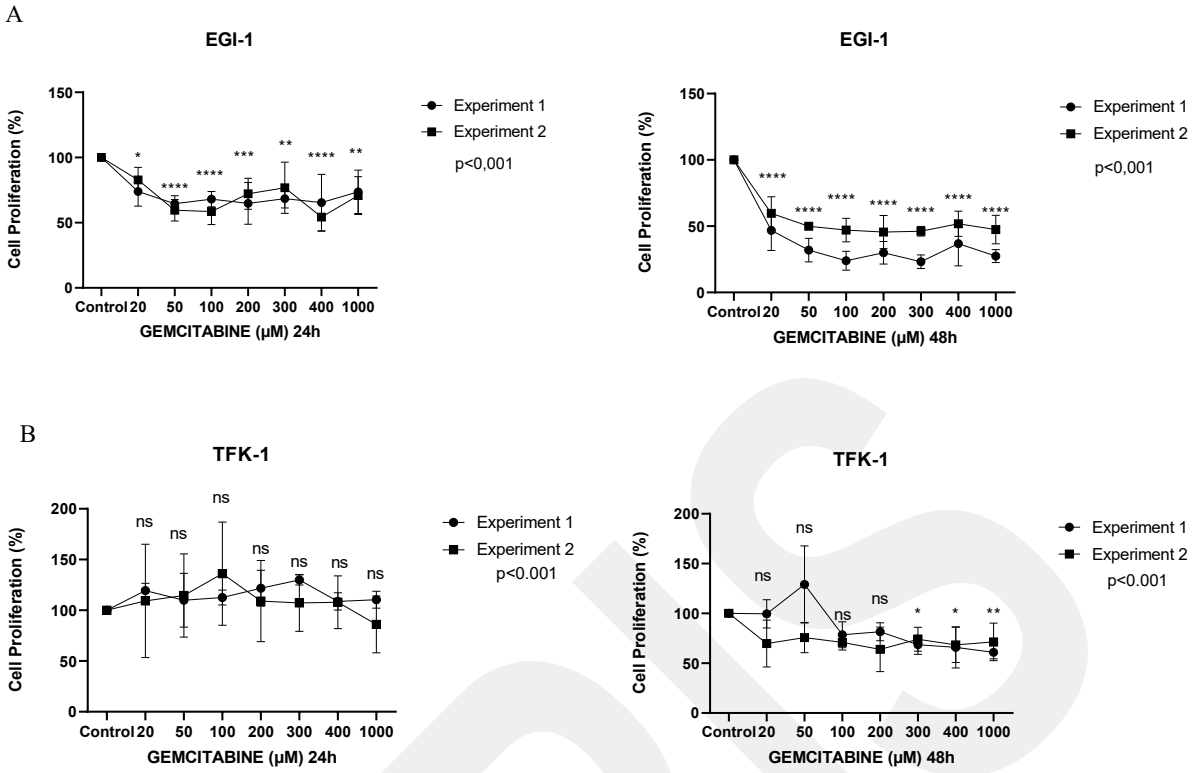


Figure 3.16 The effect of gemcitabine treatment (50 μM to 1000 μM) on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 24h, and 48h. In each experiment the number of replicates was 2, and the average of the replicates performed during each experiment were imported to GraphPad, where the statistical 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).

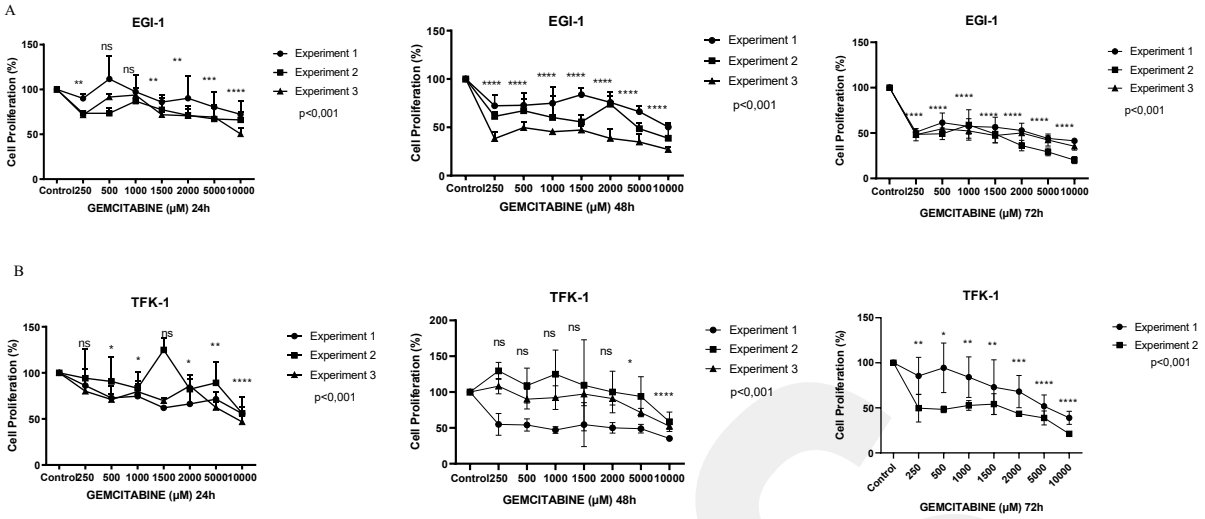


Figure 3.17 The effect of gemcitabine treatment (250 μM to 10000 μM) on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 24h, 48, and 72h. In each experiment the number of replicates was 3, and the average of the replicates performed during each experiment were imported to GraphPad, where 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).

As a result of the MTT assays performed with a various dose range of gemcitabine. The best dose range was decided between 1000 μM and 15000 μM for 48h

After all of the MTT assays to decide the IC50 value for gemcitabine, it was observed that the IC50 value for gemcitabine is 5000 μM for EGI-1 and 7500 μM for TFK-1, for 48h treatment. The cytotoxic assays indicates that treatment of EGI-1 and TFK-1 cells with 5000 μM and 7500 μM gemcitabine, respectively, for 48h have been decreased the cell number by almost 50% which have shown in the Figure 3.18.

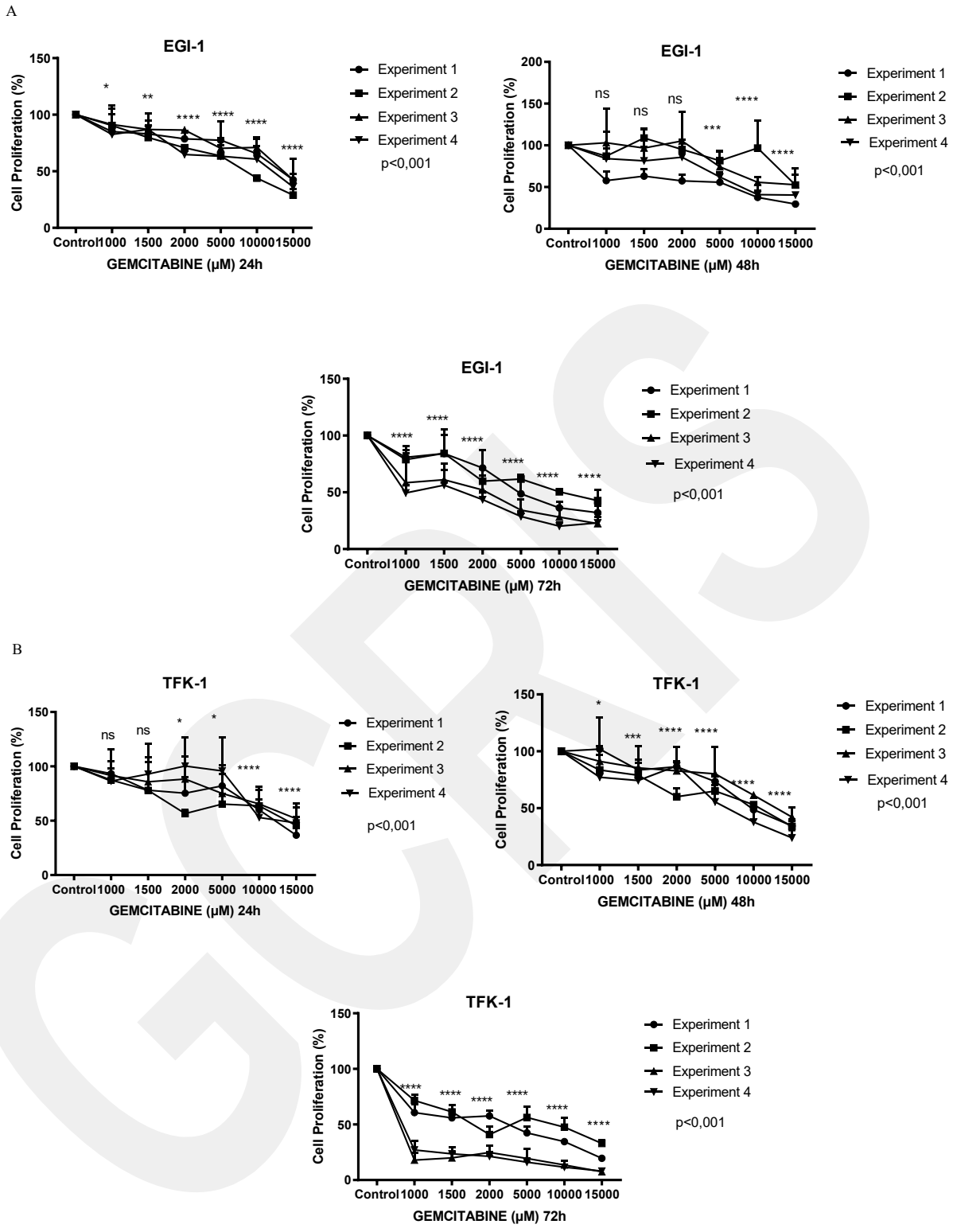


Figure 3.18 The effect of gemcitabine treatment (1000 µM to 15000 µM) on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 24h, 48h, and 72h. In each experiment the number of replicates was 3, and the average of the replicates performed during each experiment were imported to GraphPad, where 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).

In this set of experiments, drug dose has been determined for IC50 and the doses were much more than the doses used in the studies that we mentioned previously.

Intending to generate resistance cells, we started to treat EGI-1 and TFK-1 parental cells with the lowest dose of gemcitabine (0,1 μM) and incubated them for proliferation, after 24h cells were treated with 0,1 μM gemcitabine. The TFK-1 and EGI-1 cells were cultured with the same concentrations of gemcitabine 3 times. After each triplet of the treatment, the concentration was duplicated and TFK-1 and EGI-1 cells were treated with increased doses of the gemcitabine.

Same as for cisplatin, comparison experiment was performed for gemcitabine, this experiment aimed to observe how previously unaffected parental cells would change when the resistant cells were programmed to be tolerated.

The treatment of gemcitabine was performed for EGI-1 and TFK-1 parental and 0,2 μM gemcitabine-resistant cells. When 0,2 μM gemcitabine was applied to control (parental) and gemcitabine-resistant cells of both TFK-1 and EGI-1 cell lines, almost all of the parental (never exposed to cisplatin) cells died, while 0,2 μM gemcitabine-resistant lines proliferation was increased and cells healthy.

In the figure 3.19, cell confluency and number of cells were clearly have shown that generating resistance against cisplatin is achieved for 0,2 μM gemcitabine for both EGI-1 and TFK-1 cells.

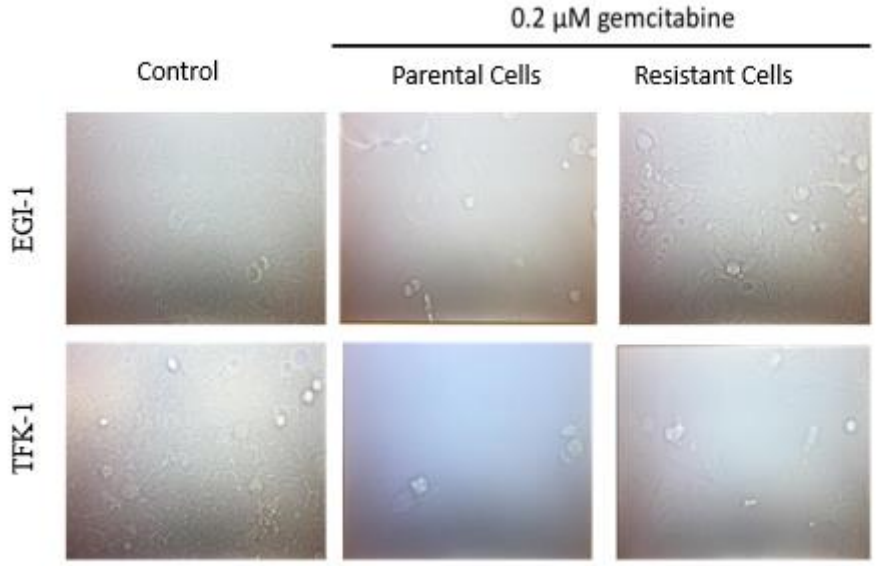


Figure 3.19 The effect of gemcitabine treatment (0,2 μM) on the cell proliferation of EGI-1 and TFK-1 parental cells and EGI-1 and TFK-1 0,2 μM gemcitabine resistance cells cells for 48h.

In this experiment, it is observed that the results were the same as we expected in general. Drug doses that did not affect the resistant cells were effective in the parental cells. For the resistant cells; it was observed that the cell proliferation without being affected by the drugs in terms of confluency, attachment, and proliferation. However, when the parent cells were exposed to the same drug doses, a considerable decrease in the cell number, an increase in the number of not attach cells, and a negative effect on the confluency formation were observed in most of the parental cells. As a result, it was observed that resistant cells gained resistance to different doses of gemcitabine.

In the second step of the comparison, since the time passed and the drug dose increased further, the same application was applied to the cells and the cell numbers were recorded at the same time.

At the time of the experiment, they were 1.6 µM in gemcitabine-resistant EGI-1 and TFK-1 cells. For this reason, both parental and resistant cells were treated with 1.6 µM gemcitabine and an IC50 value which is 5000 µM for EGI-1 and 7500 µM for TFK-1. The treatment of both doses showed a decrease in the number of viable cells, but the resistant cells showed more viability compared to the parental cells. In gemcitabine-resistant EGI-1 and TFK-1 cells, as a result of the treatment with 5000 µM and 7500 µM gemcitabine to the resistant EGI-1 and TFK-1 cells, respectively, a very low mortality rate was observed compared to control cells (Figure 3.20-Figure 3.21). This result was very promising, because living resistant cells receiving the same dose of drug were 3 times more than living parental cells.

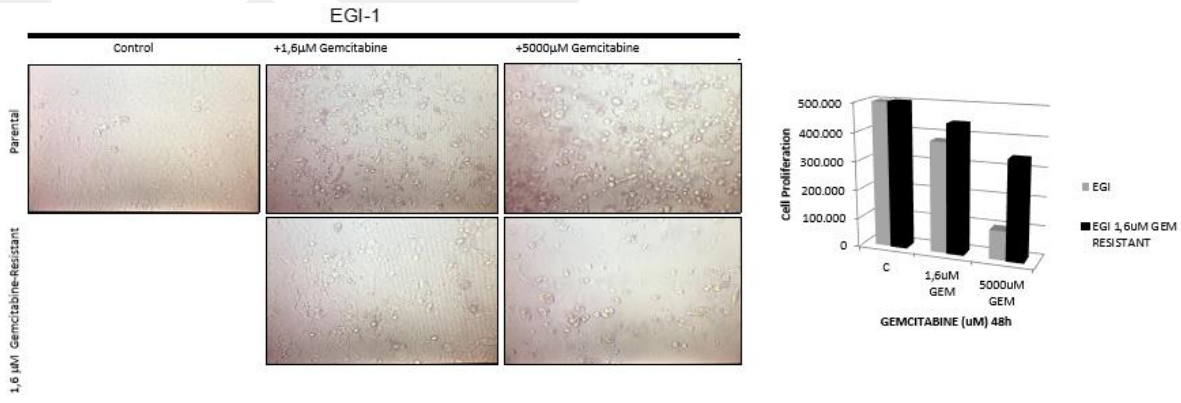


Figure 3.20 The effect of cisplatin treatment (1,6 µM and 5000 µM) on the cell proliferation of EGI-1 parental cells and EGI-1 and 1,6 µM gemcitabine resistance cells for 48h.

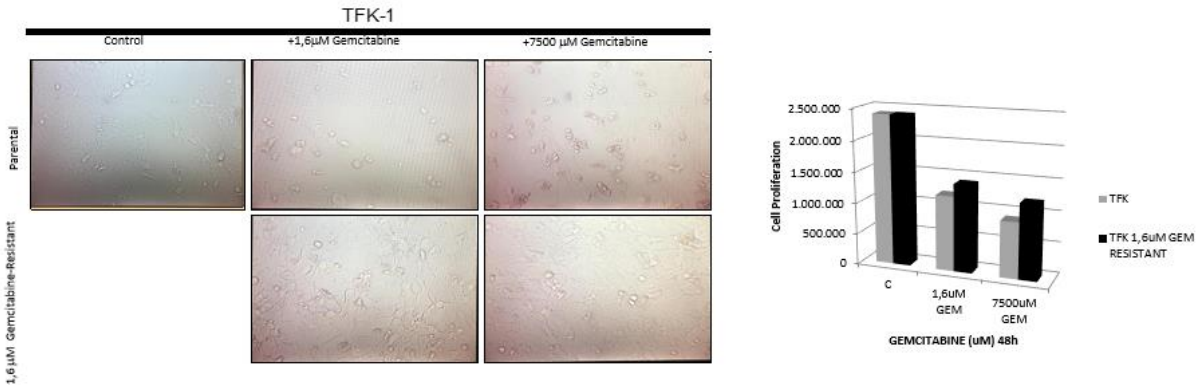
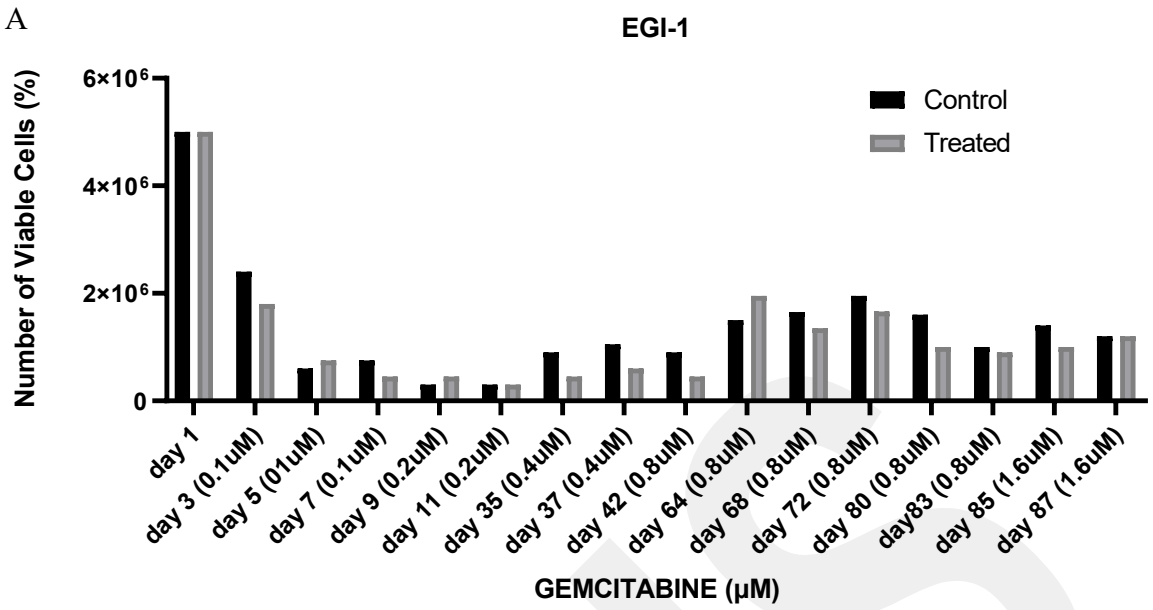


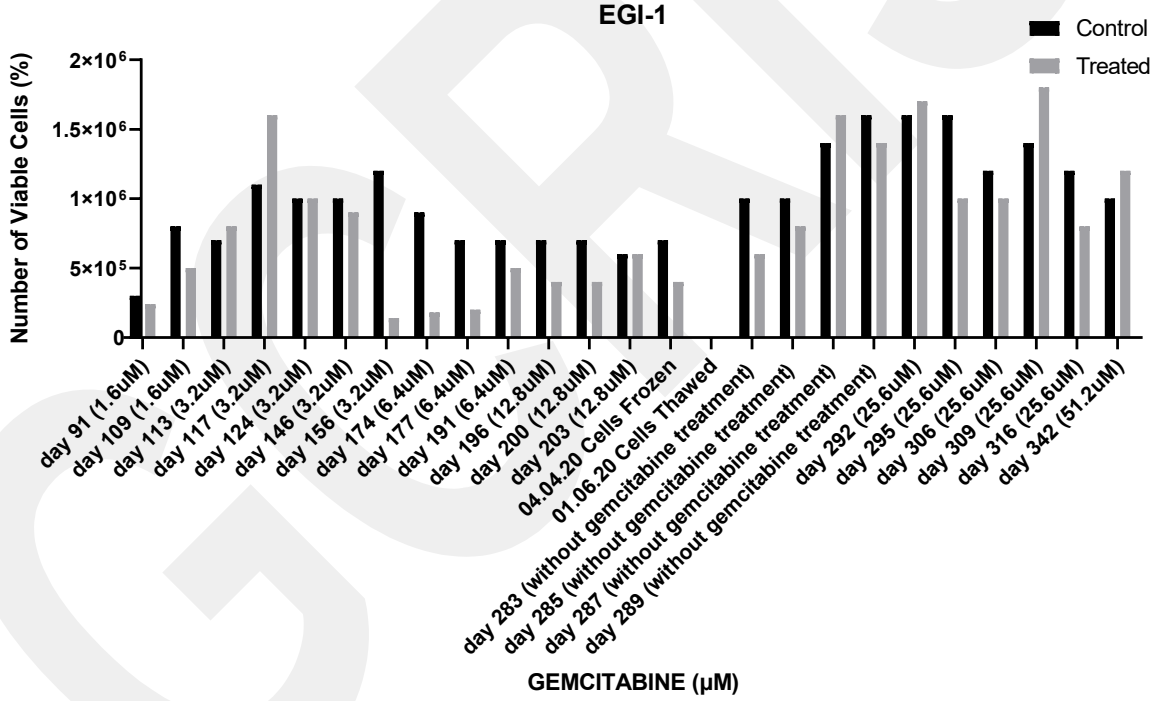
Figure 3.21 The effect of gemcitabine treatment (1,6 µM and 7500 µM) on the cell proliferation of TFK-1 parental cells and TFK-1 and 1,6 µM gemcitabine resistance cells for 48h.

With the exact purpose of checking the proliferation of the parental EGI-1 and TFK-1 cells during the generation of gemcitabine-resistant cells, the cell number of each cell line has been counted during each passage of the cells and imported via GraphPad.

As described in the Figure 3.22, the concentration of the gemcitabine has been duplicated after every 3 passages and after gemcitabine treatment, parental cells were needed time to adapted and increased their number.

A


EGI-1
Control
Treated
Number of Viable Cells (%)
6×10⁶
4×10⁶
2×10⁶
0
day 1
day 3 (0.1uM)
day 5 (01uM)
day 7 (0.1uM)
day 9 (0.2uM)
day 11 (0.2uM)
day 35 (0.4uM)
day 37 (0.4uM)
day 42 (0.8uM)
day 64 (0.8uM)
day 68 (0.8uM)
day 72 (0.8uM)
day 80 (0.8uM)
day83 (0.8uM)
day 85 (1.6uM)
day 87 (1.6uM)
GEMCITABINE (µM)


EGI-1
Control
Treated
Number of Viable Cells (%)
2×10⁶
1.5×10⁶
1×10⁶
5×10⁵
0
day 91 (1.6uM)
day 109 (1.6uM)
day 113 (3.2uM)
day 117 (3.2uM)
day 124 (3.2uM)
day 146 (3.2uM)
day 156 (3.2uM)
day 174 (6.4uM)
day 177 (6.4uM)
day 191 (6.4uM)
day 196 (12.8uM)
day 200 (12.8uM)
day 203 (12.8uM)
04.04.20 Cells Frozen
01.06.20 Cells Thawed
day 283 (without gemcitabine treatment)
day 285 (without gemcitabine treatment)
day 287 (without gemcitabine treatment)
day 289 (without gemcitabine treatment)
day 292 (25.6uM)
day 295 (25.6uM)
day 306 (25.6uM)
day 309 (25.6uM)
day 316 (25.6uM)
day 342 (51.2uM)
GEMCITABINE (µM)

B

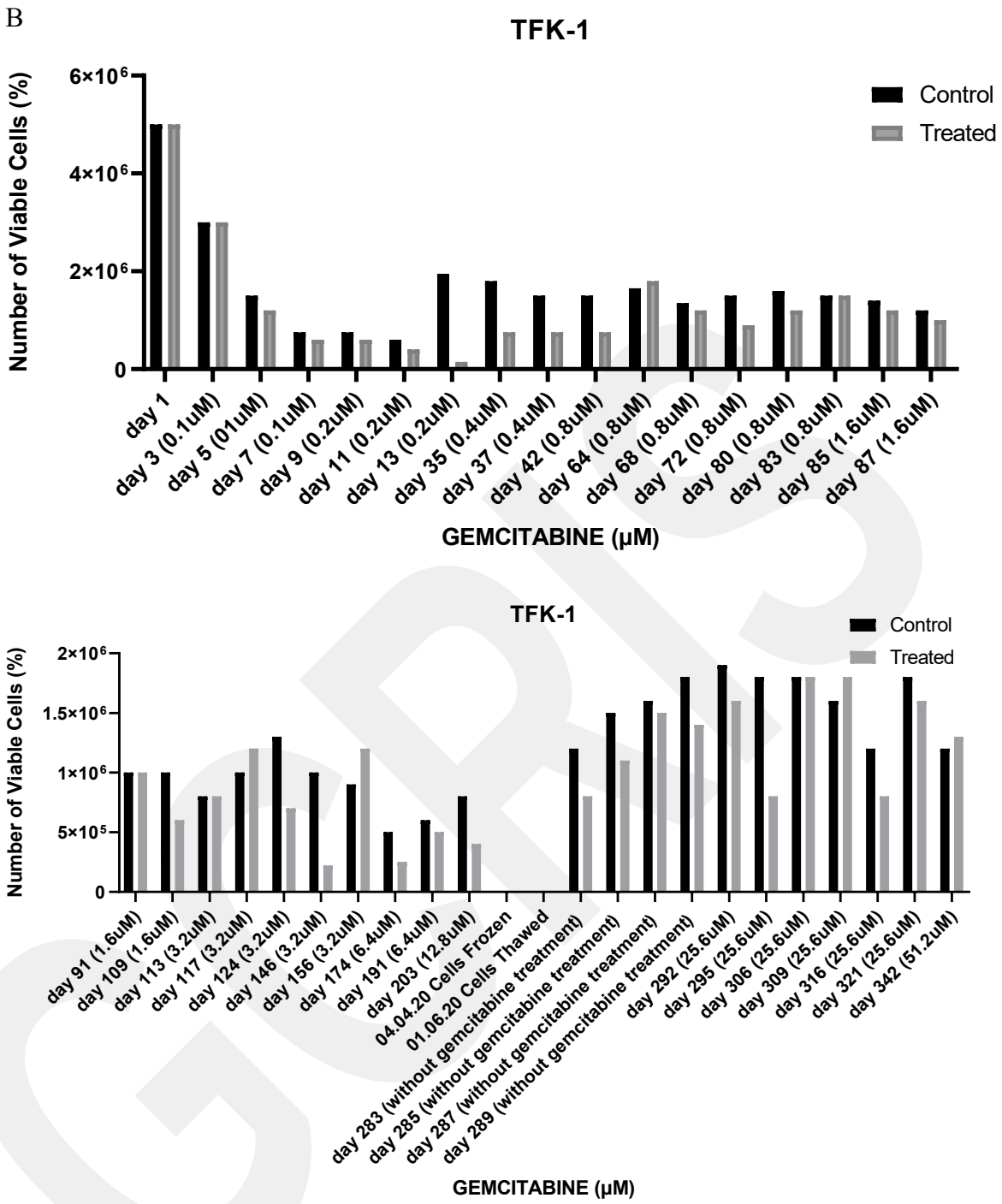


Figure 3.22 The effect of gemcitabine treatment on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells during the generation of resistant cells. The number of viable cells during each passage was imported to GraphPad.

Since the generation of gemcitabine-resistant EGI-1 and TFK-1 cells was harder than the cisplatin-resistant cells because of the extremely high IC50 of gemcitabine for both cell lines. We stopped the treatment of gemcitabine for both the EGI-1 and TFK-1 cell lines. According to the slow proliferation and adaptation rate against gemcitabine,

both EGI-1 and TFK-1 cells were frozen and new method for the generation have been started. We tried to generate gemcitabine-resistant cells in a different way depending on the literature. [192].

In this experiment, parental cells were directly exposed to gemcitabine at high drug concentrations and aimed to select cell clones resistant to this drug concentration at the end of the experiment [192]. EGI-1 and TFK-1 cells were treated with high and increased concentrations of gemcitabine. EGI-1 and TFK-1 parental cells were plated into 6 well plates as a triplet plate layout, for each well 300.000 cells were plated and after 24h treated with (100 µM, 250 µM, 500 µM, 1000 µM) different doses of gemcitabine. Subsequently, cells were washed with 1X PBS and the old medium was replaced with a fresh medium.

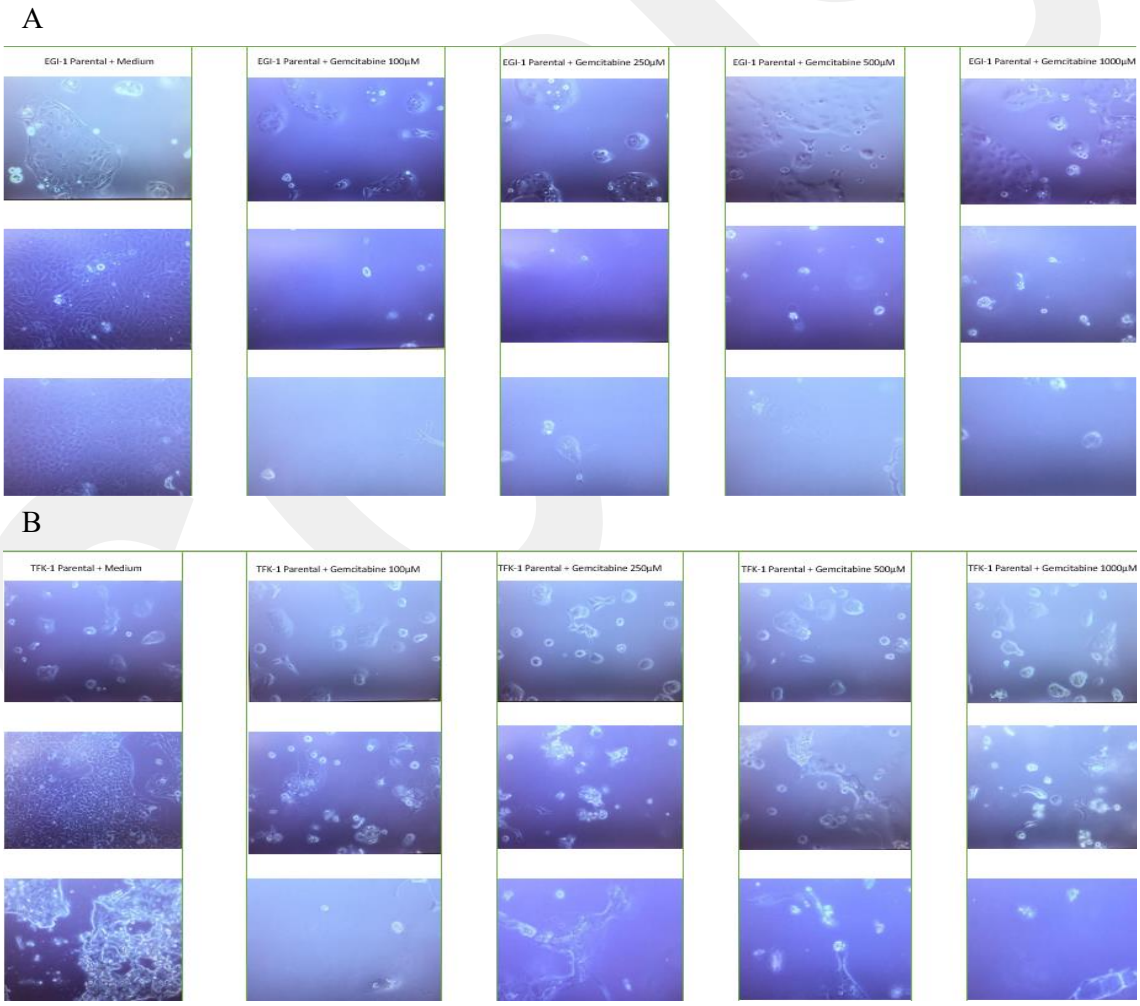


Figure 3.23 The effect of gemcitabine treatment on the cell proliferation of (A) EGI-1 cell treatment with 100 µM, 250 µM, 500 µM, 1000 µM of gemcitabine. (B) TFK-1 cell treatment with 100 µM, 250 µM, 500 µM, 1000 µM of gemcitabine during the generation of resistant cells.

Unfortunately, both EGI-1 and TFK-1 cells were not healthy and could not be lived or increased their number of cells under these treatments after a few passages with a high treatment dose of gemcitabine (Figure 3.23).

3.3 The effect of cisplatin treatment on the proliferation of HCC cell line

In order to understand the effect of cisplatin treatment on the proliferation of HCC cell line called HepG2, we treated the HepG2 parental cell with increased doses of cisplatin. Doses have been chosen according to the previous studies that include cisplatin treatment [193]. For the purpose of finding the most effective range of doses, several cytotoxic assays were performed for 24, 48, and 72 hours.

The cisplatin doses started with a low concentration and were followed by increased concentrations (1-15 μM).

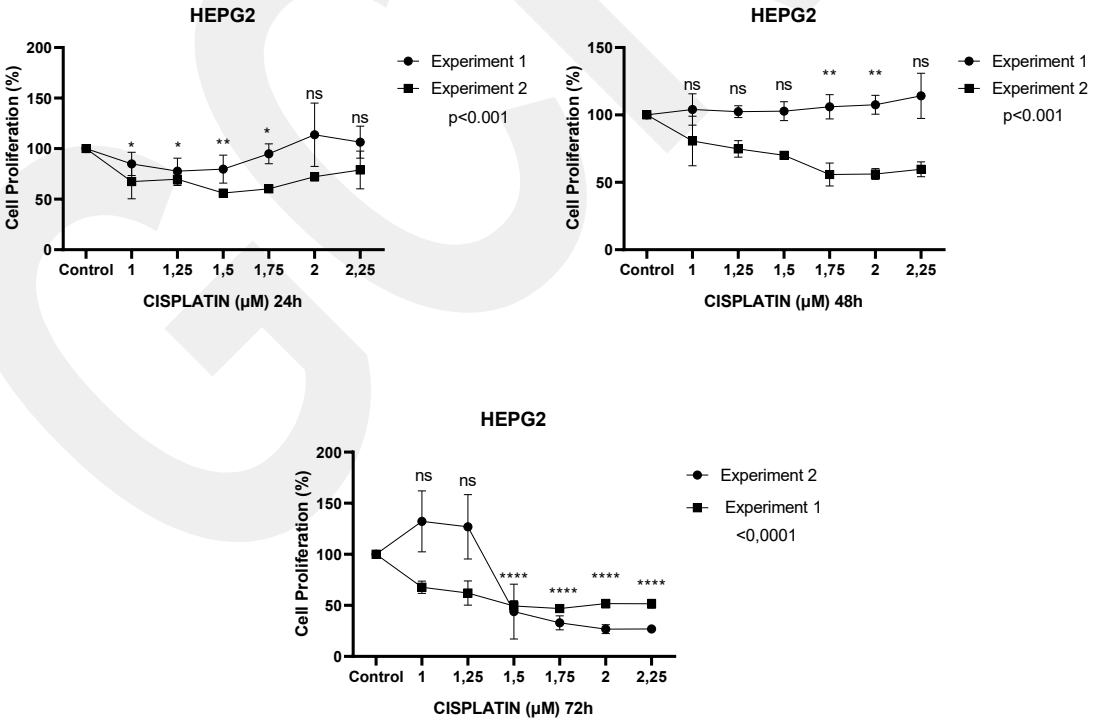


Figure 3.24 The effect of cisplatin treatment (1 µM to 2,25 µM) on the cell proliferation of HCC cells for 24h, 48h, and 72h. In each experiment the number of replicate 2, and the average of the replicates performed during each experiment were imported to GraphPad, where the statistical analysis was done on different independent experiments (n=3) and, (ns=P>0.05, *= P ≤ 0.05, **= P ≤0.01, ***= P ≤ 0.001, ****= P ≤ 0.0001).

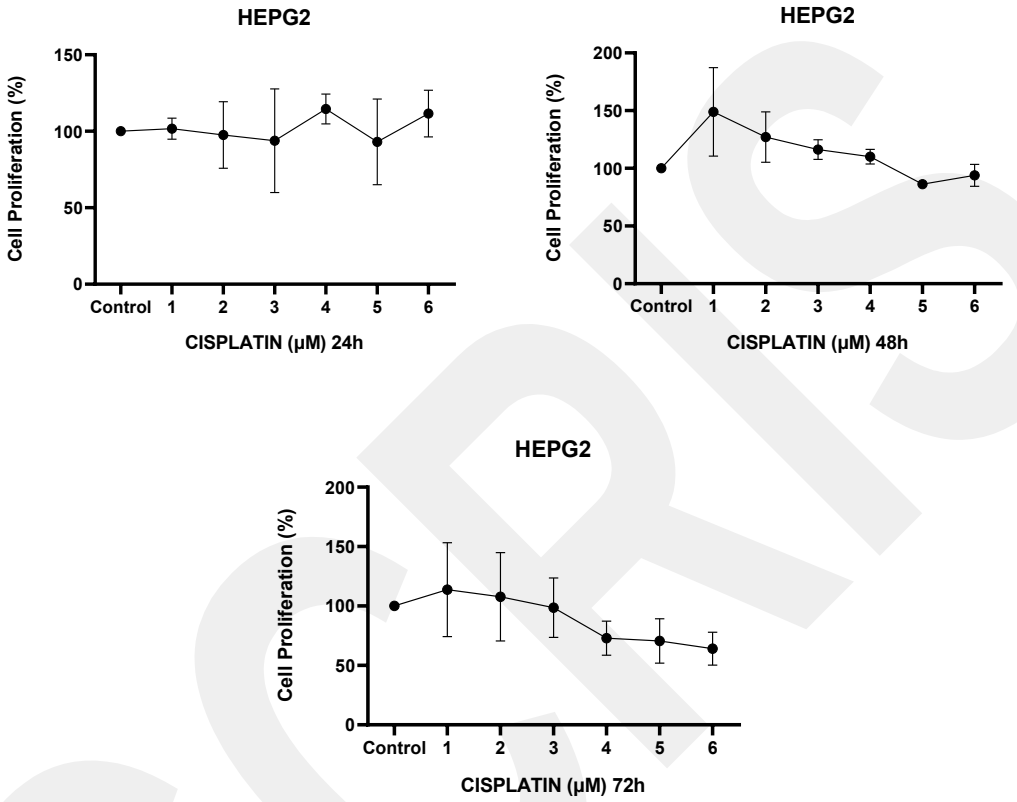


Figure 3.25 The effect of cisplatin treatment (1 µM to 6 µM) on the cell proliferation of HCC cells for 24h, 48h, and 72h. The average of the 3 replicates performed during each experiment were imported to GraphPad.

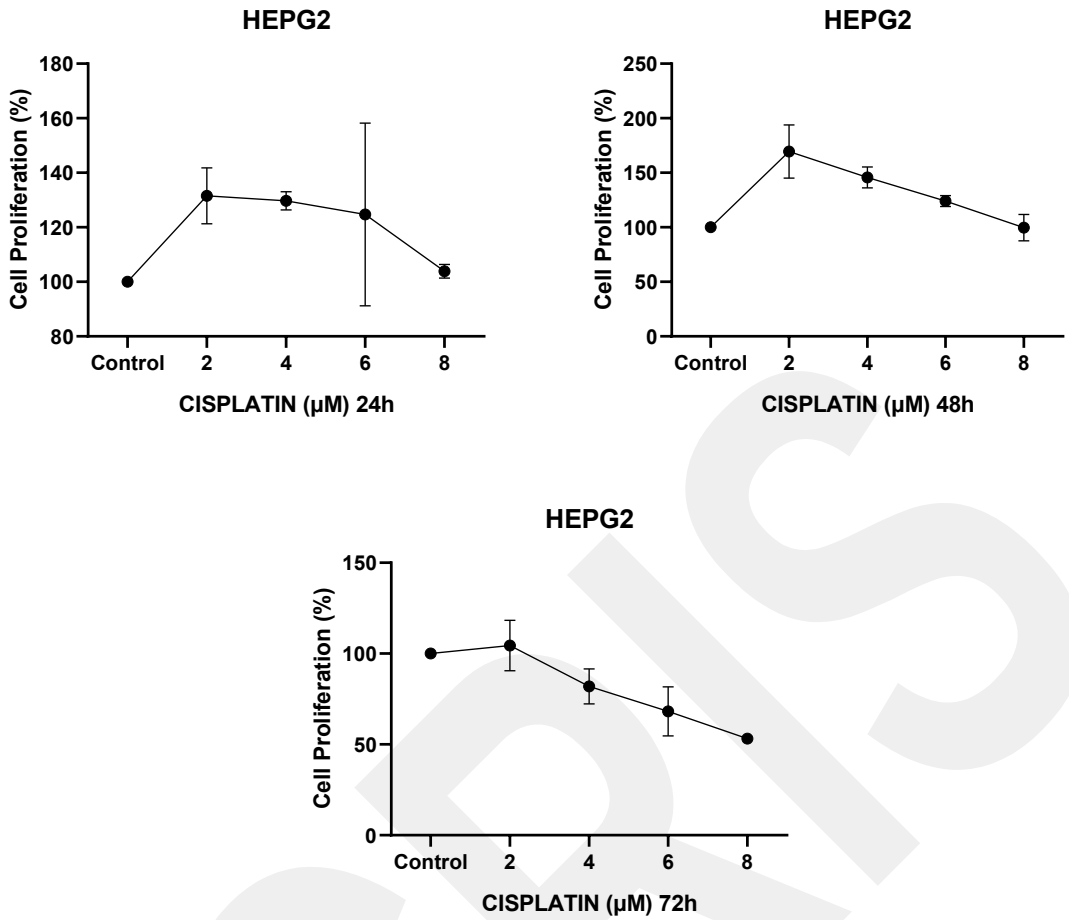


Figure 3.26 The effect of cisplatin treatment (2 µM to 8 µM) on the cell proliferation of HCC cells for 24h, 48h, and 72h. The average of the 3 replicates performed during each experiment were imported to GraphPad.

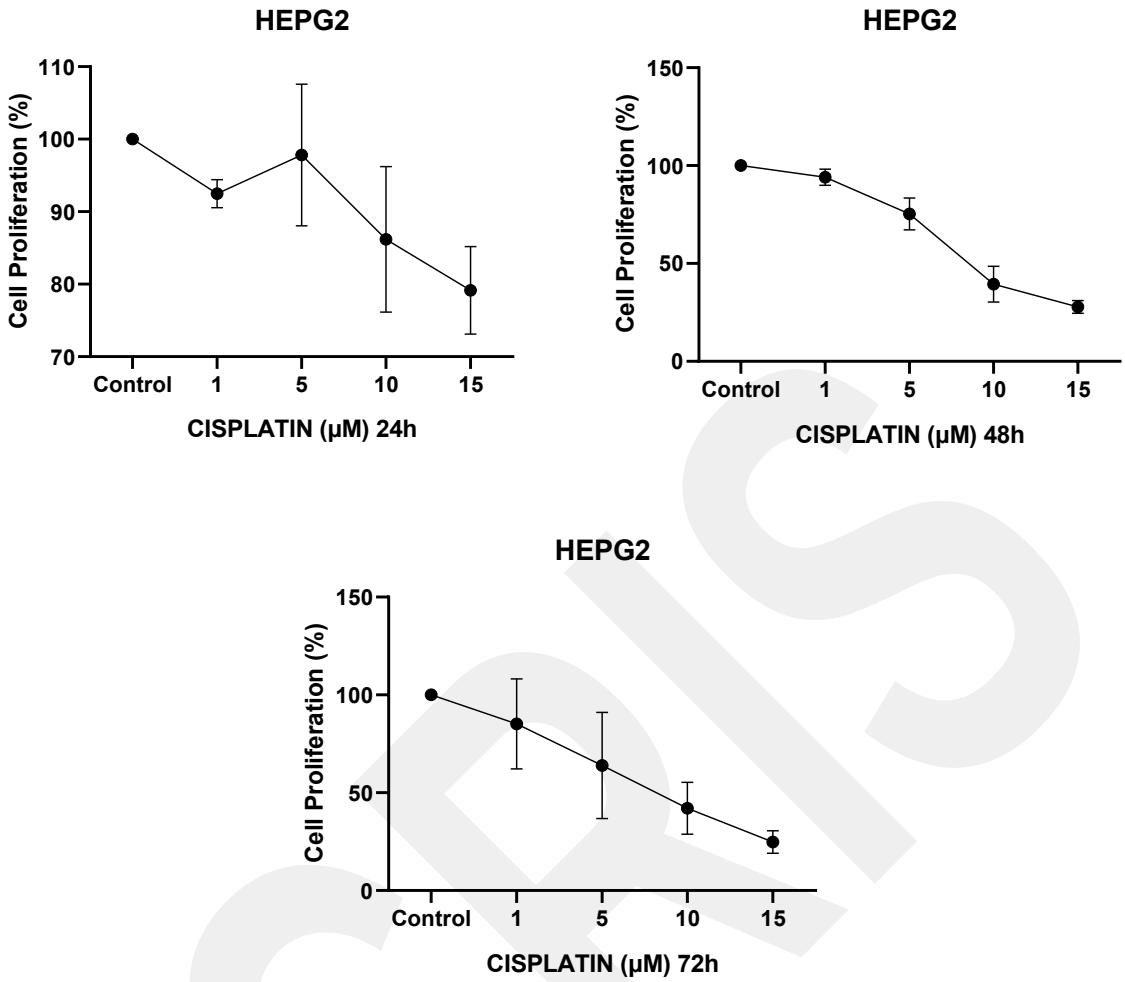


Figure 3.27 The effect of cisplatin treatment (1 µM to 15 µM) on the cell proliferation of HCC cells for 24h, 48h, and 72h. The average of the 3 replicates performed during each experiment were imported to GraphPad.

After all of the MTT assays to check the effect of cisplatin on HCC cell line (HepG2), it was observed that 10µM concentrations of cisplatin treatment for 48h, as an IC50 value for HepG2 cells. The cytotoxic assays indicate that treatment of HepG2 cells with 10 µM cisplatin for 48h have been decreased the cell number by almost 50% which have shown in the Figure 3.27.

3.4 The effect of gemcitabine treatment on the proliferation of HCC cell line

In order to understand the effect of gemcitabine treatment on the proliferation of HCC cell line called HepG2, we treated the HepG2 parental cell with increased doses of gemcitabine. Doses have been chosen according to the previous studies that include gemcitabine treatment [194]. For the purpose of finding the most effective range of doses, several cytotoxic assays were performed for 24, 48, and 72 hours. The gemcitabine doses started with a low concentration and were followed by increased concentrations (0,5-2 μM).

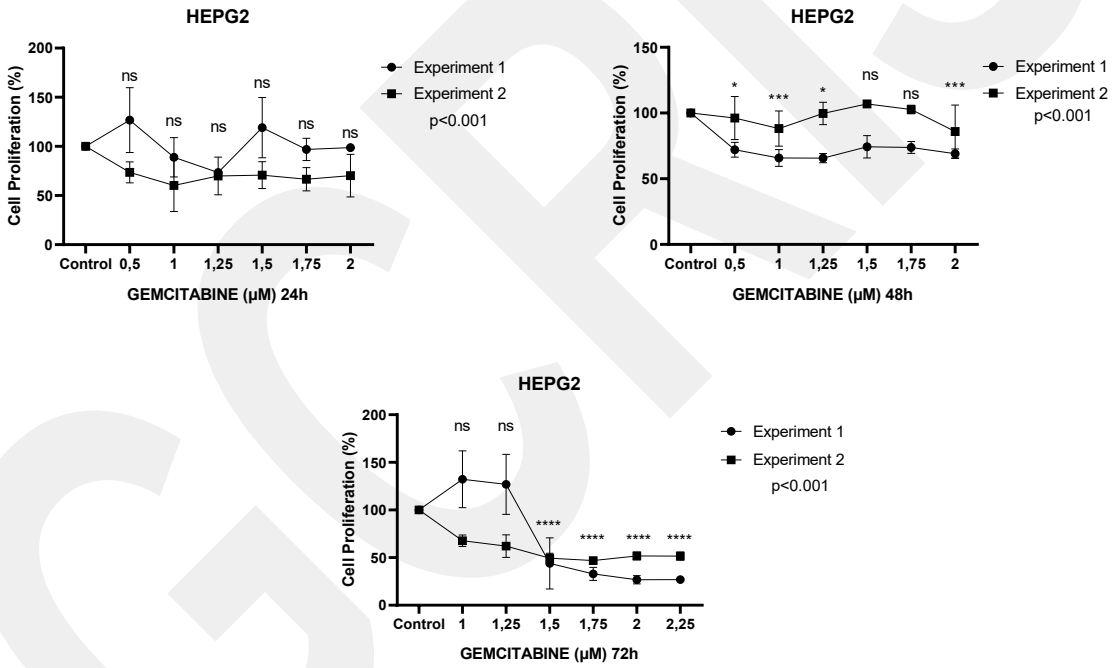


Figure 3.28 The effect of gemcitabine treatment (0,5 μM to 2 μM) on the cell proliferation of HCC cells for 24h, 48h, and 72h. In each experiment the number of replicates was 3, and the average of the replicates performed during each experiment were imported to GraphPad, where the statistical analysis 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).

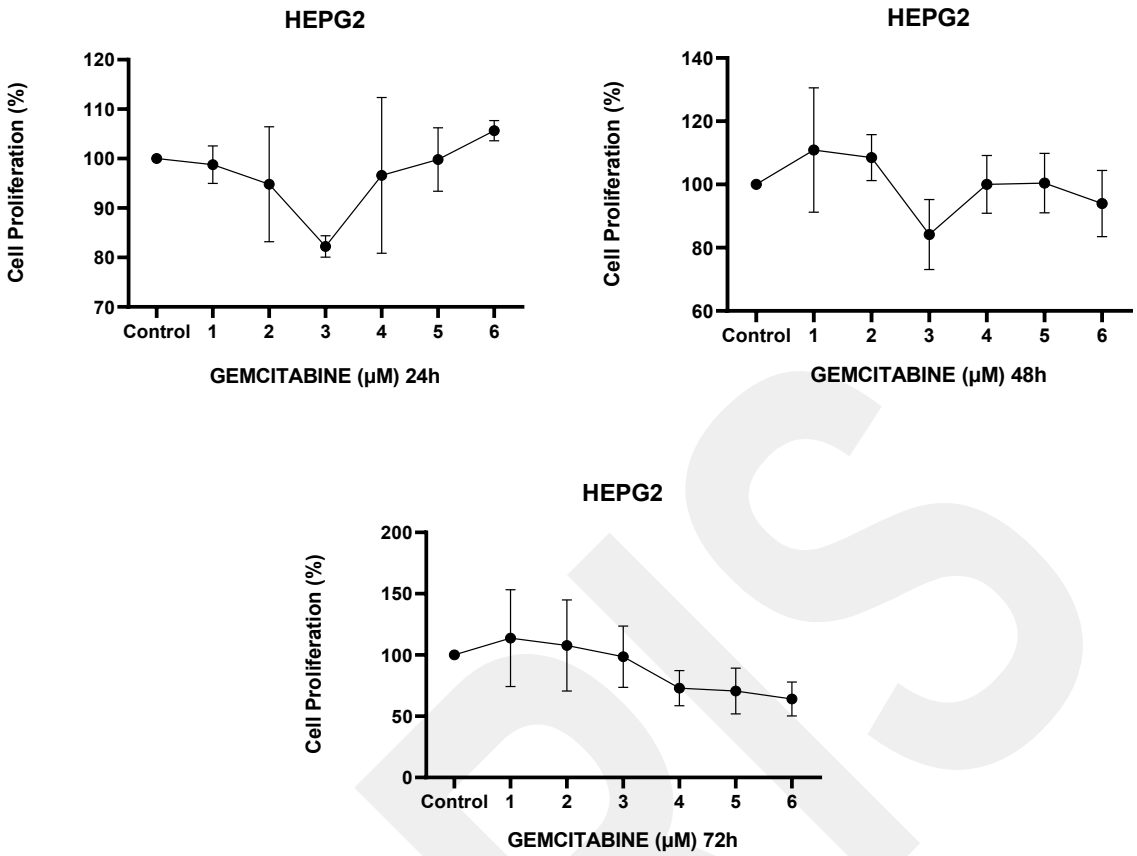


Figure 3.29 The effect of gemcitabine treatment (1 µM to 6 µM) on the cell proliferation of HCC cells for 24h, 48h, and 72h. The average of the 3 replicates performed during each experiment were imported to GraphPad.

After all of the MTT assays to check the effect of gemcitabine, it was observed that performed concentrations of gemcitabine treatment for 48h were not decreased the cell number by 50% which has been shown in the figure. On the other hand, the cytotoxic assays indicate that treatment of HepG2 cells with 6 µM gemcitabine for 48h have been decreased the cell number by almost 50% which have shown in the Figure 3.29.

3.5 The effect of Autophagy manipulation on the proliferation of chemoresistant CCA cell lines

Nocodazole is one of the microtubule-depolymerizing agents that increases autophagosome number and causes inhibition of autophagy-mediated protein degradation and endosome autophagosome fusion [159,160,163].

In order to manipulate the autophagy pathway, the inhibition of autophagy achieved with autophagosome-lysosome fusion using Nocodazole [158-163].

According to the experiments performed in our laboratory before, we decided on the IC50 value of Nocodazole, 2,15μM and 2,89μM for 48h, for EGI-1 and TFK-1, respectively.

3.6 The effect of HDAC inhibition on the proliferation of chemoresistant CCA cell lines

Histone deacetylase (HDAC) is essential for both chromatin remodeling and gene expression. The dysregulation in the function of HDACs in cancer can lead to the repression of genes mostly involved in the regulation of proliferation, differentiation, angiogenesis, metastasis, and migration. In humans, the HDAC family consists of eighteen enzymes classified into four isoform, based on homology to yeast HDACs [95,195,196].

HDACis have promising anticancer characteristics against solid and hematological malignancies with relative resistance in normal cells. HDACis have a neoplastic effect via triggering both mitochondria-mediated apoptosis and caspase-independent autophagic cell death [95].

Several HDACis are undergoing Phase I, II and III clinical trials in a variety of human cancers.

Trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA or vorinostat), LAQ-824/LBH 589, valproic acid (VPA), phenylbutyrate, and MS- 275 are the most well-known HDAC inhibitors [95,196-198]**.**

For the purpose of inhibition of HDACs, SAHA (suberoylanilide hydroxamic acid), MS-275, and FK-228 (romidepsin) [95,99,100,164-167] have been used.

3.6.1 The effect of SAHA treatment on the cell proliferation of EGI-1 and TFK-1 cells for 48h

In order to inhibit the HDACs in both parental and resistant EGI-1 and TFK-1 cells, we treated parental and resistant EGI-1 and TFK-1 cells parental cells with increased doses of SAHA. Doses have been chosen according to the previous studies that include SAHA treatment in CCA cell lines [97,199].

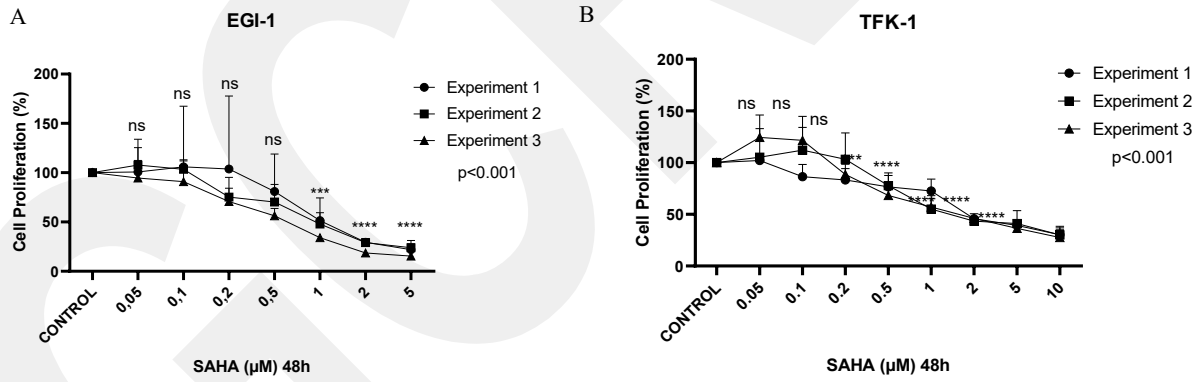


Figure 3.30 The effect of SAHA treatment (0,05 μM to 5 μM and 0,05 μM to 10 μM) on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 48h. In each experiment the number of replicates was 3, and the average of the replicates performed during each experiment were imported to GraphPad, where the 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).

After all of the MTT assays to decide the IC50 value for SAHA, it was observed that 0,43μM and 2,25μM for EGI-1 and TFK-1, respectively, concentrations of SAHA treatment for 48h, as an IC50 value for EGI-1 and TFK-1 cells. The cytotoxic assays have shown that treatment of EGI-1 and TFK-1 cells with 0,43μM and 2,25μM SAHA for 48h

have been decreased the cell number by almost 50% which have shown in the Figure 3.30.

3.6.2 The effect of MS-275 treatment on the cell proliferation of EGI-1 and TFK-1 cells for 48h

In order to inhibit the HDACs in both parental and resistant EGI-1 and TFK-1 cells, we treated parental and resistant EGI-1 and TFK-1 cells parental cells with increased doses of MS-275**.** Doses have been chosen according to the previous studies that include MS-275 treatment in CCA cell lines [167].

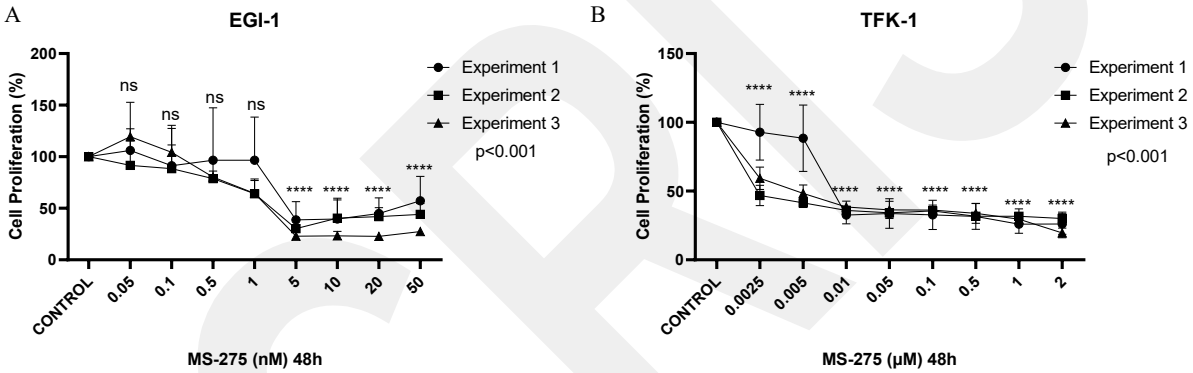


Figure 3.31 The effect of MS-275 treatment (0,05 nM to 50 nM and 0,0025 µM to 2 µM) on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 48h. In each experiment the number of replicates was 3, and the average of the replicates performed during each experiment were imported to GraphPad, where 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).

After all of the MTT assays to decide the IC50 value for MS-275, it was observed that 0,53nM and 0,0035µM for EGI-1 and TFK-1, respectively, concentrations of MS-275 treatment for 48h, as an IC50 value for EGI-1 and TFK-1 cells. The cytotoxic assays have shown that treatment of EGI-1 and TFK-1 cells with 0,53nM and 0,0035µM MS-275 for 48h have been decreased the cell number by almost 50% which have shown in the Figure 3.31.

3.6.3. The effect of Romidepsin treatment on the cell proliferation of EGI-1 and TFK-1 cells for 48h

In order to inhibit the HDACs in both parental and resistant EGI-1 and TFK-1 cells, we treated parental and resistant EGI-1 and TFK-1 cells parental cells with increased doses of Romidepsin. Doses have been chosen according to the previous studies that include Romidepsin treatment in CCA cell lines [200].

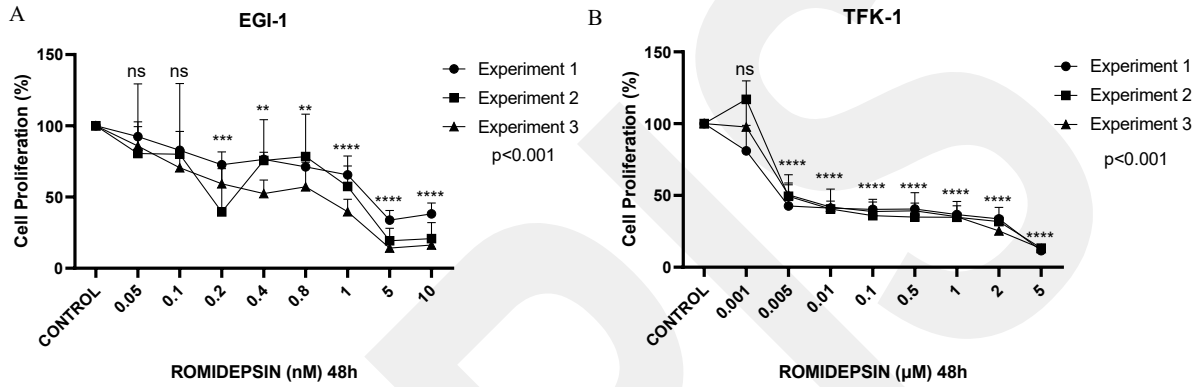


Figure 3.32 The effect of Romidepsin treatment (0,05 nM to 10 nM and 0,001 µM to 5 µM) on the cell proliferation of (A) EGI-1 and (B) TFK-1 cells for 48h. In each experiment the number of replicates was 3, and the average of the replicates performed during each experiment were imported to GraphPad, where 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).

After all of the MTT assays to decide the IC50 value for Romidepsin, it was observed that 0,74nM and 0,0037µM for EGI-1 and TFK-1, respectively, concentrations of Romidepsin treatment for 48h, as an IC50 value for EGI-1 and TFK-1 cells. The cytotoxic assays have shown that treatment of EGI-1 and TFK-1 cells with 0,74nM and 0,0037µM Romidepsin for 48h have been decreased the cell number by almost 50% which have shown in the Figure 3.32.

3.7 Combination treatment of autophagy and HDAC inhibition and its effect on the proliferation of chemoresistant CCA cell lines

3.7.1 The effect of SAHA and Chloroquine combination treatment on the cell proliferation of EGI-1 and TFK-1 cells for 48h

Autophagy is a complex process and able to be regulated at so many levels by different proteins, this allows chemical inhibition at the different levels of autophagy. Chloroquine (CQ) is a lysosomotropic agent that has been used to inhibit autophagy by perturbing lysosomal function [201]. Chloroquine has been shown to block autophagosomal degradation and inhibit autophagy via blocking lysosomal acidification. According to the experiments performed in our laboratory previously, we decided to use chloroquine with the value of IC50, which is and for EGI-1 and TFK-1 respectively.

In this experiment, we aimed to inhibit autophagy with Chloroquine and inhibit HDACs with SAHA at the same time.

Combination treatment of these two inhibitors has been performed for EGI-1 parental and resistant cells as followed concentrations; the IC30 value of SAHA (0,43 μM), the IC30 value of Chloroquine (5,14 μM), and the combination of IC30 values of both of them, for TFK-1 parental and cisplatin-resistant cells as followed concentrations, the IC30 value of SAHA (2,25 μM), and the combination of IC30 value of SAHA and increasing concentrations of Chloroquine (10 μM to 500 μM).

In EGI-1 parental cells, it is observed that compared to the control, parental cell proliferation was 50%, 100%, and 55% for the treatment of IC30 value of SAHA, the IC30 value of Chloroquine, and the combination of IC30 values of SAHA and Chloroquine, respectively.(Figure 3.33)

In EGI-1 cisplatin-resistant cells, it is observed that compared to the control, parental cell proliferation was 56%, 13%, and 37% for the treatment of IC30 value of SAHA, the IC30 value of Chloroquine, and the combination of IC30 values of SAHA and Chloroquine, respectively.(Figure 3.33)

In TFK-1 parental cells, the combination treatment of SAHA and Chloroquine was more effective than the treatment of cisplatin-resistant TFK-1 cells.

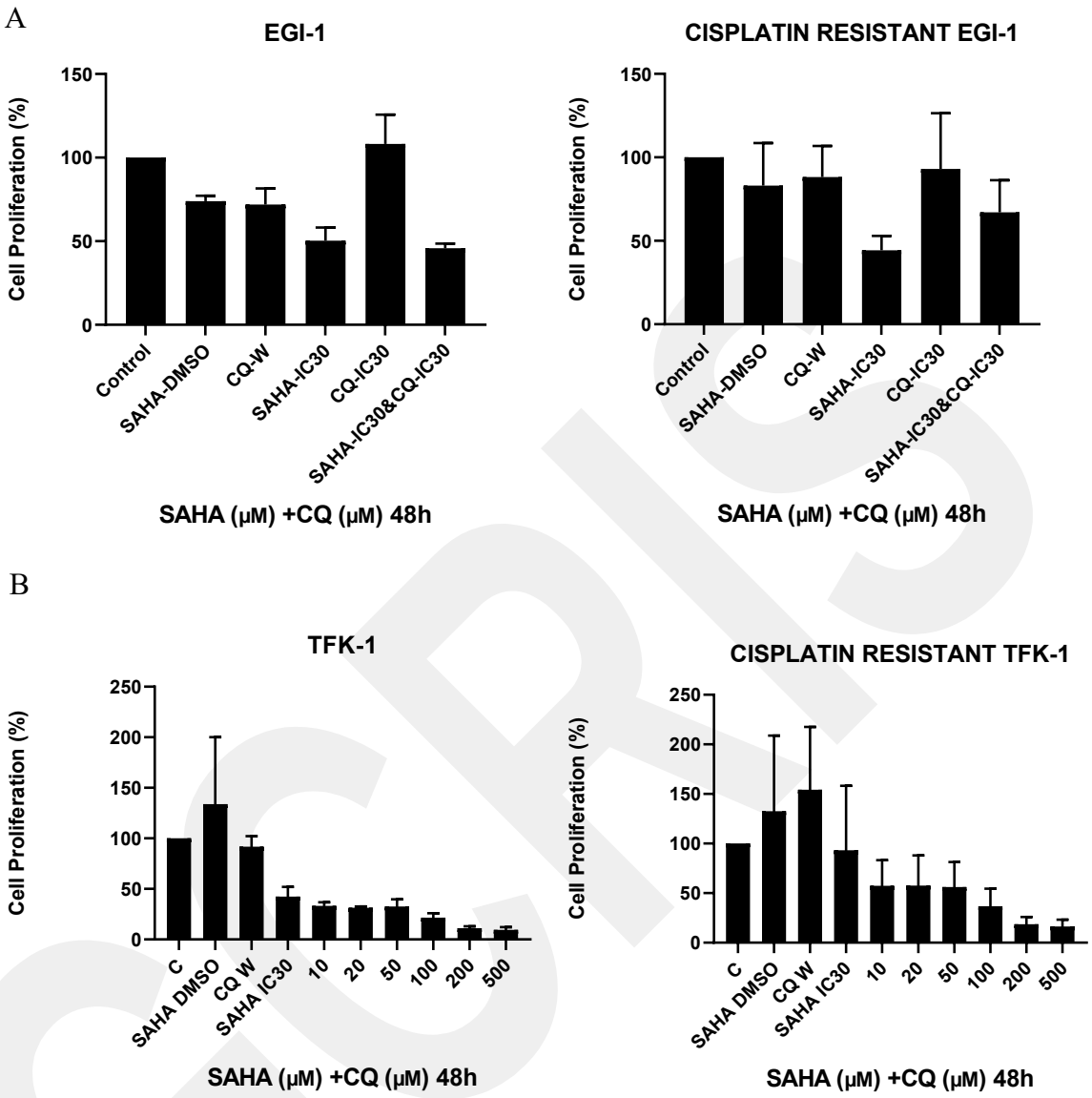


Figure 3.33 The effect of SAHA (µM) and Chloroquine (µM) combination treatment on the cell proliferation. (A) The proliferation of EGI-1 and cisplatin resistant EGI-1 cells after treatment with SAHA-DMSO (µM), Chloroquine-Water (µM), SAHA-IC30 (µM), Chloroquine-IC30 (µM), and combination of SAHA-IC30 (µM) and Chloroquine-IC30 (µM) for 48h , (B) The proliferation of TFK-1 and cisplatin resistant TFK-1 cells after treatment with SAHA-DMSO (µM), Chloroquine-Water (µM), SAHA-IC30 (µM), combination of SAHA-IC30 (µM) and Chloroquine (10 µM, 20 µM, 50 µM ,100 µM ,200 µM,500 µM) for 48. Cell proliferation was imported to GraphPad.

As a result, these percentages have shown that the combination of HDAC inhibition and autophagy blocking (IC30 value of SAHA and IC30 value of Chloroquine) results in

a synergetic combination for cell death which makes it is better than only blocking the autophagy pathway.

3.7.2 The effect of Nocodazole combination treatment with SAHA, MS-275, and Romidepsin on the cell proliferation of EGI-1, cisplatin resistant EGI-1 and TFK-1, cisplatin-resistant TFK-1 cells for 48h.

For the exact aim of check the synergistic effect of combination treatment on the proliferation of parental EGI-1, TFK-1, cisplatin-resistant EGI-1 and TFK-1 cells, the cells were treated with Nocodazole alone, SAHA alone, MS-275 alone, Romidepsin alone, and a combination of Nocodazole and SAHA, Nocodazole and MS-275, and Nocodazole and Romidepsin for 48h. IC30 doses of Nocodazole (2,15 μM for EGI-1 and 2,89 μM for TFK-1), IC30 doses of SAHA (0,43 μM for EGI-1 and 2,25 μM for TFK-1), IC30 doses of MS-275 (0,53 nM for EGI-1 and 0,0035 μM for TFK-1), and IC30 doses of Romidepsin (0,74nM for EGI-1 and 0,0037 μM for TFK-1). The concentrations were determined with the help of the cell proliferation assays performed previously.

When the EGI-1 were treated with, Nocodazole alone, the cell proliferation decreases approximately 40% of cells compared to control, SAHA alone, the proliferation of cells decreases by around 30% of cells compared to control, MS-275 alone, the proliferation of cells decreases by around 80% of cells compared to control, and Romidepsin alone, the proliferation of cells decreased by around 80% of cells compared to control. On the other hand, the combination treatment of Nocodazole with MS-275 and Romidepsin, respectively, lead to a considerable decrease in EGI-1 cells proliferation compared to the single treatment of each inhibitor (Figure 3.34).

When the cisplatin-resistant EGI-1 cells were treated with, Nocodazole alone, the proliferation of cells decreases approximately 20% of cells compared to control, SAHA alone, the proliferation of cells decreases almost 30% of cells compared to control, MS-275 alone, the proliferation of cells decreases around 85% of cells compared to control, and Romidepsin alone, the proliferation of cells decreased almost 90% of cells compared to control. Additionally, the combination treatment of Nocodazole with MS-275 and Romidepsin, respectively, lead to a further decrease in cisplatin resistant EGI-1 cells proliferation compared to the single treatment of each inhibitor (Figure 3.34)

When the TFK-1 cells were treated with only Nocodazole, the proliferation of cells decreases around 50% of cells compared to control, SAHA alone, the proliferation of cells decreases by around 50% of cells compared to control, MS-275 alone, the proliferation of cells decreases almost 30% of cells compared to control, and Romidepsin alone, the proliferation of cells decreased approximately 40% of cells compared to control (C). In addition to that, the combination treatment of Nocodazole with MS-275 and Romidepsin, respectively, lead to a further decrease in cell proliferation compared to the single treatment of each inhibitor (Figure 3.35).

When the cisplatin-resistant TFK-1 cells were treated with, Nocodazole alone, the proliferation of cells decreases almost 40% of cells compared to control, SAHA alone, the proliferation of cells decreases by around 30% of cells compared to control, MS-275 alone, the proliferation of cells were not decreased compared to control, and Romidepsin alone, the proliferation of cells decreased approximately 50% of cells compared to control (C). On the other hand, the combination treatment of Nocodazole with SAHA and Romidepsin, respectively, lead to a further decrease in cisplatin-resistant TFK-1 cells proliferation compared to the single treatment of each inhibitor (Figure 3.35)

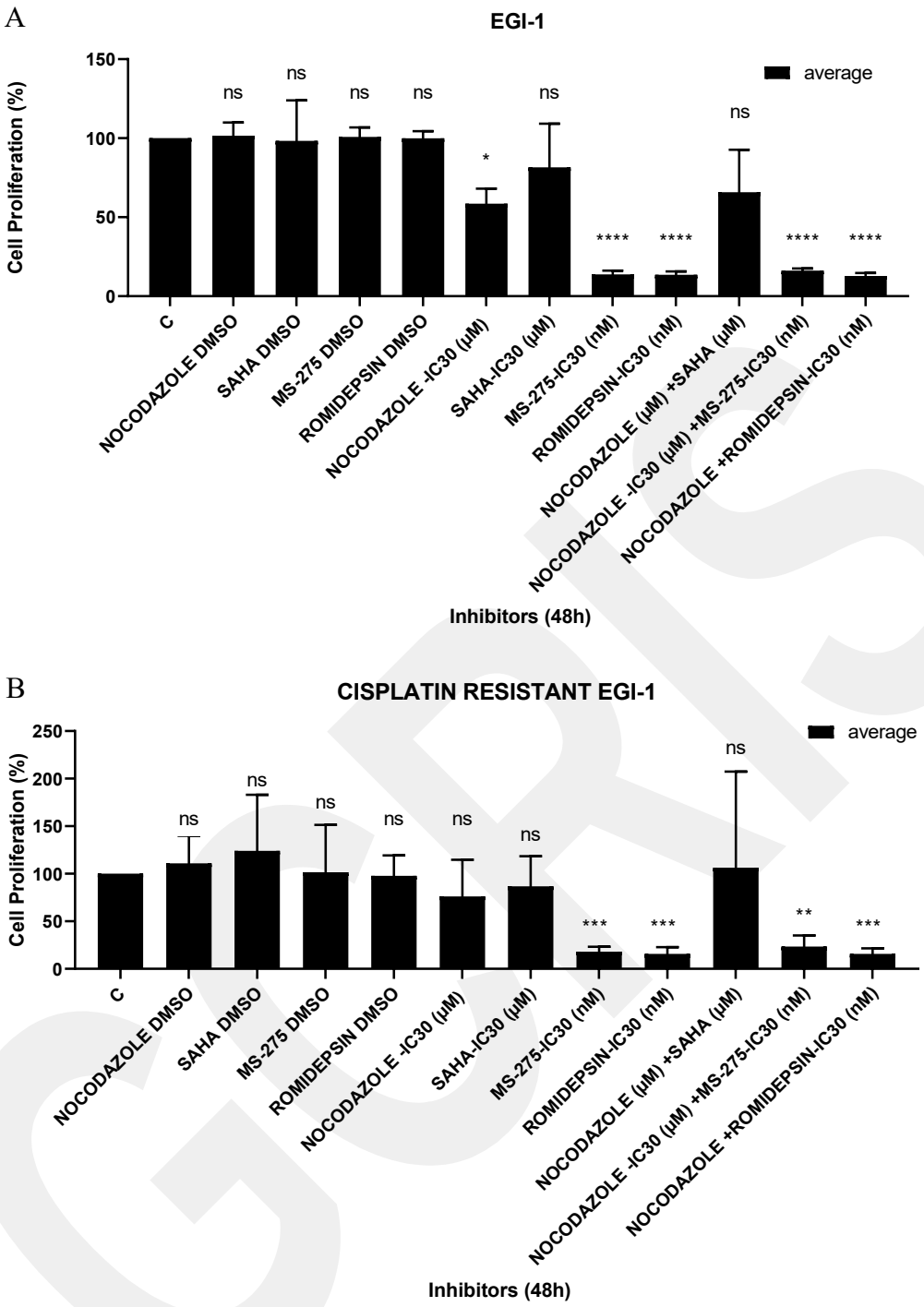


Figure 3.34 (A) The proliferation of EGI-1 parental cells after treatment with Nocadozole- DMSO (µM), SAHA-DMSO (µM), MS-DMSO (nM), Romidepsin-DMSO (nM), Nocadozole-IC30 (µM), SAHA-IC30 (µM), MS-275-IC30 (nM), Romidepsin-IC30 (nM), combination of Nocadozole-IC30(µM) and SAHA-IC30 (µM), combination of Nocadozole-IC30 (µM), and MS-IC30 (nM), combination of Nocadozole-IC30 (µM), and Romidepsin-IC30 (nM), for 48h.
(B) The proliferation of EGI-1 cisplatin-resistant cells after treatment with Nocadozole- DMSO (µM), SAHA-DMSO (µM), MS-DMSO (nM), Romidepsin-DMSO (nM),

Nocadozole-IC30 (µM), SAHA-IC30 (µM), MS-275-IC30 (nM), Romidepsin-IC30 (nM), combination of Nocadozole-IC30 (µM) and SAHA-IC30 (µM), combination of Nocadozole-IC30 (µM), and MS-IC30 (nM), combination of Nocadozole-IC30 (µM), and Romidepsin-IC30 (nM), for 48h. Cell proliferation was imported to GraphPad.

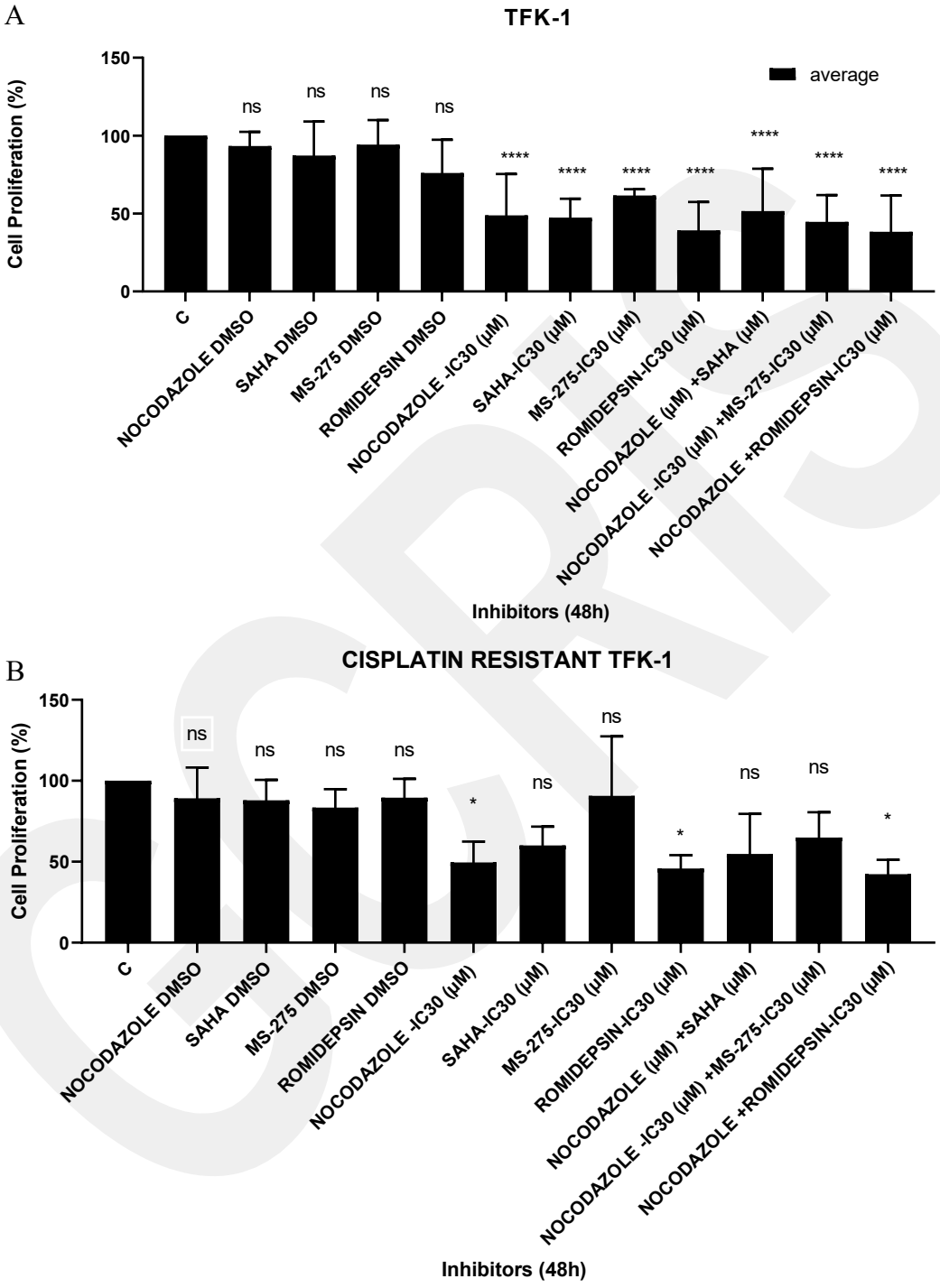


Figure 3.35 (A) The proliferation of TFK-1 parental cells after treatment with Nocadozole- DMSO (µM), SAHA-DMSO (µM), MS-DMSO (µM), Romidepsin-DMSO (µM), Nocadozole-IC30 (µM), SAHA-IC30 (µM), MS-275-IC30 ((µM), Romidepsin-IC30 (µM), combination of Nocadozole-IC30(µM) and SAHA-IC30

(µM), combination of Nocadozole-IC30 (µM), and MS-IC30 (µM), combination of Nocadozole-IC30 (µM), and Romidepsin-IC30 (µM), for 48h.
(B) The proliferation of cisplatin resistant TFK-1 parental cells after treatment with Nocadozole- DMSO (µM), SAHA-DMSO (µM), MS-DMSO (µM), Romidepsin-DMSO (µM), Nocadozole-IC30 (µM), SAHA-IC30 (µM), MS-275-IC30 ((µM), Romidepsin-IC30 (µM), combination of Nocadozole-IC30(µM) and SAHA-IC30 (µM), combination of Nocadozole-IC30 (µM), and MS-IC30 (µM), combination of Nocadozole-IC30 (µM), and Romidepsin-IC30 (µM), for 48h. Cell proliferation was imported to GraphPad.

This suggests that dual treatment of autophagy inhibitor (Nocodazole) and different HDAC inhibitors (SAHA, MS-275, and Romidepsin) lead to a further decrease in the proliferation of parental and cisplatin-resistant CCA cell lines compared to individual treatments. Thereby, suggesting that these treatments can sensitize cisplatin-resistant CCA.

3.8 Autophagy Inhibition Lead to an Increase in Cell Death in Cisplatin-Resistant EGI-1 and Cisplatin Resistant TFK-1 CCA Cell Lines

Several methods help in the identification of apoptotic cells. Depending on the reduced DNA content and changes in morphology, nuclear condensation apoptotic cells are recognized and these cells can be detected by different methods such as flow cytometry, Trypan Blue, or Hoechst staining. Changes that happen in the plasma membrane composition are detected via the appearance of phosphatidylserine on the plasma membrane. Phosphatidylserine reacts with Annexin V-fluorochrome conjugates. A combination of Annexin V-fluorochrome conjugates and propidium iodide (PI) staining, show the difference between the early and late apoptotic events [202].

Followed the determination of anti-proliferative effects of inhibitors, depending on the proliferation test results, the IC30 and IC50 values of Nocodazole and Romidepsin were determined and the Cisplatin-Resistant EGI-1 cells and Cisplatin-Resistant TFK-1 cells were treated with Nocodazole and Romidepsin for 48h.

Following the 48h treatment with both Nocodazole and Romidepsin, the results have been demonstrated the increase in the early apoptotic cell/control at combination

treatment of IC30 values on Cisplatin-Resistan EGI-1 cells. In Cisplatin-Resistant TFK-1 cells, the treatment of IC30 values of Nocodazole and Romidepsin has shown the increase in necrotic cell/control at combination treatment (Figure 3.36)

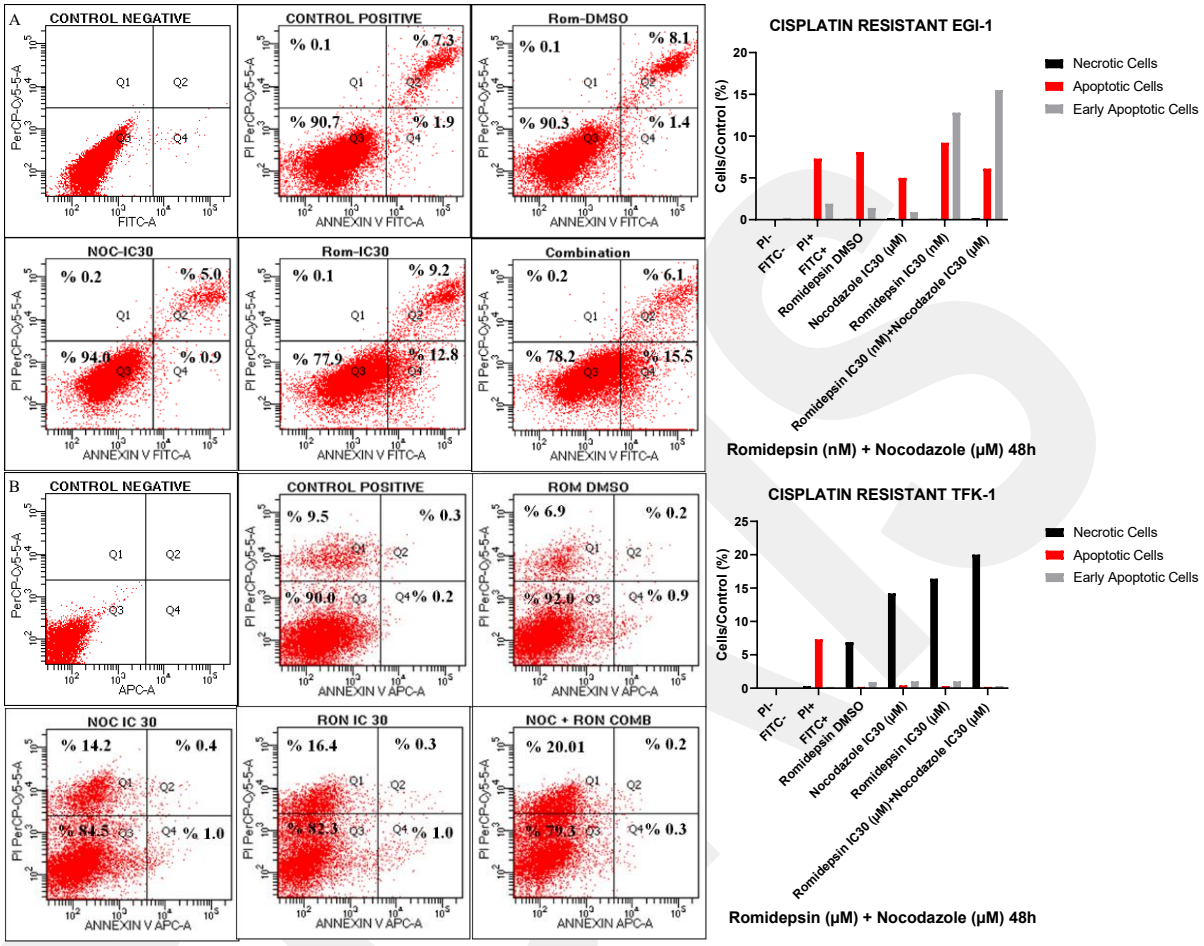


Figure 3.36 Apoptotic analysis of Cisplatin-Resistan EGI-1 and TFK-1 cells by flow cytometer
(A) Determination of necrotic (Q1), apoptotic (Q2), and early apoptotic (Q4) percentage after 48h with IC30 values of Nocodazole (2,15 µM) and Romidepsin(0,74 nM) treatment. The graph shows the quantification of necrotic, apoptotic and, early apoptotic cell percentage of Cisplatin-Resistant EGI-1 cells per control after Nocodazole and Romidepsin treatment. (B) Determination of necrotic (Q1), apoptotic (Q2), and early apoptotic (Q4) percentage after 48h with IC30 values of Nocodazole (2,89 µM) and Romidepsin (0,0037 µM) treatment. The graph shows the quantification of necrotic, apoptotic and, early apoptotic cell percentage of Cisplatin-Resistant TFK-1 cells per control after Nocodazole and Romidepsin treatment. Cell proliferation was imported to GraphPad.

Following the 48h treatment with both Nocodazole and Romidepsin, the results have been indicated the increase in the early apoptotic cell/control at combination treatment of IC50 values on both Cisplatin-Resistant EGI-1 and TFK-1 cells. In

Cisplatin-Resistant EGI-1 and TFK-1 cells, the treatment of IC50 values of Nocodazole and Romidepsin indicated the increase in the early apoptotic cell/control at combination treatment of IC50 values on Cisplatin-Resistant EGI-1 and TFK-1 cells (Figure 3.37).

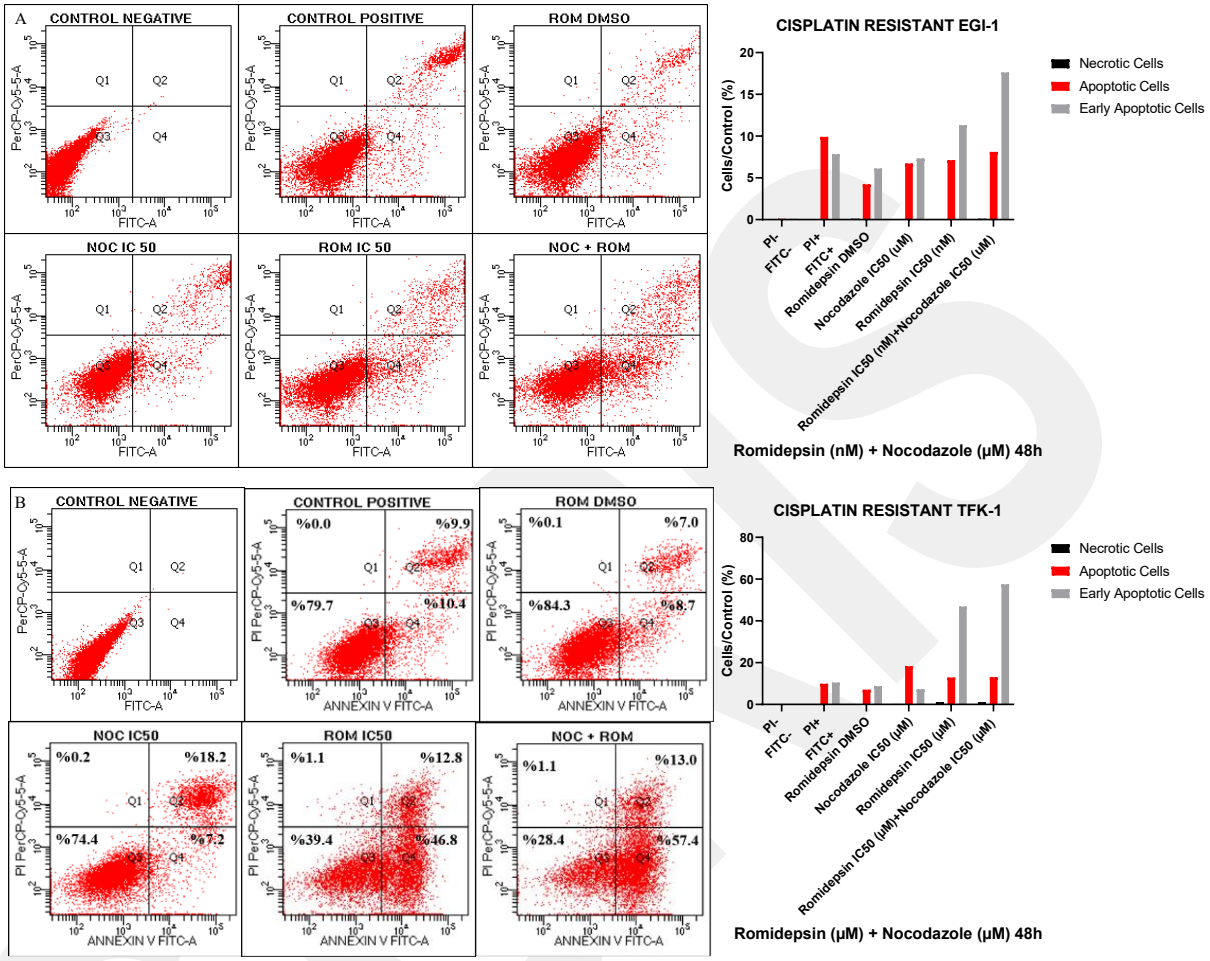


Figure 3.37 (A) Determination of necrotic, apoptotic, and early apoptotic cell percentage after 48h with IC50 values of Nocodazole (0,20 µM) and Romidepsin (0,005 nM) treatment.The graph shows the quantification of necrotic, apoptotic and, early apoptotic cell percentage of Cisplatin-Resistant EGI-1 cells per DMSO control after Nocodazole and Romidepsin treatment. (B)Determination of necrotic, apoptotic, and early apoptotic cell percentage after 48h with IC50 values of Nocodazole (0,30 µM) and Romidepsin (0,25 µM) treatment. The graph shows the quantification of necrotic, apoptotic and, early apoptotic cell percentage of Cisplatin-Resistant TFK-1 cells per DMSO control after Nocodazole and Romidepsin treatment. Cell proliferation was imported to GraphPad.

With the purse of understanding and explaining the decrease in cell proliferation which is observed after autophagy and HDACs inhibition, the cell cycle profile of the cells has been checked via the cell cycle analysis method.

PI flow cytometric assay is one of the flow cytometric analysis methods that can follow the changes in the cell cycle after a variety of changes such as drug treatments. PI is one of the fluorogenic dyes that can bind stoichiometrically to nucleic acids, thus, the DNA content of the cell can be detected via fluorescence emission [203].

Following the 48h treatment with both Nocodazole and Romidepsin, the results have shown approximately the same results at combination treatment of IC30 values on cisplatin-resistant EGI-1 and other treatments on Cisplatin-Resistant EGI-1 cells. Following the 48h treatment with both Nocodazole and Romidepsin, the results have been demonstrated the decrease in the G0/G1 phase of the cell cycle at combination treatment of IC30 values on Cisplatin-Resistant TFK-1 cells (Figure 3.38).

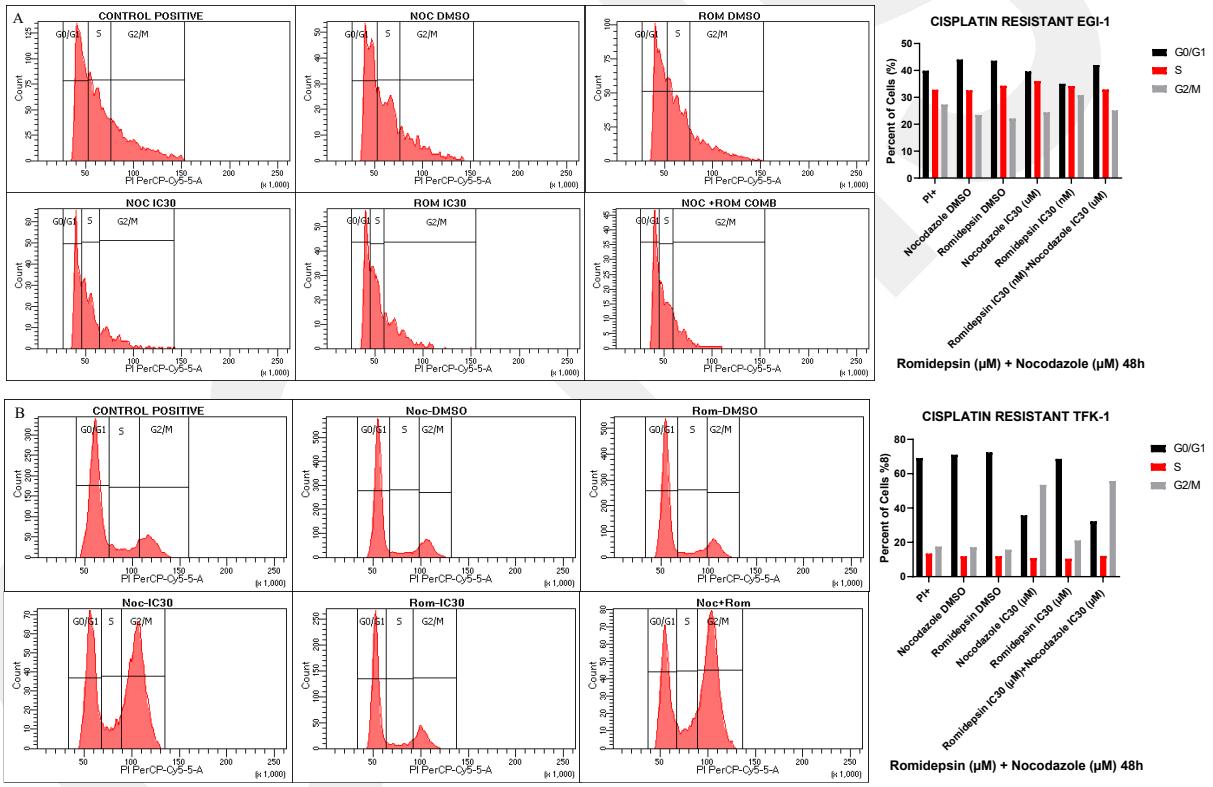


Figure 3.38 Cell cycle analysis of Cisplatin-Resistant EGI-1 and TFK-1 cells by flow cytometer.
(A) PI staining of Cisplatin-Resistant EGI-1 cells after 48h with IC30 values of Nocodazole (2,15 µM) and Romidepsin (0,74nM) treatment.The graph shows the quantification of G0/G1, S, and G2/M cell percentage of Cisplatin-Resistant EGI-1 cells per control after Nocodazole and Romidepsin treatment. B) PI staining of Cisplatin-Resistant TFK-1 cells after 48h with IC30 values of Nocodazole (2,89 µM) and Romidepsin (0,0037 µM) treatment. The graph shows the quantification of G0/G1, S, and G2/M cell percentage of Cisplatin-Resistant TFK-1 cells per control after Nocodazole and Romidepsin treatment. Cell proliferation was imported to GraphPad.

3.9 The Effect of Combination of Autophagy and HDAC inhibition on the Molecular Markers of the Autophagy

With the purpose of understanding the effect of autophagy and HDAC inhibition on Cisplatin-Resistant CCA cells, firstly, we decided to check the molecular markers of autophagy such as the expression of the Phosphatase and tensin homolog (PTEN). The TFK-1 CCA cell line has been shown to express PTEN and have an active AKT/mTOR/PI3K pathway in several studies [156,204,205]. With the purpose of understanding this effect, we performed western blot assays. We checked the level of the PTEN protein expression. For this, we used the IC30 value of HDAC inhibitors and the IC30 value of autophagy inhibitor called Nocodazole.

Histone acetylation is a crucial epigenetic modification, these modifications on chromatin architecture can lead to changes in the regulation of gene expression via making the chromatin transcriptionally active or inactive by opening or closing the structure of the chromatin structure. It plays a main role in essential processes for cell proliferation such as cell cycle progression and differentiation [206].

To check the HDAC inhibition at a molecular level, the H3 and acetylation of the H3 level were detected via western blotting. Since the acetylation or deacetylation of H3 can lead to the overexpression of cellular proteins, and in this case PTEN expression is crucial for CCA, detection of the acetylated lysines of histones H3 is important in this situation to observe the association between PTEN expression and H3 acetylation in Cisplatin-Resistant TFK-1 cells.

In this experiment, PTEN expression in Cisplatin-Resistant TFK-1 cells was shown a decrease after the combination treatment of IC30 values of Nocodazole and SAHA while single treatment of SAHA was increased the expression of PTEN. At the same time, combination treatment has shown the same results for Acetylated-H3 on TFK-1 cells. These results have been demonstrated the harmony of PTEN expression and acetylation of H3. (Figure 3.39)

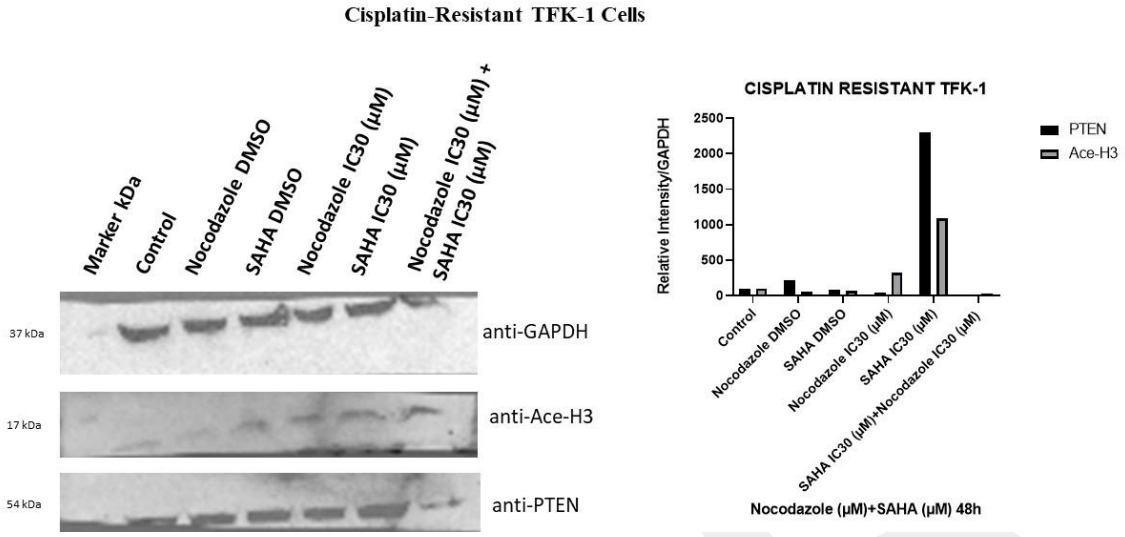


Figure 3.39 Western blot analysis of Cisplatin-Resistant TFK-1 cells after treatment with DMSO control, IC30 values of Nocodazole (2,89 µM) and SAHA (2,25 µM), alone and combinations. Representative western blot analysis of PTEN, Ace-H3, and GAPDH. GAPDH was used as an internal control and for protein loading normalization and densitometric comparison of PTEN and GAPDH expression after Nocodazole and SAHA treatments for 48h.

For the purpose of understanding the function of PTEN in Cisplatin-Resistant cells, silencing of PTEN has been performed via the knockdown process. Cisplatin-Resistant TFK-1 cells were treated with; control siRNA to check the expression of PTEN without any PTEN silencing, siPTEN with two different doses (2 µM and 5 µM) to better understand the effect of the concentration, and Cisplatin-Resistant TFK-1 cells were grown and extracted without any treatment. As a control, nuclear extraction of parental TFK-1 cells has been used.

As a result of this set of experiments, PTEN expression in Cisplatin-Resistant TFK-1 was significantly silenced by siPTEN, moreover, without any treatment, the expression level of PTEN was higher in the total extraction of Cisplatin-Resistant cells than nuclear extraction of parental TFK-1 cells. Moreover, this result have shown the contrast between PTEN and H3 expression on Cisplatin-Resistant TFK-1 cells. (Figure 3.39)

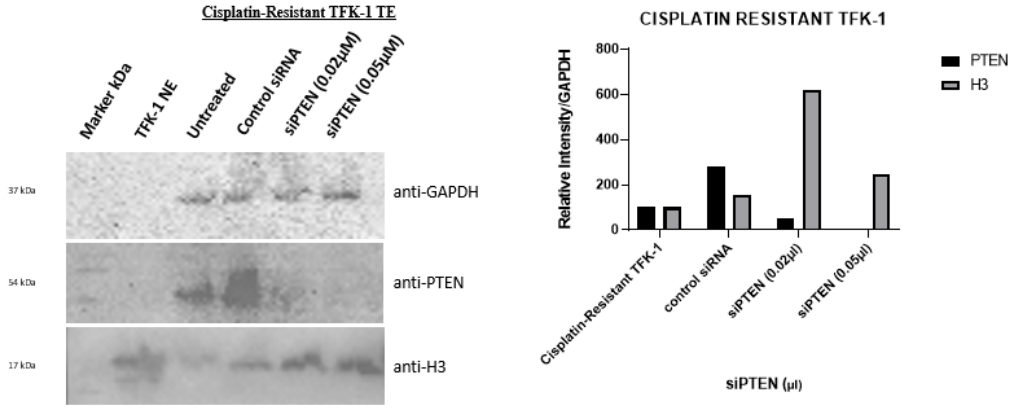


Figure 3.40 Western blot analysis of Cisplatin-Resistant TFK-1 cells, as controls; nuclear extraction of TFK-1, untreated sample, control siRNA sample, and after silence with 0,02 µM siPTEN, 0,05 µM siPTEN samples.
Representative western blot analysis of PTEN, H3 and GAPDH. GAPDH was used as an internal control and for protein loading normalization and densitometric comparison of PTEN,H3 and GAPDH expression after silencing of PTEN.(NE : Nuclear Extraction, TE: Total Extraction)

In this experimental design, Cisplatin-Resistant TFK-1 cells were treated with the combination of SAHA and Nocodazole for 48h. H3 expression in Cisplatin-Resistant TFK-1 cells was shown an increase after the combination of Nocodazole and SAHA treatment while single treatment of SAHA has decreased the expression of H3 on Cisplatin-Resistant TFK-1 cells.

When we compared the results of Ace-H3 (Figure 3.39) and this result, it is clear to observe the contrast between combination therapy of SAHA and Nocodazole, thus, their effect on the acetylation of H3. (Figure 3.41)

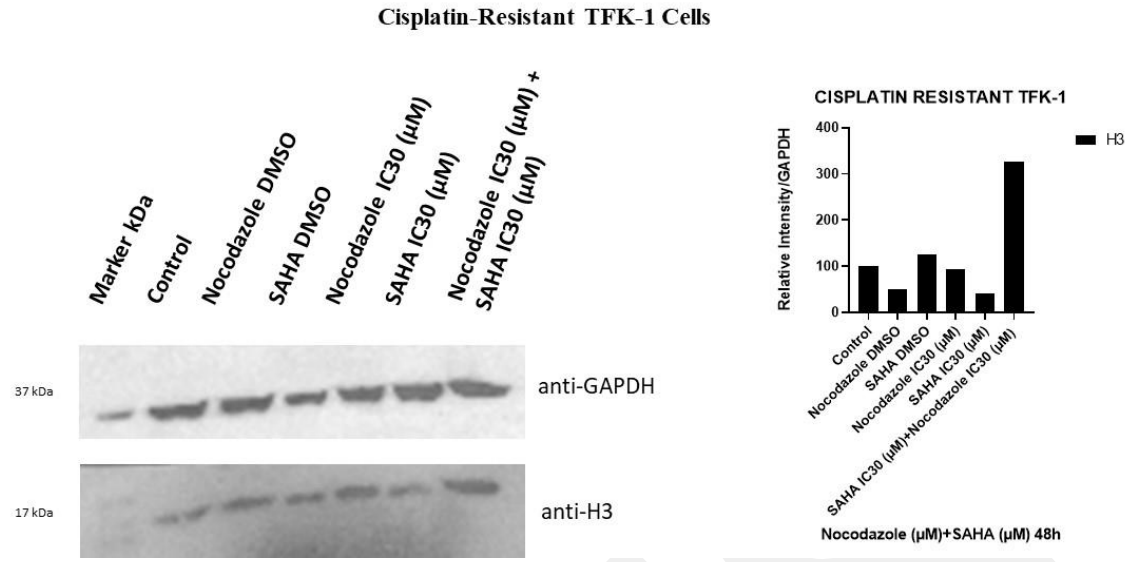


Figure 3.41 Western blot analysis of Cisplatin-Resistant TFK-1 cells after treatment with DMSO control, IC30 values of Nocodazole (2,89 µM) and SAHA (2,25 µM), alone and combinations. Representative western blot analysis of H3 and GAPDH. GAPDH was used as an internal control and for protein loading normalization and densitometric comparison of H3 and GAPDH expression after Nocodazole and SAHA treatments for 48h.

In this experiment, a different HDAC inhibitor called Romidepsin has been used for the combination therapy. Cisplatin-Resistant TFK-1 cells were treated with the combination of Romidepsin and Nocodazole for 48h. H3 expression in Cisplatin-Resistant TFK-1 cells was shown an increase after the single treatment of IC30 value of Romidepsin and combination of Romidepsin and Nocodazole treatment while single treatment of Nocodazole has decreased the expression of H3 significantly, on Cisplatin-Resistant TFK-1 cells.

When we compared the results, Ace-H3 and H3 expression under the combination therapy, H3 expression has been dramatically decreased compared to the Ace-H3 expression. Consequently, combination treatment of Nocodazole and Romidepsin increased the acetylation of H3, which is increasing the PTEN expression as explained in the previous results. (Figure 3.42)

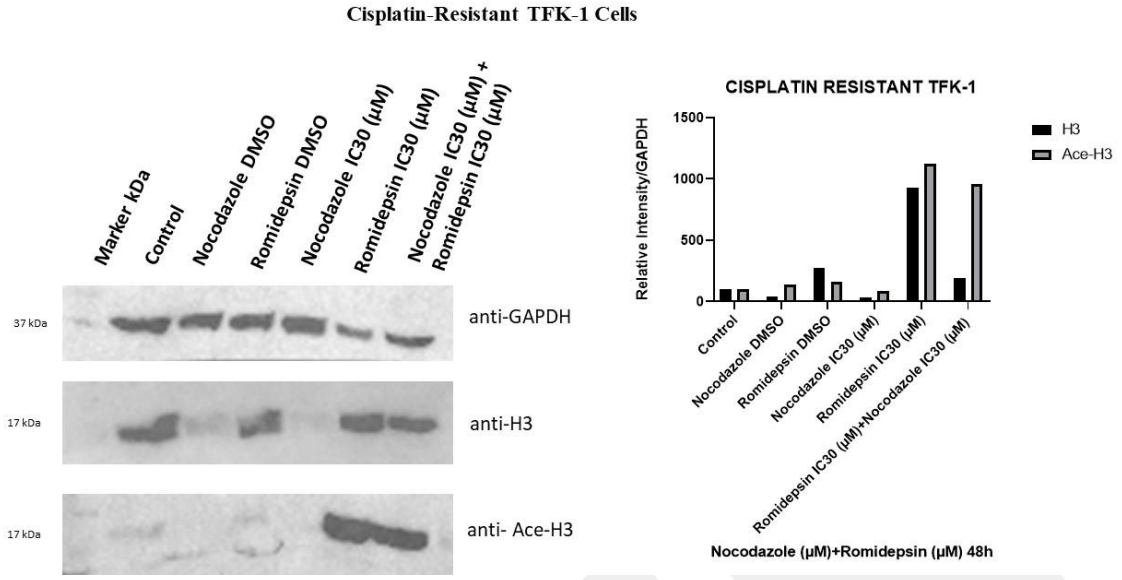


Figure 3.42 Western blot analysis of Cisplatin-Resistant TFK-1 cells after treatment with DMSO control, IC30 values of Nocodazole (2,89 µM) and Romidepsin (0,0037 µM), alone and combinations.. Representative western blot analysis of H3, Ace-H3, and GAPDH. GAPDH was used as an internal control and for protein loading normalization and densitometric comparison of H3, Ace-H3, and GAPDH expression after Nocodazole and Romidepsin treatments for 48h.

In this set of experiments, we indicated the relation between the acetylation of H3 and PTEN expression. SAHA and Nocadozole combination have shown a negative effect on Ace-H3 and PTEN expression while Romidepsin and Nocodazole combination increased the expression of Ace-H3 on Cisplatin-Resistan TFK-1 cells. Depending on the inhibitor type, both PTEN and Ace-H3 expression changed the same way. Since the expression of PTEN is crucial for chemoresistant CCA, modulation of the HDACs via HDAC inhibitors and modulating the autophagy by autophagy inhibitor led to altered the expression level of PTEN, this is a considerable definition for promising treatment methods.

3.10 The Effect of Cisplatin-Resistance on the Expression Location of PTEN Autophagic Marker

One of the important proteins which can activate the autophagic pathway is phosphatase and tensin homolog (PTEN). PTEN is one of the tumor suppressor genes and located on the chromosome and induces autophagy via conversion ability and leads to the inhibition of PI3K, this is the reason for PTEN inhibition to activate the PI3K/AKT pathway [207-209].

In normal quiescent cells, PTEN is localized in the nucleus. However, in cancer cells, PTEN is usually detected in the cytoplasm which has shown the considerable role of the nuclear localization of PTEN in tumor suppression [209]. Several studies have shown the modulatory effect of HDACs on the activation of PTEN.

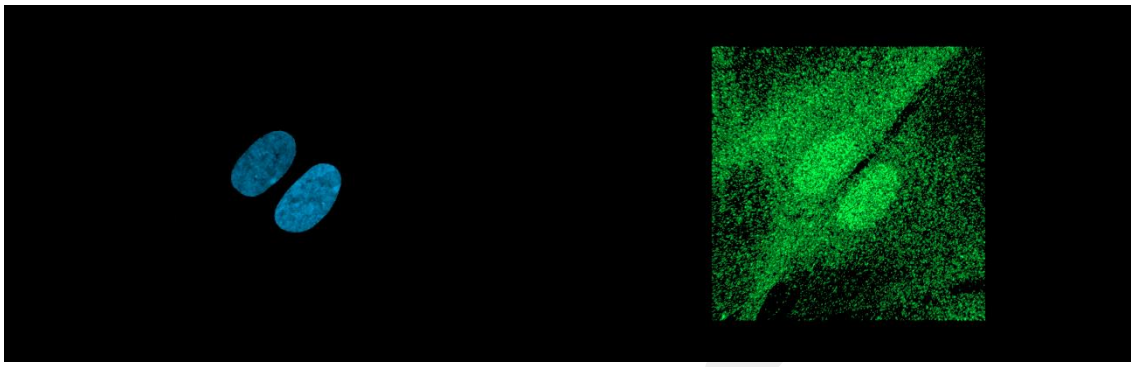
Since the existence of the association between the loss of PTEN and low autophagy, these studies also have shown the induction of the PI3K/AKT pathway which can promote resistance against chemotherapeutic drugs in epithelial ovarian cancer and breast cancer [207,210,211]. PTEN expression has been shown to sensitize breast cancer cells to the SAHA treatment [212].

In CCA patients, PTEN mutations are detected and are found to be involved in the formation of CCA [213-215]. In addition to the effect of promotion of the development of CCA, PTEN inhibition is associated with prognosis [154,213,216,217].

According to the effect of PTEN and its location in the chemoresistance CCA, we decided to check the location of PTEN in both parental and cisplatin-resistant CCA cells, for this purpose, immunofluorescent staining has been performed [218]. Localization and expression of PTEN in cisplatin sensitive and resistant cells have shown the translocation of the PTEN in the case of chemoresistance, clearly. As a control, we used human fibroblast to better understand the localization of PTEN in healthy cells. As described in Figure 3.43, resistant cells have PTEN expression in the cytoplasm while sensitive cells have in the nucleus. In order to better understand the location of the nucleus, we stained with DAPI which is the most well-known nuclear staining agent [219].

(A) Fibroblast

A **B**



(B) EGI-1

A **B**

(C) Cisplatin-Resistant EGI-1

A **B**

(D) TFK-1

A **B**

(E) Cisplatin-Resistant TFK-1

A **B**

Figure 3.43 The immunofluorescence staining of PTEN and DAPI ın (A) Human fibroblast, (B) EGI-1, (C) Cisplatin-resistant EGI-1, (D) TFK-1, (E) Cisplatin-resistant TFK-1 (A: DAPI, B: PTEN)

These results have suggested the effect of the location of PTEN expression lead to gaining mechanism against chemotherapeutic drugs via manipulating the autophagic pathway. Therefore, our combination treatment has the potential to inhibit PTEN expression in the cytoplasm on cisplatin-resistant cells, thus, decrease the chemoresistance and wipe out the chemoresistant CCA formation via HDAC and autophagy inhibition.

Chapter 4

4. Conclusions and Future Prospects

4.1 Conclusions

Cholangiocarcinoma (CCA) is an adenocarcinoma that has the characteristic of aggressive and elusive. [169,170]. CCA is the second most common primary liver tumor [172,220]. Cholangiocarcinoma has a characteristic of a poor prognosis and 5% of the patients surviving approximately 5 years [221-223]. The precise etiology of the development of CCA is still not clearly defined [222,224]. Several risk factors of CCA have been shown to cause the induction of inflammation and thereby, leading to the formation of CCA [170,222].

The majority of CCA patients have high tumor metastasis [172,173]. Surgery is an option for CCA treatment; however, relapses after surgery are a common problem. Other treatment methods can lead to specific complications of advanced disease and organ transplantation is only affect the patients with no metastasis. Additionally, the current chemotherapeutic treatments such as Cisplatin, rifampicin, mitomycin C, paclitaxel, gemcitabine, docetaxel, and 5-fluorouracil are not effective because of the multidrug resistance [169,170,174,175]. The combination of gemcitabine and cisplatin has shown poor outcomes for the treatment of CCA [157,169,170,174,220,222].

The histone deacetylase inhibitors have shown promising anticancer characters and dysregulated pathways in chemoresistance CCA such as PTEN/PI3K/AKT/mTOR can modulate in different ways [225].

With the aim of the generate cisplatin-resistant cells, EGI-1 and TFK-1 cells were treated with the lowest dose of cisplatin after each passage of the cells for 3 weeks, after each triplet of the treatment, the concentration was duplicated until the IC50 value [184,186]. During the generation of resistant cells, comparison experiments have been performed to observe the difference between parental and resistant cells. Comparison of parental and 1.6 μM cisplatin-resistant EGI-1 and TFK-1 cells have shown the ability of proliferation under 1.6 μM cisplatin treatment. This proliferation was demonstrating the acquirement of resistance mechanism after a few weeks since the generating of resistant cells was started. (Figure 3.7).

In order to continue the comparison after increased concentration of cisplatin, parental and 12,8 μM cisplatin resistant EGI-1 and TFK-1 cells were compared under the treatment of 12,8 μM and 17 μM cisplatin for 48h. The results were demonstraing the gaining of cisplatin resistance EGI-1 and TFK-1 cells. The proliferation and confluency of the resistant cells were clearly higher than the parental cells (Figure 3.8).

The generation of the resistant cells was a long process, in order to compare each treatment, parental and cisplatin-resistant EGI-1 and TFK-1 cells were counted during each passage and the results were compared with each other. With this way, we observed several parameters such as the adaptation time of the cells after each increasing dose of cisplatin, differences between the proliferation of the cells during the treatment of 3 same concentrations of cisplatin, comparison of the parental and resistant cells after each treatment (Figure 3.9). This counting of the cells demonstrated the proliferation of resistance cells after each passage and proved the permanent resistance mechanism. After achieving the IC50 value, cells were grown in a normal medium, without cisplatin treatment for 3 weeks, after, the cells were grown with 17 cisplatin for 3 weeks and finally, cells were resistant and cisplatin treatment was ended. This process was necessary to observe the temporary and permanent resistance mechanism.

In order to check the existence of resistance formation after completed cisplatin treatment, we repeated the MTT assays which we used to determine the IC50 values of cisplatin for EGI-1 and TFK-1 cells. Cisplatin doses were the same as before (5μM to 200 μM), as a result of this comparison we proved the higher IC50 value of cisplatin-resistant

EGI-1 and TFK-1 cells. This has been indicated the EGI-1 and TFK-1 cells were gained a resistance mechanism against cisplatin (Figure 3.10).

In addition to the proliferation differences of parental and cisplatin-resistant TFK-1 cells, to check the colony formation ability under the cisplatin treatment, the colony formation assay has been performed for parental and cisplatin-resistant cells. The cells were treated with an IC50 value of cisplatin (17μM) and a concentration that is more than 2 times higher than the IC50 value (50μM). After 2 weeks of incubation, the results proved the resistance against cisplatin. Moreover, resistant cells were showing proliferation in the environment consist of 50 μM cisplatin while parental cells have died almost totally (Figure 3.11).

During the generation of resistant cells, comparison experiments have been performed to observe the difference between parental and resistant cells. Comparison of parental and 0.2 μM gemcitabine-resistant EGI-1 and TFK-1 cells have shown the ability of proliferation under 0.2 μM gemcitabine treatment. This proliferation was demonstrating the gaining resistance mechanism after a few weeks since the generating of resistant cells was started (Figure 3.19).

To continue the comparison after increased concentration of gemcitabine, parental and 1.6 μM gemcitabine resistant EGI-1 and TFK-1 cells were compared under the treatment of 1.6 Mm, 5000 μM gemcitabine and 7500 μM gemcitabine for EGI-1 and TFK-1, respectively, for 48h. The results were demonstrating the gaining of gemcitabine resistance EGI-1 and TFK-1 cells. The proliferation and confluency of the resistant cells were clearly higher than the parental cells (Figure 3.20, Figure 3.21).

In the literature, the concentration of the gemcitabine was not as high as we observed in our cells [184,185,229]. Since the generation should start with a low concentration and increased after time-spaces, it took a long time to treat the cells with gemcitabine and wait until the cells can be adapted and grow normally under gemcitabine treatment. We treated and adapted the cells for 51.2 μM gemcitabine, but even the adaptation for this concentration took 342 days (Figure 3.22). Since the project and experiments depend on a timeline and achieving the IC50 value for both EGI-1 and TFK-1 cells was challenging, we decided to try another way to generate gemcitabine-resistant

cells. We treated our 25.6 μM and 51.2 μM gemcitabine resistant EGI-1 and TFK-1 cells with concentrations that are three and four times more than the resistance level of the cells, however, cells could not be lived and grown. Although many treatment experiments to achieve the IC50 value, we did not observe significant changes in the resistance level.

Lastly, we have frozen our 51.2 μM gemcitabine-resistant cells and started another procedure which was depending on the resistant subcolonies [192]. EGI-1 and TFK-1 parental cells were treated with increased concentrations of gemcitabine and after each passage, the gemcitabine treatment has been performed (Figure 3.23). Unfortunately, both of the cell lines have been decreased their cell number after each treatment and in the end, we could not reach the point that achieves the IC50 value.

Additively, the pandemic affected almost everything about the laboratory issues and specifically the generation of resistant cell lines. As mentioned previously, treatment of the cells was performed after each passage, when we had to close the laboratory for a while, we had to freeze the cells for some time. After this frozen when we thawed the cells we have been grown them without any treatment to make them healthy, stable, and ready to expose treatment. Because of this period, we continued the experiments as much as faster and after this phase of the experiments, we tried to fix the timeline.

Because of the time problem for the generation of gemcitabine-resistant cells, we decided to try our treatment hypothesis only on cisplatin-resistant EGI-1 and TFK-1 cells.

HCC is primary liver cancer, since the CCA increases the incidence and becomes the second most common primary malignancy of the liver. Because of the common location and origin of both cancer types [230], in our project, we wanted to check the cisplatin and gemcitabine effect on one of the HCC cell lines called HepG2. IC values for both EGI-1 and TFK-1 were not extremely high and approximately lower than both CCA cell lines. Therefore, we observed the effect of the treatment of cisplatin and gemcitabine on HepG2 and HCC cell line were not needed significant-high concentrations on the contrary of cisplatin and specifically gemcitabine (Figure 3.24, Figure 3.25, Figure 3.26, Figure 3.27, Figure 3.28 Figure 3.29). These results have shown the high resistance capacity of our CCA cell lines compares to one of the HCC cell lines.

During the MTT assays for both of the cell lines, some of the results have shown an increase in cell proliferation when the cell lines were exposed to increased concentrations of the drugs. Since the liver cells have a drug detoxification character, this abnormal increase in the proliferation of the cells may be related to this character.

In our hypothesis, we suggested sensitize the chemoresistance and with this way facilitate the treatment of cancer formation on chemoresistant CCA cell lines. The hypothesis was depending on the inhibition of HDACs and the modulation of the autophagic pathway at the same time. Due to the inhibition of HDACs, we expected to observe changes in the expression level of related proteins such as H3 and Ace-H3 [206]. Histones H3 is one of the main histone targets of the enzymatic activity of HDAC, Histones H3 undergo acetylation at lysine residues, acetylation occurs at multiple sites within the histone tails extending [93]. Since acetylation and deacetylation can affect the transcription which means the expression of the gene, it was important to alter the HDACs. On the other hand, PI3K/AKT/mTOR activation is a common property in CCA cases and is associated with a poor prognosis. Therefore, this activation inhibits the mTOR and activates the autophagic pathway in CCA patients. Inhibition of the PI3K/AKT/mTOR pathway can sensitize CCA against chemotherapeutic drugs such as cisplatin and oxaliplatin. This indicated that autophagy is an escape mechanism of chemotherapeutics through promoting chemoresistance in CCA, thus make this pathway a clear target for the treatment of chemoresistance [128,146,156,231].

In order to inhibit HDACs, we used different inhibitors such as SAHA, MS-275, and romidepsin [95,99,164-165]. Cytotoxic assays have been performed to better understand their effect on parental CCA cells and decide the IC30 values for cisplatin-resistant CCA cells. According to the results, we decided the IC30 values and completed the choosing HDAC inhibitors part (Figure 3.30, Figure 3.31 Figure 3.32).

The second part was deciding the autophagy inhibitor. Due to the experiments which were performed in our laboratory previously, we decided to use Chloroquine and Nocodazole which are autophagolysosomal degradation (chloroquine), and autophagosome-lysosome fusion (nocodazole) agents [139,159,163,232]. For the purpose of deciding the best autophagy inhibitor, two different autophagy modulators have been used for the treatment. Chloroquine was the first inhibitor that we used. When we performed the cytotoxic assays with Chloroquine alone and with the combination of

SAHA, since SAHA is the most well-known HDACis [233], we observed the results which were not dramatically affecting both EGI-1 and TFK-1 cisplatin-resistant cells (Figure 3.33). Therefore we performed the combination experiments with Nocodazole and HDAC inhibitors to decide the best synergetic combination. The results have shown the most harmonic effect on Nocodazole and SAHA combination and Nocodazole and Romidepsin combination (Figure 3.34, Figure 3.35).

Followed the performing of cytotoxic assays and deciding the contents of the combinations, we performed apoptosis and cell cycle analysis on cisplatin-resistant EGI-1 and TFK-1 to check the effect of combination treatment of Romidepsin and Nocodazole which were the best combination depending on the cytotoxic assays.

Firstly, apoptosis assay has been performed with the IC30 values of Romidepsin and Nocodazole. Results were positive for cisplatin-resistant EGI-1 cells, there were only early apoptotic and apoptotic cells which have shown the successful apoptotic effect of the combination treatment on cisplatin-resistant EGI-1 cells, however, cisplatin-resistant TFK-1 cells have mostly died in a necrotic way (Figure 3.36). Our purpose was to wipe out the chemoresistance CCA cells with a programmed cell death which is apoptosis. Since this is the best way and less harmful way for healthy cells, the combination treatment aim was to induce the apoptosis process [234].

In order to figure out the concentration of the inhibitors in combination therapy for cisplatin-resistant EGI-1 and TFK-1 cells, we performed the same experiment with the IC50 values of the Romidepsin and Nocodazole. This experiment set was clearly have shown only early apoptotic and apoptotic cells after the combination treatment (Figure 3.37).

As a result, for apoptosis IC50 values of Romidepsin and Nocodazole have shown the best outcomes for apoptotic cell death. For the cell cycle, cisplatin-resistant EGI-1 has not shown a significant difference between the treatments while cisplatin-resistant TFK-1 has shown a considerable arrest in the G0/G1 phase of the cell cycle. In cell cycle analysis with the cells which were treated with the IC30 values of the Romidepsin and Nocodazole, combination treatment was clearly arrested the cell cycle in G0/G1 phase. Since we were expecting to arrest the cycle in the checkpoint of the G0/ G1 phase, this

result has been demonstrated the promising effect of combination treatment (Figure 3.38).

Followed the performing of cytotoxic, apoptosis, and cell cycle assays to decide the contents of the combinations, we performed western blot analysis to check the changes in a protein level. As mentioned previously, combinations were containing Nocodazole dual treatment with SAHA and Romidepsin. For both of the combinations, we performed the western blot analysis with IC30 values for 48h on cisplatin-resistant TFK-1 cells. Results were demonstrating the relation between acetylation of H3 and PTEN expression, additionally, showing the expression differences due to the contents of the combination treatment. Several studies mentioned previously have shown the PTEN expression abnormalities and the inhibition effect of the HDAC inhibitors in CCA patients. In this set of experiments, since we were expecting the expression differences and normalize the level of expressions via combination therapy, these results were demonstrating the beneficial effect of combination therapy as we expected.

When we performed the treatment with SAHA and Nocodazole, the expression level of the Ace-H3 and PTEN were dramatically decreased while the same combination treatment increased the expression level of H3 (Figure 3.39, Figure 3.41). This result has shown the certain inhibition of PTEN via the synergetic effect of Nocodazole with SAHA. Because, as described in Figure 3.39, a single treatment of Nocodazole could not totally decrease the expression of PTEN, however, when we use Nocodazole with SAHA there was almost no PTEN expression. In addition to that, as described in Figure 3.41, SAHA and Nocodazole combination decreased the Acetylation and increased the expression level of H3, therefore, these results indicated the association between the acetylation of H3 is in direct proportion to PTEN expression (Figure 3.39, Figure 3.41). According to the inhibition effect of HDACis, the HDAC inhibitor (SAHA) should decrease the expression level of H3, therefore, increase the Acetylated H3. SAHA is a considerable HDAC inhibitor, thus made the expression of Ace-H3 increased in the single therapy of SAHA. In addition to that, in a single SAHA treatment, we observed the synergetic increase in both PTEN and Ace-H3 expression. Since the combination therapy dramatically decreased the expression level of Ace-H3 and PTEN, in this set of experiments we observed the significant effect of Nocodazole and Romidepsin combination on cisplatin-resistant TFK-1 cells, as we expected.

On the other hand, combination treatments of Romidepsin and Nocodazole have shown extremely different expression levels of H3 and Ace-H3. Single treatment of Romidepsin significantly increased both H3 and Ace-H3 expression levels while the combination treatment of Romidepsin and Nocodazole considerably decreased the expression level of H3 and approximately did not change the expression level of Ace-H3 (Figure 3.42) These results indicated the clear effect of the Romidepsin and Nocodazole combination on the expression level of H3, thus, transcriptional activation is increased depending on the combination treatment. In addition to that, the expression level of Ace-H3 almost did not affect by the combination therapy, this result has been demonstrated our hypothesis which was based on the inhibitory effect of the combination therapy only on HDACs (Figure 3.42). Romidepsin did not show an extreme decrease during a single treatment compare to the single SAHA treatment, however, the combination with Nocodazle decreased the expression level of H3 considerably. In this set of the experiment, the crucial role of the combination treatment was observed. The results were almost as we expected in the case of only H3 inhibition, however in the single treatment, the expression level of H3 was more than we expected.

With the exact purpose of understanding the function of PTEN on cisplatin-resistant TFK-1 cells, we performed a western blot analysis with the samples of the PTEN silencing experiment. To better understand the expression level of PTEN on TFK-1 and cisplatin-resistant TFK-1 cells, we silenced PTEN expression via the siRNA method. Depending on the results in Figure 3.40, nuclear extraction of TFK-1 cells did not show any significant PTEN expression while the total extraction of cisplatin-resistant TFK-1 cells was expressing PTEN. When we silenced the PTEN in cisplatin-resistant TFK-1 cells with two different concentrations (0,02 and 0,05) to decide the optimal concentration, we observed the overexpression of total PTEN on cisplatin-resistant TFK-1 cells compare to the nuclear extraction of TFK-1 cells. Moreover, results indicated the contrast expression level of PTEN and H3. While there was PTEN expression in both untreated and control siRNA samples, there was a low level of H3 expression in both samples. However, in the case of both concentrations of the PTEN silencing the expression level of H3 was increased compared to the existence of the PTEN in the environment (Figure 3.40).

Finally, in order to check the controversial effect of PTEN location in parental and cisplatin-resistant CCA cell lines, immunofluorescent staining has been performed. With this staining, we aimed to compare healthy, cancerous, and resistant cancer cells with each other to clearly define the translocation of PTEN expression. We expected to see the PTEN localization mainly in the nucleus in healthy fibroblast cells. In CCA cell lines we were expecting to see the shuttling of PTEN to the cytoplasm and more cytoplasmic PTEN in resistant cells compared to the sensitive cells. Results of the staining indicated the translocation of the PTEN expression exists in the case of chemoresistance. In Figure 3.43, comparison of the healthy cell which is the human fibroblast, parental CCA cell lines EGI-1 and TFK-1, and cisplatin-resistant CCA cells EGI-1 and TFK-1 cells stained with PTEN antibody which has FITC feature. Moreover, for the aim of deciding the exact location of the nucleus and make a comparison, DAPI, a nuclear staining agent, has been used.

Outcomes of the results demonstrated that gain of the resistance mechanism and location of PTEN expression is related to each other (Figure 3.43).

4.2 Societal Impact and Contribution to Global Sustainability

Cholangiocarcinoma is an elusive, life threatening highly aggressive adenocarcinoma and the most second common primary liver cancer type. The most crucial two features of CCA are late diagnosis poor prognosis. Treatment options such as endoscopic stenting, radiation therapy, and photodynamic therapy may be considered as monotherapy in patients with specific complications of advanced disease. Another treatment option is orthotopic liver transplantation, which is only applicable for patients with no metastatic disease (OLT) and still has a chance to relapse. In addition to these challenges, chemoresistance becomes an essential problem for the treatment of advanced diseases. Even the most common chemotherapeutic agents called cisplatin and gemcitabine combination are not effective. Since the symptoms are not clear until the late stages most patients have advanced disease with a high metastasis rate and resistance against chemotherapeutic drugs, the survival rate is dramatically decreased. The main problem, in this case, is to sensitize the resistant cells and facilitate the treatment. Single

treatment options are not enough to sensitize the chemoresistant CCA to chemotherapeutic drugs, thus, several studies include combination therapies. However, the certain definition of the mechanism of chemoresistant CCA is still not clear. Unfortunately, treatment options for CCA are not showing promising results, thereby, the need for novel therapeutics against CCA is increasing.

With the exact aim of sensitizing the chemoresistance mechanism, thus, decrease the proliferation of chemoresistant CCA cells and induce apoptosis as a cell death mechanism, we combined HDAC and autophagy inhibitors. HDACis are promising anticancer agents that have been used in clinical trials and autophagy manipulation is crucial for induce apoptotic cell death to prevent healthy cells as much as possible. With our combination treatment, we aimed to sensitize and treat chemoresistant CCA at the same time. Moreover, figure out the crosstalks between HDACs and autophagy pathway allows to develop of more personalized therapy, thus, increase the benefit for the different CCA patients and improve the life quality of these patients.

4.3 Future Prospects

In CCA, specific symptoms and abnormalities are absent during the early stages of the disease, thus, for the histological diagnosis surgical resection is necessary. In the case of both intrahepatic and extrahepatic CCA, peritoneal metastasis is found in 83% of the cases this is the reason for late detection and causing the late diagnosis. All of these challenges make complicate the treatment of the CCA. Additionally, in chemoresistant CCA, it is not possible to use chemotherapeutic drugs and apply the therapy [8,155,169,170,184].

Since single treatment of chemotherapeutic agents not effective, combinational therapies are more effective for treatment, however, the most well-known combination which is the combination of gemcitabine with cisplatin showed poor outcomes in CCA [157,169,170,174,222].

According to the previous pieces of information, signaling pathways and epigenetic factors became promising options for the treatment of chemoresistant CCA. Studies have shown the promising effect of HDAC inhibitors and different signaling pathway modulations on chemoresistant CCA and other cancer chemoresistant cancer types

[95,164,166,196].

In CCA, activation of PI3K/AKT/mTOR is a common characteristic and related with poor prognosis [156,231,235]. This active pathway, induces the pathogenic effect of autophagy in the case of CCA [128,156,157,236]. Inhibiting the PI3K/AKT/mTOR pathway sensitizes CCA cisplatin and oxaliplatin which are common chemotherapeutic drugs [145,146]. These studies have shown the escape mechanism features of autophagy against chemotherapeutics agents and promote chemoresistance in CCA. Several studies have shown that chemoresistance can be overcome via combinational therapies including both mTOR inhibitors and autophagy blockers [145,146].

In our study, we demonstrated the effect of the combination treatment includes HDAC inhibition and autophagy modulation on cisplatin-resistant TFK-1 cells. Results have shown promising outcomes for both sensitizing and inducing apoptosis which is a cell death mechanism and activated by combination therapy. For this part as a future plan, apoptosis level may increase with several combinations that include a variety of drug concentrations of the selected agents. for cisplatin-resistant cancer cells. This clarification may define the exact IC values for each agent for the purpose of apoptosis.

Generation of gemcitabine-resistant CCA and HCC cell lines is essential to check the effect of the combination treatment in the future.

Also, in the future we need to check, other autophagic markers such as ATG-5, ATG-7 which are crucial for autophagosome formation, other histones related markers such as HDAC1, HDAC2, Histones H4 to better understand the effect of HDAC inhibition and consequences of the modulations.

Since the combination treatment method can lead to a dramatic decrease in cytoplasmic PTEN expression level, thus, prevent autophagic manipulation and sensitize the resistant cells, in the future, we need to check the cellular location of PTEN after combination treatment.

Understanding, clarifying, and defining the regulation crosstalk between HDACs and autophagy in chemoresistant CCA is crucial to improve the treatment options. Manipulation and specifically the escape mechanism feature of autophagy in chemoresistant CCA is an essential need to define clearly for better treatment options. All these need to make the certain definition will allow us to develop better treatment options which are more personalized targeted therapy for chemoresistant CCA patients.

With the purpose of proving the previously mentioned in vitro data in our study, we need to analyze the gaining chemotherapeutic resistance mechanism, HDAC inhibition, and

autophagy modulation in vivo using mouse models for the carcinogenesis of chemoresistant CCA.

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CURRICULUM VITAE

2013 – 2018	B.Sc., Molecular Biology and Genetics, Inönü University, Malatya, TURKEY
2019 –Presents	M.Sc., Bioengineering, Abdullah Gül University, Kayseri, TURKEY