2018

Study of Anti-proliferative Activity of Cucurbitacins Inspired Estrone Analogs on Hepatocellular Carcinoma

Sara Elgazwi
South Dakota State University

STUDY OF ANTI-PROLIFERATIVE ACTIVITY OF CUCURBITACINS INSPIRED ESTRONE ANALOGS ON HEPATOCELLULAR CARCINOMA

BY

SARA ELGAZWI

A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

Major in Biochemistry

South Dakota State University

2018

STUDY OF ANTI-PROLIFERATIVE ACTIVITY OF CUCURBITACINS INSPIRED ESTRONE ANALOGS ON HEPATOCELLULAR CARCINOMA

SARA ELGAZWI

This dissertation is approved as a creditable and independent investigation by a candidate for Doctor of Philosophy in Biochemistry degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Fathi T. Halaweish, Ph.D.
Dissertation Advisor Date

Douglas Raynie, Ph.D
Head, Department of Chemistry & Biochemistry Date

Dean, Graduate School Date

CONTENTS

ABBREVIATIONS

Apaf-1 Apoptotic protease activating factor 1

dd H_2O Double distilled water

DMSO Dimethylsulphoxide

DNA Deoxyribonucleic acid

EGFR Epidermal Growth Factors

EMEM Eagle's Minimum Essential Medium

ERK Extracellular Signal-Related Kinase

FDA Food and Drug Administration

HBV Hepatitis B virus

HCC Hepatocellular Carcinoma

HCV Hepatitis C virus

MAPK Mitogen Activated Protein Kinase

MDR Multidrug Resistance

MEK MAPK/ERK kinase

MRP Multidrug resistance protein

MRP2 Multidrug resistance proteins 2

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NaCl Sodium chloride

PARP Poly ADP ribose polymerase

pERK1/2 Phosphorylated Extracellular-Regulated Kinase ½

RT Room temperature

SDS Sodium dodecyl sulfate

SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Tris 2-Amino-2-hydroxymethyl-propane-1,3-diol

TEMED Tetramethylethylenediamine

VEGF Vascular epithelial growth factor

β-ME β-Mercaptoethano

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

STUDY OF ANTI-PROLIFERATIVE ACTIVITY OF INSPIRED ESTRONE ANALOGS ON HEPATOCELLULAR CARCINOMA

SARA ELGAZWI

2018

Hepatocellular carcinoma (HCC) is considered the third leading cause of death from cancer. Overall survival rate is significantly low, due to the emerging resistance to chemotherapeutic agents and lack of selectivity. Recent studies have demonstrated that epidermal growth factor receptor (EGFR) is a promising molecular target for cancer therapy, especially HCC. Current studies showed that cucurbitacins are potent anticancer compounds which target EGFR. This prompted us to investigate the anti-proliferative activity of novel cucurbitacins inspired estrone analogs (CIEA) against sensitive and resistant HepG2 cell lines. Anti-proliferation activity of 20 CIEA analogs were examined against HepG2 using MTT assay and showed that antiproliferative activity of analogs MMA132, and MMA102 IC_{50} are 2μM, and 3 μM respectively in comparison to Erlotinib 25 μM. Study of the mechanism of anti-proliferation effects of these novel analogs was elucidated. Western blot analysis showed that MMA132, and MMA102 significantly inhibit EGFR/pEGFR, RAF/pRAF, MEK/pMEK, and ERK/PERK. Cell cycle analysis on HepG2 cell line revealed that MMA132 and MMA102 arrested the cells at G1 phase and inhibited the HepG2 cell migration after 24 hr. MMA132 induced apoptosis through activation of caspase 3,9 and inhibition of PARP.

Treatment of HepG2-R (Erlotinib resistant) with MMA132 and MMA102 showed that these two novel drug candidates still possessing potent anti-proliferation activities against HepG2-R. Further characterization of the anti-proliferation of these lead compounds was demonstrated through mapping the change in EGFR signaling pathway (ERK, pERK, RAS, AKT and MEK) by western blot, cell cycle analysis, demonstrated that MMA132 and MMA102 stop the cell cycle of HepG2-R at G2 phase and inhibited cell migration after 48hrs. HepG2-R cell line significantly expressed MRP2 in comparing to sensitive cells. Moreover, MK571(MRP2 inhibitor) showed an inhibitory

effect on resistant HepG2-R cancer cell lines. Combination of MMA132 with MK571 (13 µM and 15 µM respectively) showed a significant increase in the cytotoxicity of MK571 from 18.5 µM to 10 µM. In conclusion, our study documented the discovery of novel estrone analogs as potential drug candidates for treatment of HCC and promising chemotherapeutic agent toward HepG2 resistant to erlotinib.

Chapter One

1. General Introduction and Background

1.1 Hepatocellular Carcinoma

Hepatocellular carcinoma is the sixth most common cancers with about 500,000 people diagnosed each year, and it's reported as a third largest cause of cancer –related death[1,2]. The treatment is challenging, 5-years survival rate is estimated to be less than 5%[3].The most common causes of death among HCC patients are recurrence, metastasis, and the development of new primary tumors[4].

1.2 Causes for Hepatocellular carcinoma

Many risk factors are responsible and play role in HCC progress. Induced Hepatitis C, B virus (HCV, HBV) infection are associated with the highest HCC incidence in persons with cirrhosis, the data about HCC risk is still limited but factors like older age, male sex, the severity of compensated cirrhosis, and sustained activity of liver disease are important predictors of HCC. More studies are needed to demonstrated the mechanism of factors such as HBV genotype/mutant, occult HBV, HIV confection and other risk factors that cause HCC (e.g., obesity, diabetes)[5]. (**Figure 1.1)**

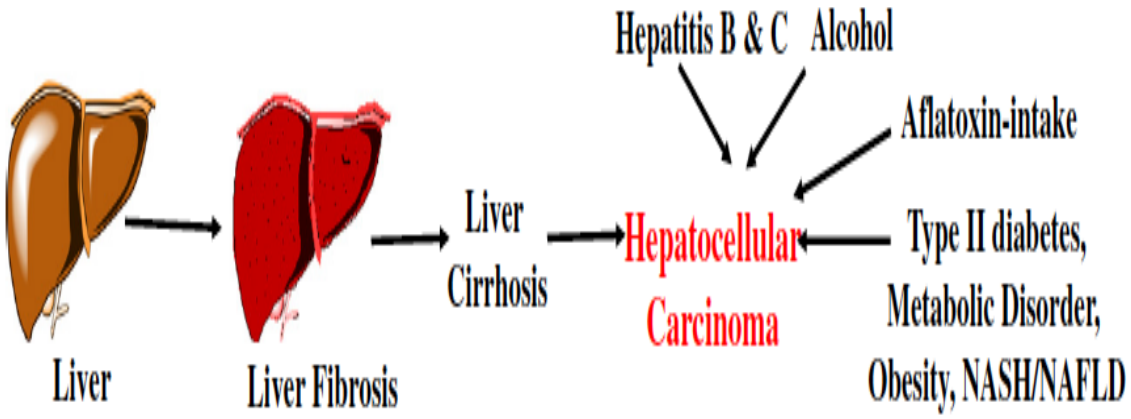

Figure 1.1 Causes of Hepatocellular carcinoma (HCC).

1.2.1 Virus induced hepatocarcinogenetic

Approximately 2 billion and 170 million people around the world infected by hepatitis B virus (HBV) and hepatitis C virus (HCV). There are many different mechanisms to promote HCC by HBV and HCV, by target and binding to growth factors and thus promote cellular growth, survival and by pass the DNA-damage checkpoints. HBV is one of the Hepadnaviridae families, which is an enveloped DNA virus. It is a very common viral disease, about 320,000 people die annually due to complications of HBV infection. Asia and Africa reported to have the highest HBV incidence worldwide [6].In addition, HCV is a member of the Flaviviridae family which is a single-stranded RNA non-cytoplasmic type virus. It is a contagious virus that can contribute to infection by direct blood contact, perinatal from mother to fetus, and in rare cases, by sexual intercourse. China, Egypt, and Pakistan have the highest number of HCV cases worldwide[7,8]. HCV has been reported to have biological properties compared to HBV, HCV is an associated hepatocarcinogenetic. Because HCV has a better tendency (60-80%) to produce chronic infections compared to HBV, which has only a 10% tendency. This tendency is connected to HCV's ability to generate a fast rate of replication errors that can cause immune evasion.

1.2.2 Alcohol induced hepatocarcinogenetic

Alcohol consumption is one of the significant causal factors for HCC. Chronic alcohol addiction causes pre-inflammatory cytokines by activating and disturbing the monocyte later will lead to an abnormal evaluation circulating endotoxin concentration. These bring about hepatocyte damage[9]. In the case of chronic ethanol toxicity, the hepatocyte demonstrates a high sensitivity to the TNFα cytotoxicity effect, which is a clear sign of chronic hepatocyte disturbance, activation of the stellate cell, liver cirrhosis, and eventually[10]. However, alcohol can affect and damage the liver via a process called oxidative stress. Three mechanisms can be explaining the connection between oxidative stress and the liver damage that leads to HCC. First, oxidative stress induces the progression of cirrhosis and fibrosis, which are considered the main causes of HCC[11]. Second, oxidative stress that results from ethanol toxicity has a relevant effect on the HCC-signaling cascade, such as a decrease in the tyrosine phosphorylation of the signaling transducer and the activator of transcription I (STATI)[12].Third, oxidative stress in some cases increases the rate of telomere shortening which may interrupt the DNA replication process and cause HCC[13]. In addition, Alcoholic liver disease (ALD) it's a factor leads to cirrhosis and later HCC because (ALD) cause liver injuries such as steatosis, steatohepatitis and consequently HCC. But generally HCC doesn't develop in absence of cirrhosis, just in heavy alcohol intake without cirrhosis it the obvious risk factor in some HCC patients[14].

1.2.3 Obesity and type 2 diabetes

Obesity has been found to be a primary risk factor for some cancers including breast cancer, endometrial cancer, colon cancer, renal cell carcinoma, esophageal adenocarcinoma, and HCC. Extensive studies have confirmed a strong link between obesity and HCC **(Figure 1.2)**[15]**.** The obesity and type 2 diabetes are the factors causing the development of non-alcoholic fatty liver disease (NAFLD) and progress of HCC. In United State the number of NAFLD associated with HCC was reported to increase to 9%. Beside the role of obesity in HCC, genetic factors also have an essential role in metabolic syndrome of the obese patients and subsequently to HCC. Increasing the free fatty acids from triacylglycerol (TG), in obese case will cause the release of tumor necrosis

factor (TNF-α) from recruited macrophages and decrease secretion of adiponectin which considered as an insulin sensitizer. So, in the end hepatic lipogenesis is increased due to induced TG content leading to development of insulin resistance which will cause stimulation of the transcription factors (SREBP1, ChREBP1) which play role in promotion of hepatocarcinogenetic.

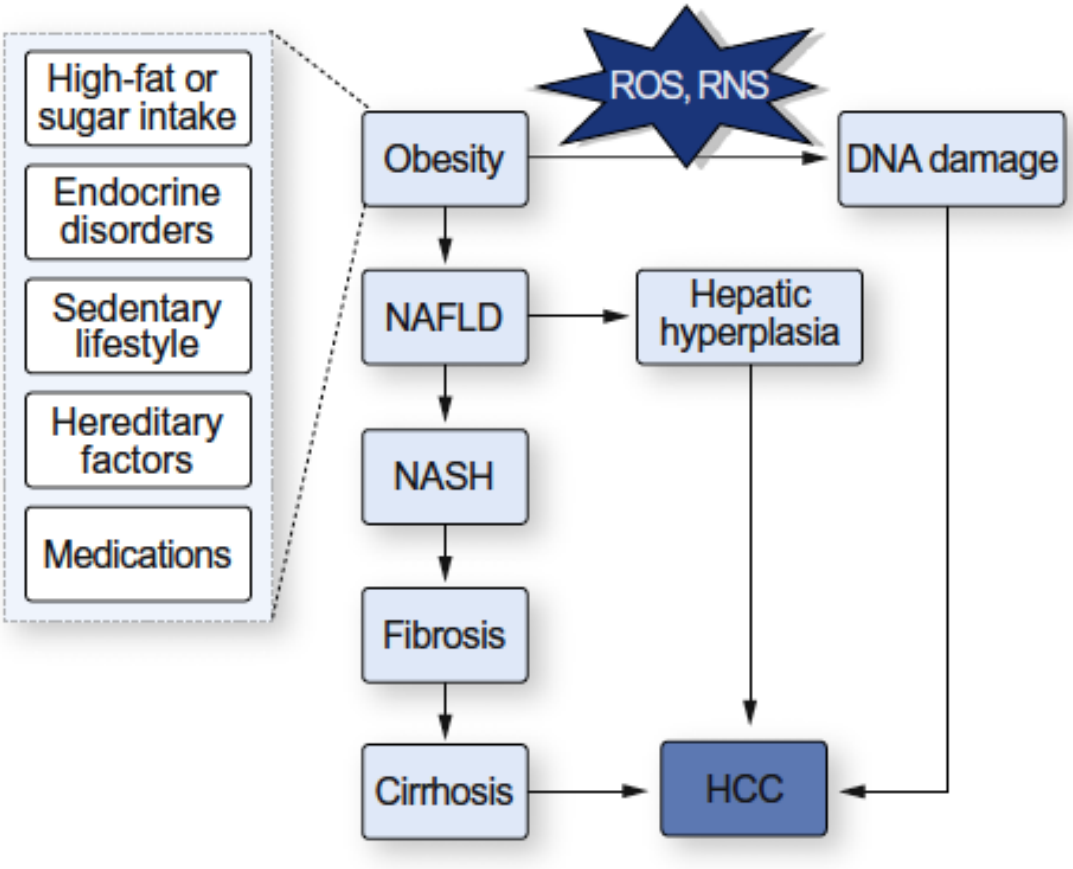

Figure 1.2 Three putative mechanisms for obesity-induced and obesity-promoted hepatocarcinogenesis

1.2.4 liver Cirrhosis

Liver cirrhosis is consider as the main reason for HCC development, which is mainly caused by the most two common liver's viral infection, HBV and HCV[16],[17].The development of cirrhosis usually occurs when patients have a chronic liver disease over a

period of years, which can be distinguished by a decrease in hepatocyte proliferation as a sign of liver damage. This causes an increase in the fibrous tissue and a disturbance of liver cells that leads to the development of liver cancer. Table 1.1 shows the annual percentage rate of HCC from cirrhosis caused by viruses or liver complications[16].

While these results show HCC progression by liver cirrhosis caused via different liver diseases, the development of HCC may also include additional mechanisms[18,16]. Many studies have found the exact mechanism associated with HCC development from liver cirrhosis, but only a few possible mechanisms have been proposed, including micro- and macro-environmental changes that induce cellular proliferation and telomerase dysfunction[19].

Table 1.1 Annual Percentages of HCC Incidences Caused by Liver Cirrhosis.

Underlying disease	Annual incidence (%)
HCV	1-8
HBV	1-15
Alcohol liver disease	1
Nonalcoholic steatohepatitis	2.6
Hemochromatosis	2-6
Primary biliary cirrhosis	2
Autoimmune hepatitis	<0.2

1.2.5 Fungi-induced hepatocarcinogenetic

Mycotoxins are considered a fungal secondary metabolite with different toxic effects. Aflatoxin B1 one of many mycotoxins is a food contaminant produced by the fungi. In addition, it is also known as a carcinogen and is involved in p53 mutation and induction of HRAS oncogene mutation. AFB1 is activated when it is absorbed in the blood circulation and metabolism to AFB1-exo-8, 9-epoxide. The active metabolite binds to DNA and damages it, so it can be distinguished by the activation of the P53 protein[1]. This DNA damage has been founded in 30- 60% of HCC patients in AFB1 epidemic regions[2].

1.3 Diagnosis of HCC:

The main challenge in HCC remains the early diagnosis which allows potential treatment approaches. Various tests used to diagnose HCC, include imaging, histology and serological tests. Imaging tests, such as ultrasound (US), computerized tomography (CT) and magnetic resonance imaging (MRI) can be used to detect hepatic nodules[20],[21]. Monitor and control of cirrhosis in patients of any etiology may decrease tumor-related mortality [22]. Beside the cirrhosis the value of α-fetoprotein (AFP) and newer biomarkers such as lectin-bound AFP (AFP-L3) as surveillance needed to define their significance[23]. In general, gene expression profiles, proteomic and recent progress in metabolomics can act as potential biomarkers. This will help in the identification of HCC development and may serve for monitoring therapeutic response[22].

1.4 Stages of Hepatocellular carcinoma:

Staging of hepatocellular carcinoma is a critical step in management of patients and HCC treatment. Because any of these stages should be linking with treatment indication and this should be based on strong scientific data[24]. The Barcelona Clinic Liver Cancer staging system is concluded in **figure (1.3)**[25] which is important to treatment of HCC patients based on this staging system[26].

The BCLC recognizes the following five stages of HCC:

1.4.1 Very early stage (BCLC 0)

This stage can start in patients who have single HCC smaller than 2 cm in cirrhotic liver without clinically related portal hypertension. Because these small tumors have a very low chance of microscope dissemination, especially if they were related to indistinctly nodular type[27].

1.4.2 Early stage (BCLC A)

In this stage patients have single HCC or nodules up to three or less than 3cm. The function of the liver is defined by Child-Pugh A and Child-Pugh B status not reaching the criteria for transplantation[25].

1.4.3 Intermediate stage (BCLC B)

This stage is characterized by those patients with multifocal/large disease who are asymptomatic and do not present vascular invasion or extrahepatic spread Liver function[25].

1.4.4 Advanced stage (BCLC C)

For this stage is formed by those patients with extrahepatic spread, vascular invasion and / or mild cancer-related symptoms. Liver function in this stage is not well confirmed as a prognostic predictor, in addition the presence of ascites may a worse prognosis[28] .

1.4.5 End stage (BCLC D**)**

This include those patients with severe impairment of liver function (Child-PughC) who are not allowed for liver transplantation and those who they have heavy impaired physical condition as established by an ECOG performance status >2[28].

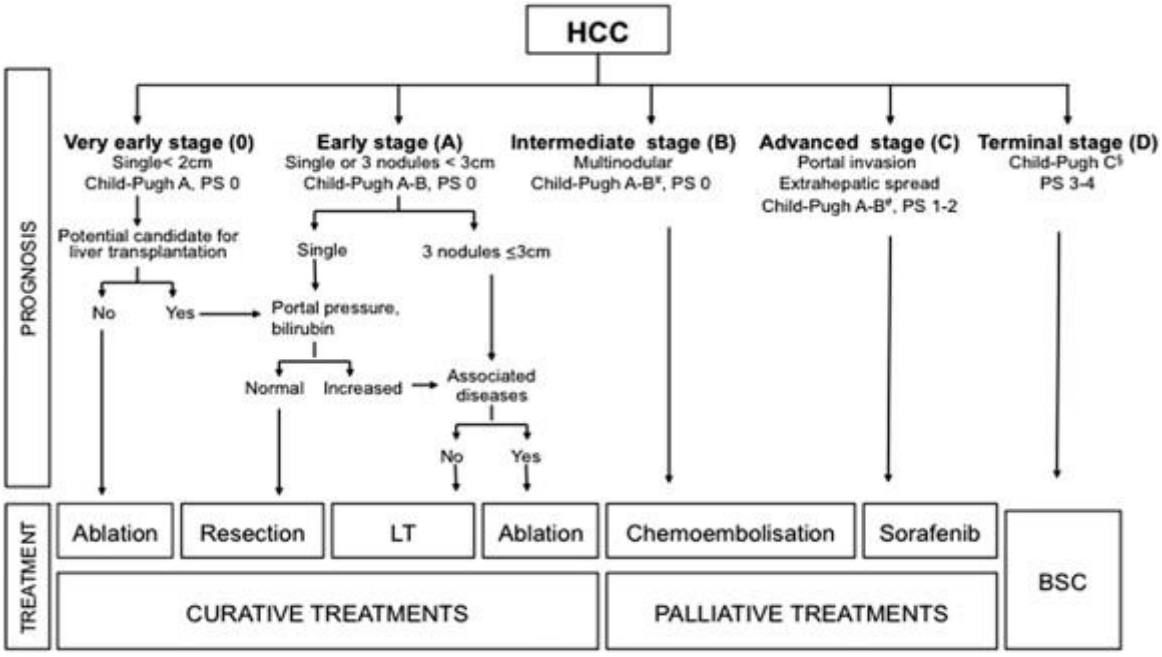

Figure 1.3 The Barcelona -Clinic Liver Cancer (BCLC)staging system for HCC.

1.5 Current treatment of HCC:

Treatments for HCC depend on the extent (stage) of the disease as well as the age, overall health, the type of local medical resources and personal preferences, there are various treatment choices available for HCC[26]. Treatment of HCC is divided into two types; curative and palliative. The curative treatment for HCC treatment include ablation, surgical resection and liver transplantation; these usually provide a high percentage of treatment response which increases the survival rate. For the second type, the palliative options of HCC treatment, such as chemotherapy and radiotherapy, do not tend to provide a high rate of response compared to the curative options, but they can improve the survival rate of HCC patients in general[29].

1.5.1 Surgical Resection

HCC patients with a non-cirrhotic liver, an early stage of HCC and the liver health and function are good candidates for the resection surgery. The process of liver resection it is done by removing the specific part of the liver that has the tumor mass, along with a small range of liver tissues around the mass, leaving the healthy part of the liver to renew the whole organ. Among all the HCC treatments choices, surgical resection for the early stage of HCC is considered the best choice because it provides complete extirpation of the tumor mass and keeps the liver function regeneration[30]. But, treatment with surgical resection has some limitations for curing HCC. First, some clinical tests should be made on the liver to ensure that the remaining part of the liver has the ability to renew the liver function. For example, HCC patients with liver cirrhosis are not suitable for surgical resection. Second, removing the liver tumor mass will not remove the tumor completely, that means will increase the chances of generating a de novo primary tumor mass. The statistical studies indicate that 75-80% of HCC patients who have had the tumor mass removed will survive. Third, if the patient has several tumors the chance of treating HCC by surgical resection will be small. In addition, the HCC cases that diagnosed at the late stages with the association of liver cirrhosis will make surgical resection an impractical option[22].

1.5.2 Liver Transplantation

Liver transplantation is considered one of the best curative treatment options for HCC patients with one tumor nodule less than 5 cm or up to three nodules each less than 3 cm, especially in the early stages[22], since it removes the whole liver including its tumor masses. It thus provides a solution for the underlying cirrhosis. On the other hand, the main problem with liver transplantation is the spread of the tumor to another organ. There are criteria Knows as the Milan criteria, which are criteria used to distinguish HCC patients who are suitable for liver transplantation. These criteria include: a solitary tumor < 5cm in diameter, total of 3 lesions <3cm in diameter, and no spread of the tumor to the other organs or vessels. The Milan Criteria increased the survival rate to 80% and returning survival rate to 83% after liver transplantation[31].

1.5.3 Local Ablation

Local ablation is usually performed in early-stage disease for those who are not candidates for resection or transplantation[22]. Ablation therapy provides local management of the HCC cancer cells with only a small influence on the neighboring cells and other hepatic tissues. The three types of ablation therapy include radiofrequency ablation (RFA), percutaneous ethanol injection (PEI) and microwave ablation (MWA). RFA uses an electrical current with a high frequency to deliver heat to the liver tissues and cause coagulative necrosis, however, RFA will continue to be the first-line therapy in nonsurgical patients with small lesion until more effective technique are established. PEI mainly causes liver cell dehydration, which is responsible for the death of the exposed liver tissues, also produces coagulative necrosis. Unlike RFA, MWA generates heat by applying an electromagnetic source to the liver tissues that can cause the tumor masses to die[30].That is why chemotherapy is another option for HCC treatment.

1.5.4 Chemotherapy

The name chemotherapy is referred to a systemic therapy using small molecule drugs to target various signaling pathways. However, treatment of HCC with chemotherapy agents has not been very promising to date and suffers from the drawbacks of dose-limiting

toxicity, developing of multidrug resistance and unfavorable side-effect such as other cancers. since they have not increased the survival rate. The major reason for this is multidrug resistance associated with most of the drugs available[32]. Multiple goals are possible when using chemotherapy to treat HCC patients, including curing cancer, slowing cancer growth, and treating cancer symptoms[33]. In general, four anti-cancer drugs types are available, including alkylating agents, antineoplastic agents, intercalating agents and molecular target anti-cancer agents. However, the Food and Drug Administration has approved only a few drugs for the treatment of HCC (Sorafenib and Erlotinib), which are considered molecular target anti-cancer agents[34].

1.5.5 Radiotherapy

Radiotherapy(RT) is another option for the treatment of HCC. Its application of radiotherapy has increased recently decades, and also the studies using the latest technologies, such as stereotactic body radiotherapy (SBRT) or proton therapy has increased. Many researchers have reported effective clinical outcomes for patients with HCC using RT. This therapy can achieve necrosis by killing the tumor cells in a small liver tumor mass. However, radiotherapy has some risks for the patients, such as causing abdominal injuries or extensive hepatitis. For these reasons, the use of this technique for treating HCC is very limited[35].

1.5.6 Treatment Strategies of HCC by Molecular Target

Treatment of HCC can be done by either liver transplantation or surgical resection if diagnosed in its early -stages. However, majority of the HCC cases are discovered in the late stages, which cause poor survival rate [36]. The main reason for this decreasing in the survival rate is the lack of effective chemotherapeutic agents that can cure HCC in its late stages. Many studies have demonstrated that only 10-20% of HCC patients respond to the chemotherapy treatments, with toxicity and cellular resistance to available chemotherapeutic agents being the main problem to successful treatment [37]. Searching for new active molecular target anti-cancer agents for HCC has become a popular area for research due to the urgent need to overcome the toxicity and cellular resistance problems. In addition, molecular targets is essential for the discovery of a therapeutic treatment that

overcomes the resistance and decreases the side effects [38]. Hepatocellular carcinoma (HCC) is a diverse and complex tumor with many variations in genome. Irregular activation of several signaling cascade has been shown by previous research. For example epidermal growth factor receptor (EFGR), RAS/extracellular signal-regulated kinase, phosphoinositol 3-kinase/mammalian target of rapamycin (MTOR), hepatocyte growth factor/mesenchymal-epithelial transition factor, Wnt, Hedgehog, and apoptotic signaling[39] .

1.5.7 Disrupted Signaling Pathways and Targeted Therapies

The molecular aberrations described protein kinase as the main targets for liver cancer therapy. Description of the whole protein kinases pathway a few years ago has helped the discovery of new oncology drug .The key signal transduction pathways involved in the pathogenesis of HCC are Wnt-βcatenin pathway, EGFR-RAS-MAPKK pathway, c-MET pathway, IGF signaling, Akt/mTOR signaling, and VEGF and PDGFR signaling cascades **Figure (1.4)[40].** In addition, targeted therapies developed for these pathways are summarized in **Table(1.2)**[39].

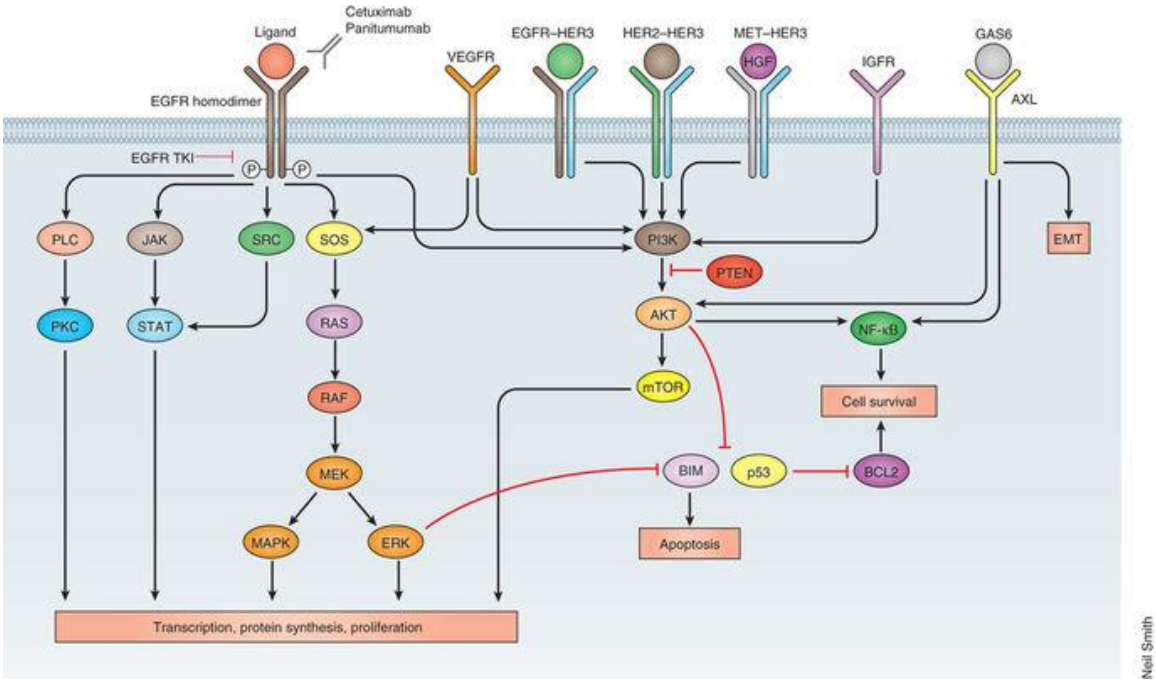

Figure 1.4 Activation of EGFR leads to downstream signaling pathways that ultimately drive tumor proliferation or impair apoptosis.

Table 1.2 Molecular Targeted Agents in Clinical Development in Cancer:

Cancer cell function	**Target**		**Agent (type)**
Signal transduction	Growth factor receptor	EGFR	Gefitinib (TKI), Erlotinib (TKI), Cetuximab (mAb), Panitumumab (mAb)
		HER	Trastuzumab (mAb), Lapatinib (TKI)
		PDGFR	Imatinib (TKI), Sunitinb (TKI), Sorafenib (TKI)
		FLT3	Lestaurtinib (TKI), PKC 412 (TKI), sunitinib
	Intracellular signaling	RAS	Farnesyl transferase inhibitor tipifarnib.
		RAF	Sorafenib.
		MEK	Vandetanib, AZD6244.
		MTOR	Temsirolimus, everolimus, rapamycin.
Angiogenesis	Growth factor	VEGF	Bevacizumab (mAb)
	Growth factor receptors	VEGFR (1–3)	Sorafenib, sunitinib, Britain, cediranib, Valatanib, IMC1121B (mAb)
		PDGFR	Sorafenib, imatinib, sunitinib.
Apoptosis	Intrinsic pathway	BCL2	GX15-070, oblimersen
	Extrinsic pathway	Apo2L/TRAI	Mapatumumab, Apomab, AMG-655, rhApo/TRA

1.5.8 Growth Factor Receptor Signaling**:**

1.5.8.1 EGFR-Ras-MAPKK Pathway

The EGFR is a member of a family of four related receptors (Her2/Neu, ErbB3, and ErbB4) that upon ligand binding trigger tyrosine kinase activity and consequently initiate signal transduction. The function of EGFR classically occurs because of point mutations, amplification, or increase in ligand-receptor interaction[41]. Activate of the RAS/MAPK signaling pathway and induce transcription of genes of the AP1 family, such as c - fos and c - jun , which play an important role for cell proliferation it's done by the ligands EGF, hepatocyte growth factor (HGF), PDGF, and VEGF[39]. In addition, effective blockade of the EGFR signaling pathway can be achieved using monoclonal antibodies against EGFR (cetuximab) or ErbB2/Her2/neu (trastuzumab). In HCC, Ras/MAPK pathway activation might cause aberrant upstream signals (EGFR signaling, IGF signaling) or inactivation of tumor suppressor genes by aberrant methylation[42].

Therefore, inhibition of the EGFR-TK signaling cascades are promising potential approach for the treatment of hepatocellular carcinoma (HCC). In the signaling cascade, both upstream or downstream targets of EGFR can be used for the treatment of any cancer in general and HCC specifically[43،44]. Many organic compounds, such Erlotinib, have shown potent inhibitory activity against EGFR by inhibiting its phosphorylation; these are known chemotherapeutic agents for the treatment of HCC. Erlotinib (Tarceva) is a very active and selective inhibitor of the EGRF-TK protein. It has an advantage over most of the anti-cancer agents in that it can be taken orally, inhibits cellular proliferation and causes cell cycle arrest at the G1 phase. In addition, it has been approved as an active drug for pancreatic and lung cancer by the FDA but is still in Phase II clinical trials as an anti-cancer drug for HCC[45].

1.5.8.2 Vascular Endothelial Growth Factor (VEGF**)**

HCC is as hypervascular cancer and has a large amount of tumor vascularity. VEGF is connected to the angiogenesis of various cancer types and HCC is one of them whose progression is enhanced by VEGF[46]. HCC and all other tumor masses need blood

vessels to survive and enlarge, as a result these blood vessels are considered abnormal since they are responsible for the high fluid pressure inside the tumor mass. Therefore, VEGF as a targeting agent may cause a decrease in the tumor vessels' supplies and their sinuosity and consider to be a promising target therapy, which leads to a decrease in the internal pressure of the tumor mass. All of these processes will lead to normal blood vessels [47]. In 2005, Gerber et al confirmed the ability of anti-VEGF drugs, in combination with other anti- cancer agents, to cause a fast decrease in the internal vessel pressure of the tumor mass, which resulted into faster targeting of the agents to the tumor mass, a decrees in the tumor size and an increase in the survival rate [48]. A lot of agents have been designed to treat HCC by targeting VEGF or VEGFR. Some of them have been proven effective, such as Erlotinib **Figure (1.5),** and some are still in clinical trials to verify their pharmacokinetic profiles. Erlotinib is one of the first molecular target drug approved by the Food and Drug Administration (FDA) for the treatment of HCC. It is considered a multi-kinase agent that stops tumor cell proliferation by inhibiting different molecular targets, including VEGFR and PDGFR tyrosine kinases, which produces an anti-angiogenic effect. In addition, it targets the downstream cascades such as the Raf/MEK/ERK signaling pathway[49, 50] **Figure (1.5).**

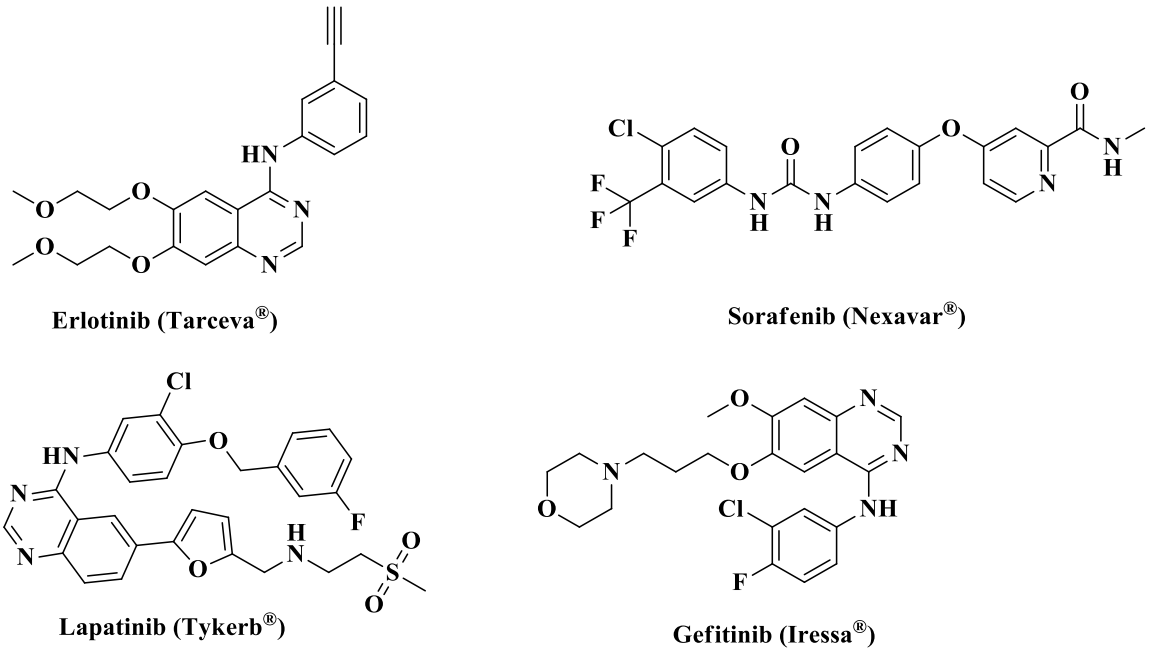

Figure 1.5 Structures of known Chemotherapeutics that target EGFR for treatment of HCC**.**

1.5.8.3 Mitogen Activated Protein Kinase (MAPK) Pathway

MAPK includes, in its downstream, four main kinases Ras, Raf, MEK and ERK **Figure (1-5)** which connect to each other by phosphorylation. They play role in cell division, growth and regulation. These downstream proteins are connected to the upstream receptors such as PDEFR, EGFR, and VEGFR **Figure(1.5)**[51,52]. The MAPK pathway is an essential player in the growth and survival of HCC cells, which makes it a promising target for the treatment of HCC[39, 53] . ABT-100 is one of the anti-cancer agents in phase II clinical trials that inhibits the farnsylation process of the protein Ras by inhibiting the enzyme farnesyl transferase, later leads to a decrease in tumor cell growth[51,54] . The family of the protein Raf includes three members: A-Raf, B-Raf, C-Raf. Hyperactivation of C-Raf (wild type) in various cancer types, including HCC, was the only reported one, which makes it a valuable target for treating HCC[55]. Sorafenib is an approved HCC chemotherapeutic agent that inhibits B-Raf, C-Raf, FGFR, PDGFR and VEGFR[56]. The family of the protein MEK includes two subunits, MEK1 and MEK2have been reported that overexpression of MEK1 and MEK2 lead to an activation of ERK1 and ERK2; in the case of HCC, this could be

happening in different percentages. For more, it has been proven in vitro studies that the addition of a MEK1 or MEK2 inhibitor to HepG2 or Hep3B HCC cell lines will inhibit the autophosphorylation and cause cell apoptosis. MEK inhibitors, including include Selunetinib, RDEA119 and ASCO2010, are still in phase II clinical trials[57].

1.5.8.4 PI3K/Akt/mTOR Pathway

The PI3K/Akt/mTOR pathway has a critical role in carcinogenesis[58]. Akt can be activated through tyrosine kinase receptor (EGF or IGF signaling) or through organized activation of PI3K or loss of function of the tumor suppressor gene PTEN by epigenetic preventing or somatic mutations. In spite of the role of pAkt in HCC still more investigation, recent studies have suggested a worse estimate for tumors with activated Akt[59].MTOR is consider as an important mediator of the PI3K-Akt pathway, which acts as a central regulator of cell growth and proliferation, by sensing nutritional status and allowing progression from G1 to S phase[41]. The mTOR pathway is activated in a subset of HCCs, and its blockade with rapamycin or everolimus inhibits growth in HCC cell lines, and in experimental models [42]. Many novel compounds (MTOR inhibitor) are recently being tested in early clinical trials. These molecules (rapamycin and analogs) are already approved as immunosuppressive treatments after liver transplantation[60].

1.5.9 Relationship of Multiple-Drug Resistance and Chemotherapy

Cancer is a serious disease that ranks on the second position among diseases that lead to death, in 2015 have been found death of one person in every five or six people in the western countries. It is also responsible for 8.2 million of deaths around the world. Every year more than 14.1 million cancer cases are revealed, mainly in the developing countries. For more, people who live with cancer count more than 32.5 million[61], [62].

When the cancer cells start to be abnormally fast divided cancer is known as serious genetic disorder. So, if the abnormal divided cells untreated, it will affect the other tissues of the body and lead to death. The main available treatments of cancer are chemotherapeutic agents which can either stop or slow the abnormal fast division of the cells[63,64]. The major challenge associated with the available chemotherapeutic agents is the

drug resistance which involved with 30%- 80% of cancer patients[65]. Therefore, there is an urgent need to find a new agent to overcome the chemotherapeutic resistance.

Drug resistance in cancer cells is not related to one anticancer drug, but the whole available chemotherapeutic agents within the same family can be influenced by the same mechanism. Some cancer cells that developed drug resistance can be resistance to other types of drugs that are different in their mechanism of action and structure. This process defined as multidrug resistance (MDR). This phenomenon might clarify the failure drug combination to overcome the cancer cell resistance[66]. There are two main clinical times of MDR; first one start at the time of treatment and the second is already present at the time of the diagnosis[67].

Various biological reactions represent the first defense for the cells include:

1. Activation of cellular elimination process, cellular uptake process and metabolic reactions to inactivate the drug process inside the cell, all of these processes will cause the decrease of the chemotherapy concentration inside the cell **(Figure 1.6)**[68,69].
2. Changes of the drug delivery to the targeted tissues because of different reasons including poor pharmacokinetics profile of the drug such as absorption, distribution, metabolism and excretion (ADME)[70], [71].
3. Enhance the process of DNA repair[71].
4. Structural modification of the targeted tissues[72].

All the previous mechanism are extracellular factors that increase the cell anticancer drug resistance. However, the cellular factors that affect the drug presence inside the cell play a main role for the cell resistance to anticancer drugs through biochemical changes in the tumor cells and this process include transport-based MDR known as ATP-binding cassette (ABC).

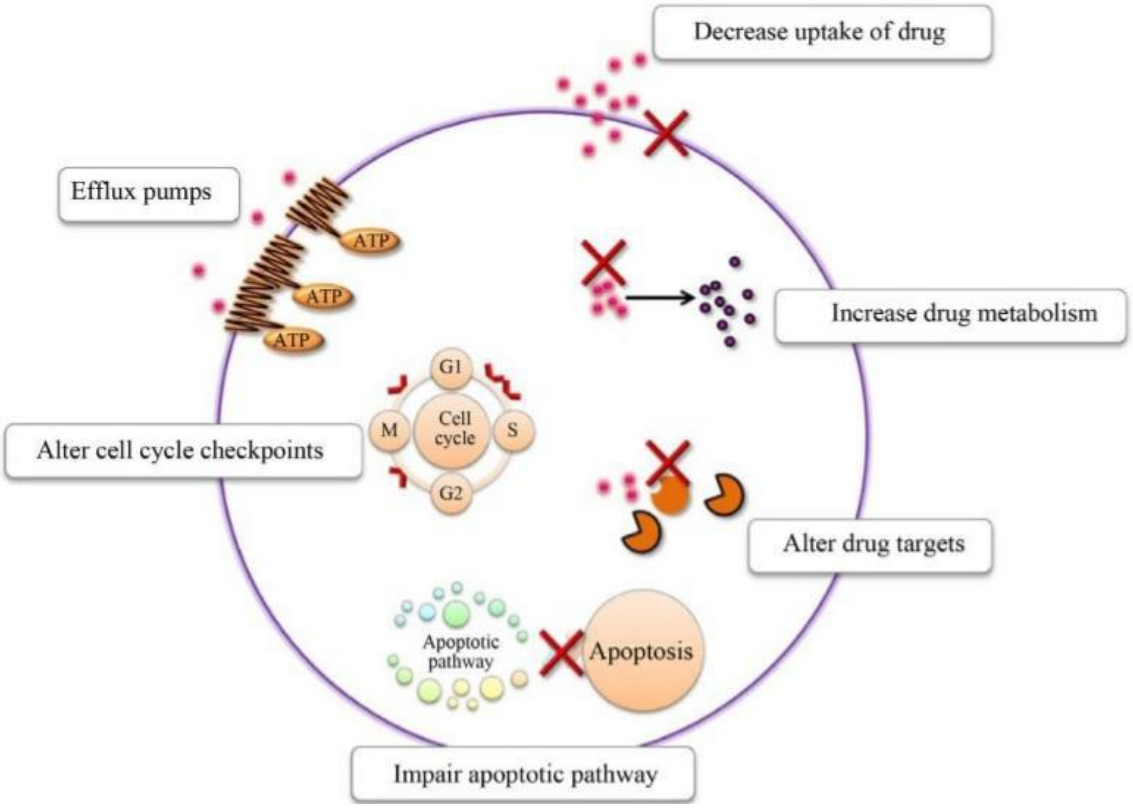

Figure 1.6 Mechanism Involved in increase Drug Resistance towards cancer chemotherapeutic drugs.

Different transporter proteins are located in the lipophilic membrane of the cell, which play essential role in the pharmacodynamics and pharmacokinetics of various drugs. Therefore, massive studies on the transporters have been conducted to identify their locations, functions, structures, selectivity and cellular distribution[73]. Cellular MDR decreases the intracellular concentration of the drugs by enhancing the ATP-dependent efflux pumps, which is one of the family membranes of ATP-binding cassette (ABC)[74]. ATP-binding cassette is considered as the largest transmembrane protein family that demonstrated wide range of specificity. There are about 49 known human ABC genes, which are categorized into 7 subfamilies starting with ABC and end with ABCG and the classification were relay on the arrangement of the domain and similarity of the sequence[66].Many products resulted from the metabolic reactions, lipids and various types of chemotherapeutic agents are pumped out of the cells through ABC-transporter utilizing ATP-energy dependent movement processes[75]. The chemotherapeutic agents that

commonly affected by MDR process are hydrophobic containing drugs, natural products such as docetaxel and paclitaxel, anthracyclines (daunorubicin, doxorubicin), anti-microtubule alkaloids (vincristine), antimetabolic agents (6-mercaptpurin, methotrexate, gemcitabine, fluorouracil), epipodophyllotoxin (teniposide and etoposide) and RNA elongation inhibitors (actinomycin-D)[76,77].

1.5.10 Role of ABC Transporters in Cancer Chemotherapy

Multidrug resistance protein 1 (MRP1)/ ABCC1, MRP2 and breast cancer resistance protein (BCRP)/ ABCG2/MXR/ABCP are responsible for MDR[78]. These proteins play key role in recognition and transport of a large number of structurally diverse compounds. Many studies have shown that inhibiting these ABC transporters can prevent MDR[79]. The MRP subfamily is, the C subset of the ABC transporter superfamily and it is composed of thirteen members, and nine of these are primarily involved in MDR[80].

Functional characterization, localization, and cloning studies have shown that, these nine MRPs have been established as ATP dependent efflux transporters for endogenous substances and xenobiotics. The other three members of the MRP subfamily, namely ABCC7/cystic fibrosis transmembrane conductance regulator (CFTR), ABCC8/sulfonylurea receptor 1 (SUR1), and ABCC9/SUR2, have no role in conferring MDR. The role of ABCC7 is to regulated chloride channel, whereas ABCC8 and ABCC9 are intracellular ATP sensors and regulate the specific K+ channel permeability[77]. The nine main MRPs can be divided into two groups on the basis of structural topology. One has a common ABC transporter structure and is composed of two membrane spanning domains (MSD) with nucleotide binding domains (NBD1 and NBD2) in between **(Figure 1.7)**[81]. These can be referred to as short (MRPs) and include MRP4, MRP5, MRP8, and MRP9 (ABCC4, 5, 11 and 13, respectively). The other group, which includes MRP1,2,3,6and7 (ABCC1,2,3,6and7, respectively), have an additional MSD (MSD0) and are referred as(MRP)[82].

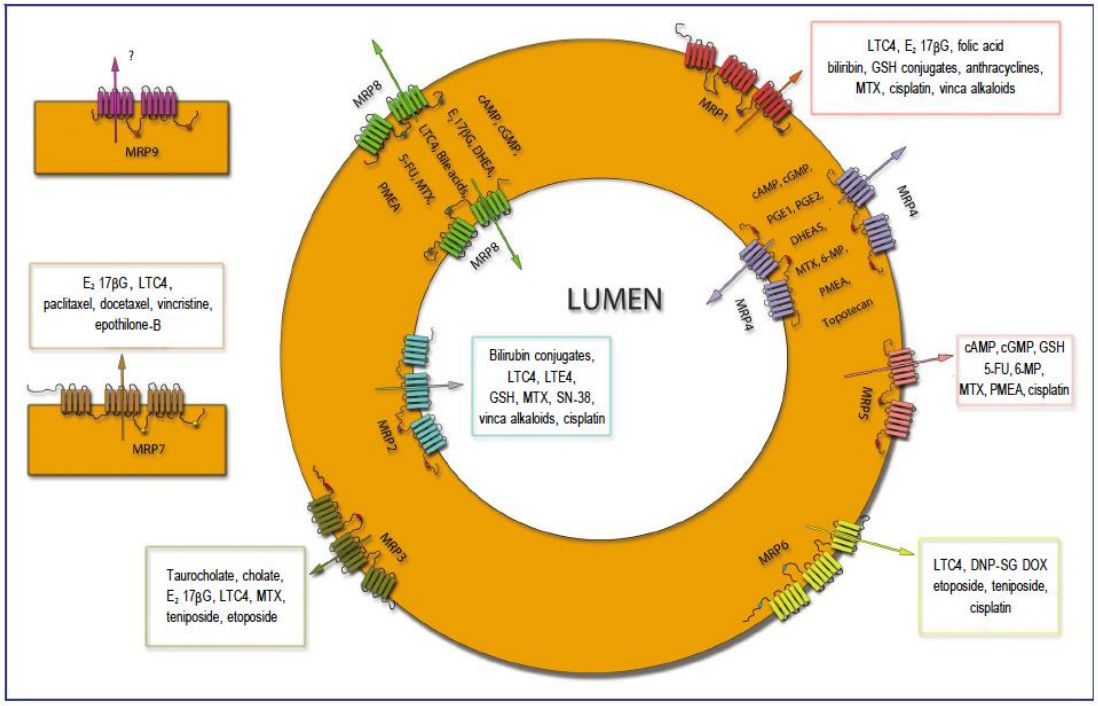

Figure 1.7 The Location of short (MRP4, MRP5, MRP6, MRP8 and MRP9) and long form (MRP1, MRP2, MRP3, and MRP7).

1.5.11 Natural products as potential source of biological agents:

Natural products are a good and affordable source for new drug entities. Different vaccines and biologics have been inspired from natural products structure. Around 48.6% of the anticancer drugs are natural products or derived from many natural products[83].

Later on, many advances in biological screening techniques have allowed the study of the biological mechanisms and chemical profiles of living systems, which has encouraged researchers to investigate the pharmacological effects of natural compounds[84]. Studies are done to clarify the natural products' synergistic impacts and their clinical effects on the individual body, which could help provide novel curative approaches to different diseases[85] Including vinblastine, etoposide, paclitaxel, and camptothecin. Plants have been considered as a source for medicaments because of their availability and it was given either in a crude extract or a pure ingredients[86]. One of the most studied natural product that has

many biological activities is cucurbitacins[87].

1.5.11.1 Cucurbitacin

Cucurbitacins natural compounds that extracted mostly from the plant of Cucurbitaceae family such as *Ecballium Elaterium*, *Genystlus keithii, Cayaponia Tayuya*, *Citrillus Colocynthic, Trichosanthes Kirilowii* and *Ecballium Elaterium*[85]. Cucurbitacins are highly oxidized tetracyclic triterpenoid. They are characterized by their bitterness and toxicity[88].

Isolation of cucurbitacin have been achieved from different parts of the plant, including the seed, roots, rhizomes and aerial parts of Cantaloupe, Watermelon, Pumpkin, Honeydew Melon, Spaghetti Squash and Crenshaw Melon[89]. Several studies have confirmed the medicinal activity of the cucurbitacins and their clinical effects. Until this time cucurbitacin are still used as a treatment in some parts such as Asia, Africa and South America. Many biological activities have been associated with cucurbitacins and their glycoside derivatives[89,90.] Recently, cucurbitacins B, C, Q, and E showed antiproliferative activity on 8 different cancer cell lines, such as HepG2, MDA-MB-468, MCF-7 and A549. Several animal studies demonstrated that cucurbitacins anticancer activity through apoptosis stimulating activity the inhibition of the Janus kinase (JAK), signaling marker and activator transcription3 (STAT3) signaling[90,91].

1.5.11.2 Structure of cucurbitacins

Cucurbitacins are tetracyclic triterpenoid steroidal carbon skeleton **(Figure 1.8).** It is believed that other cucurbitacins are derived mainly from the metabolism of cucurbitacin B or E by enzymatic reactions[92]. For example, the metabolism of cucurbitacin B produce cucurbitacin A, C, D, F, G and H, while cucurbitacin E metabolism gives cucurbitacin I, J, K, and L. In addition, cucurbitacin B and D can be reduced to 23, 24-dihydrocucurbitacin B or 23, 24-dihydrocucurbitacin D[93]. The presence of dimethyl group at C4, isopropyl group at C24 and unsaturated are considered as a unique feature for cucurbitacins. For more, there are methyl groups at C9, C13, C14 and C20. Cucurbitacins could be exist in plants as glycosylated or nonglygosylated[94].

The cucurbitacins four-ring system similar to the structure of a steroid ring[94].the Two common difference between cucurbitacins and steroids structures, are that cucurbitacins having a gem-dimethyl group at C-4 and a C-10 methyl in C-9. Cucurbitacins' main structures share common features, such as: 1) the double bond between C-6 and C-5; 2) a high level of oxidation due to the substitutions of many carbons (C-16, C-11, C-3, C-2) by oxygen atoms; 3) the presence of hydroxyl groups, α at C-16 and β at C-20 and C-25; and 4) α-β-unsaturated ketone in the side chain located at C-22, C-23 and 11 C-24. an aromatic ring is a common feature in some of the cucurbitacin derivatives, such as Fevicordin A[95]. Additionally, cucurbitacins can be found as free glycone structures or glycosidic structures by a β-linkage to the hydroxyl moiety from monoside at C-2, C-3 and C-25 or from bidesmosides at C-26 or C-27[96].

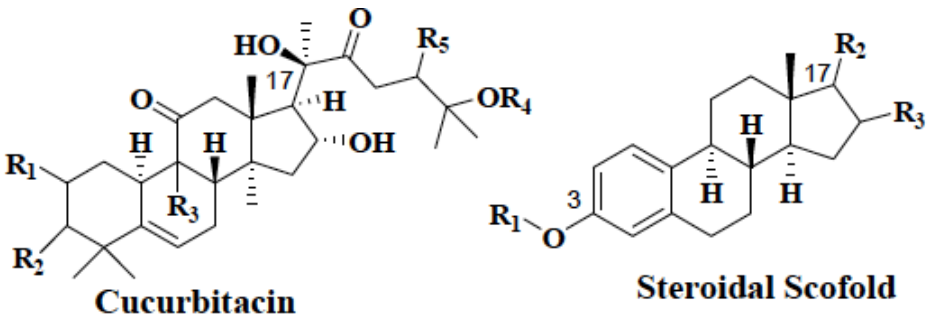

Figure 1.8 General structures of cucurbitacin and steroid.

1.5.12 Biological activities of cucurbitacin**:**

1.5.12.1 Cucurbitacins Activity as Anti-inflammatory Compounds

Cucurbitacins anti-inflammatory have been proven via targeting many biological targets to decrease the inflammation. The cyclooxygenase (COX) enzymes playing an important role in anti-inflammatory activity of cucurbitacins, especially cyclooxygenase2 (COX-2). In comparing the non-steroidal anti-inflammatory drugs (NSAIDs) and cucurbitacins, the inhibitory rate for COX-2 by cucurbitacins is less than the NSAIDs; for example, at 100mM concentrated cucurbitacins B, D, E and I showed inhibitory rates of 32, 29, 35 and 27%, respectively, compared to the COX-2 of NSAIDs such as Refeoxib, Ibuprofen and Naproxen. The same study has proved the selectivity of cucurbitacins

towards COX-2, since they do not show any activity on the cyclooxygenase-1 (COX-1) enzyme[97].

1.5.12.2 Cucurbitacins Effect on Filamentous-Actin

Many studies confirmed the activity of cucurbitacins E, I and B on the cytoskeleton, particularly on F-actin. One of these studies proved that some cucurbitacins derivatives including cucurbitacin E, stimulate actin cytoskeleton disturbance[98]. This disturbance connected with the effect of cucurbitacins on the actin-proliferative action in prostate cancer cell line. One of the features of cucurbitacin E is that it has a selective inhibition on F-actin depolymerization, but not on monomeric globular G-actin, by forming a covalent bond with CYS 257 amino acid residue[99].

1.5.12.3 Mitogen Activated Protein Kinase (MAPK) Pathway Activation by Cucurbitacins

MAPK pathway is considered one of the key parts in the cellular process, which has signaling transducing cascade including Ras/B-Raf/MEK/ERK **(Fig1.5).** A limited research studies have mentioned the potential biological activities of cucurbitacins targeting the MAPK pathway. Chean et al. confirmed that cucurbitacin B inhibits the STAT3 and RAS/B-Raf/MEK/ERK cell downstream signaling cascade using the K562 leukemia cell line[100]. One more study has been done by Salama and Halaweish via Utilizing computational semi-flexible molecular docking, MTT cell viability assay and binding immune assay, demonstrated the ability of several types of cucurbitacins to target MAPK signaling pathway using mutant B-Raf cell lines[101].

1.5.12.4 Cucurbitacins as Potential Modulator for Epidermal Growth Factor Receptors (EGFR**)**

EGFR, in human cancer has been proven to be involved in the mutation and deletion of the cell upstream and downstream targets, which makes EGFR a promising biological target for different types of human cancer[102]. Hollbro et al confirmed that ErbB receptors, a member of the GFR family, are a promising targets for the treatment of different kinds of cancer[103]. The activation of EGFR and its downstream cascade has increased the survival

rate of patients in the late stages of hepatocellular carcinoma as reported by Liovet et al[104].

1.5.13 CUCUS-Inspired Estrone Analogs (CIEA)

Cucurbitacins is a group of steroidal-triterpene tetracyclic natural products, which reported for their anti-cancer activities[105]. However, cucurbitacins have been reported for their potent activities, synthesis of these compounds is challenging due to the complexity of the carbon skeleton and functionalities of these compounds. Recently many studies in Halaweish's group started to study cucurbitacins targeting epidermal growth factor receptor (EGFR)[100].

Molecular modeling and docking methods were applied to find potential affinity between cucurbitacins and EGFR along with downstream proteins cascade including Ras, Raf, MEK and ERK **(Figure1.4).** In addition, more studies such as cytotoxicity, western blot and ELISA were used to prove the molecular docking studies results. Cucurbitacins confirmed to have activities against different cancer cell lines; however, their activities don't show specificity or selectivity toward their biological targets. Gastrointestinal toxicity is one of the side effects involved with cucurbitacins subjections due to their cellular activations[106]. Strong cytotoxicity of cucurbitacins in in-vivo model toward renal carcinoma demonstrated narrow safety and have been withdrawn from preclinical studies due to their fatal activity[107].The broad biological activities of cucurbitacins, non-selectivity and toxicities are due to their complex chemical structure[108].

Cucurbitacins inspired estrone compounds (CIEA) was accomplished in Halaweish's group targeting melanoma[109]. The success in Halaweish's group of utilizing the molecular docking inspired us to model novel analogues to target hepatocellular carcinoma (HCC).

Due to the similarity in chemical structure, particularly the cyclopentane and the four-ring system, redesigning and mimicking cucurbitacins utilizing steroids may improve their biological activities and selectivity. Cucurbitacins side chain, which contain α-β-unsaturated ketone, is significant pharmacophore for their biological activities. In addition, the presence of C-16 hydroxyl group increased the chance of forming H-bond with C-24

ketone, which may enhance the electrophilicity of the α-β-unsaturated ketone[110,111] .

1.5.14 Targeting Epidermal Growth Factor Receptor (EGFR):

Many studies have been done on cucurbitacins as promising EGFR inhibitors is not favorable sometimes due to availability and limitation. This but has encouraged us to identify the most significant pharmacophores of cucurbitacins in order to synthetically modify their main skeleton to increase the selectivity toward EGFR and minimized their undesirable side effects[101].Limited researches and studies on the structure modifications of cucurbitacins have been conducted including quantitative structure activity relationship (QSAR) studies of semi synthetic of cucurbitacins by Bartalis and Halaweish[111].

Several CIEA have been synthesized by small modifications at C-2 of estrone main structure by installing sulfamate moiety, changes the biological properties of the estrone dramatically by blocking the estrogenic activity and performing anti-proliferative activity in breast cancer cells[112]. In 2016, Bodnar et al, proved that triazole substitution at C-3 position of estrone enhanced the biological activity as anti-cancer with IC50=0.3-0.9μM [97]. Ahmed et al. 2014 confirmed a potent inhibitory activity of MAPK pathway toward treatment of melanoma by a substitution on C-17 of the estrone skeleton structure[109]. Subsequently, by utilizing molecular modeling, series of modified estrone at C-25, C-17, C-16, C-11 and C-3 positions were designed and developed to target Epidermal Growth Factor Receptor (EGFR) toward treatment of Hepatocellular Carcinoma (HCC).

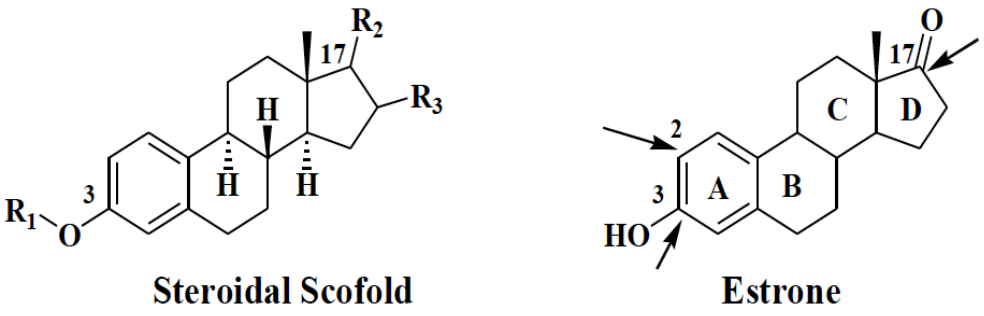

Figure 1.9 Significant positions for biological activities in estrone main structure.

In our group the molecular docking result on the crystal structure of EGFR showed an outstanding binding affinity with the CUCUS-inspired estrone analogues containing various functional groups compare to the known EGFR inhibitor, Erlotinib. Modified estrone at C-17 with isopropanol enon side chain, methoxy group at C-3 and double bond at C-16 and C-17 position such as MMA102 and MMA132 demonstrated various binding mods with EGFR binding pocket. MMA132 and MMA102

MMA102 and MMA132 are diastereomers to each other and showed varieties in the binding mode with the receptor. MMA132, which possess the stereochemistry of cucurbitacin D side chain, showed an outstanding binding mode with EGFR by forming H-bond with MET:769: A, which is same amino acids residues that erlotinib binds to in EGFR to induce anti-cancer activity by H bonding with the same amino acid MET:769-A; also, MMA132 perform hydrophobic interactions with the amino acids residues inside the binding pocket. While MMA102 which has the opposite stereochemistry of cucurbitacin D demonstrated less binding affinity toward the EGFR binding site only with a hydrophobic interaction mode with the EGFR binding pocket. This result proved the significant of assembling the enone side chain with the stereochemistry of cucurbitacin D **(Figure 1.10).**

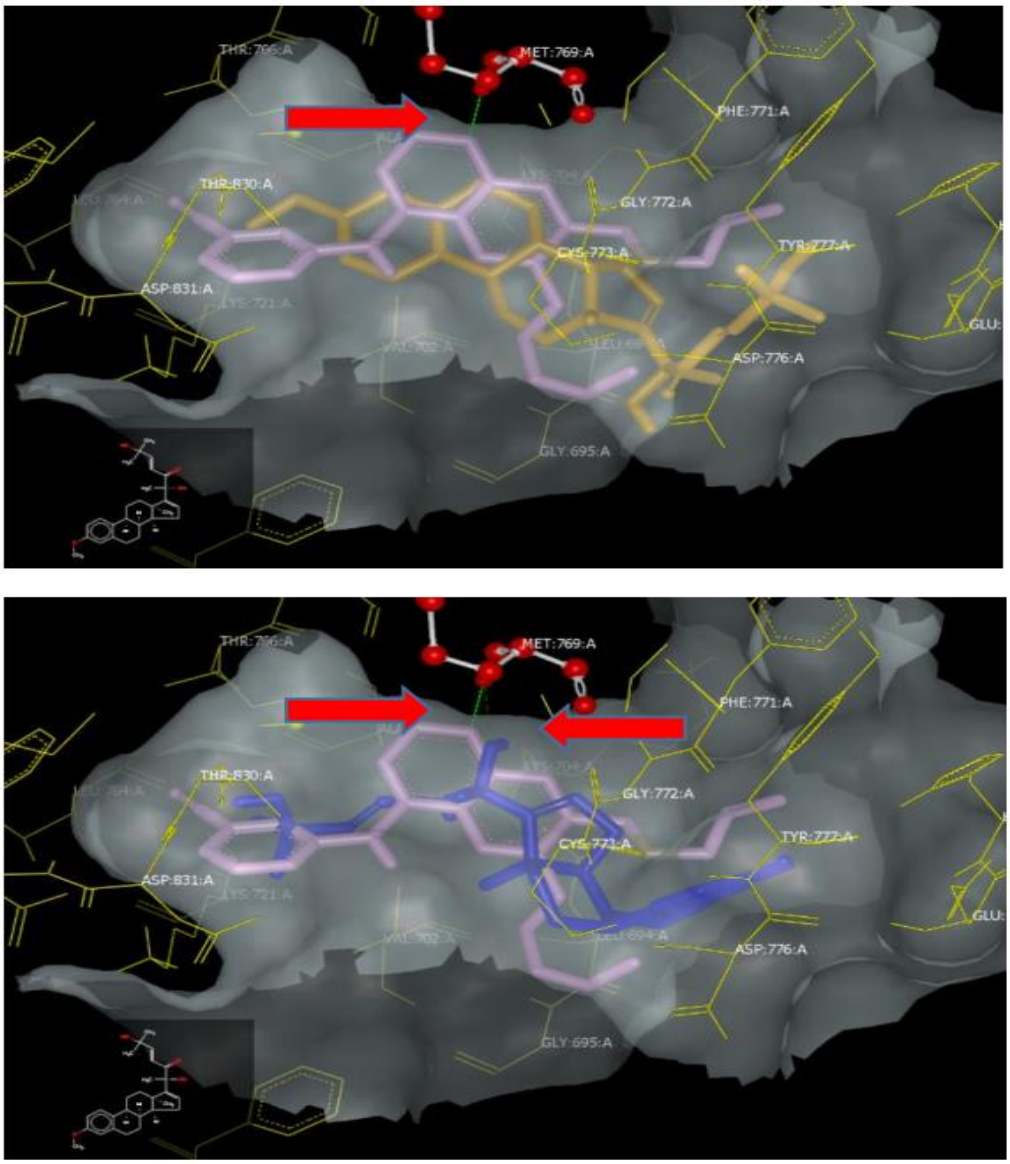

Figure 1.10 Visual representation of A) MMA102 (orange) B) MMA-132 (blue) in the EGFR ATP binding site along with Erlotinib (purple).

1.6 Project Objectives:

This project is focused on using cucurbitacins inspired analog as a semi-synthesized natural product that has inhibitory activity toward the epidermal growth factor receptor (EGFR) and develop it as drug candidates for the treatment of hepatocellular carcinoma (HCC). No many studies have defined the activity of cucurbitacins toward EGFR in HCC cell line. In addition, no cucurbitacin-like compounds have been synthesized and

biologically evaluated targeting HCC. The main objectives of this project are:

Aim1: Studying the cytotoxicity of natural products or semisynthesized natural products against Hepatocellular Carcinoma cell line

Aim2: Study of the effect of CIEA chemosensitization of HCC/HepG2 resistance cell line to chemotherapy drugs (Erlotinib**)**

Aim3: Study the role of multidrug resistance associated protein (MRPs) against sensitive HEPG2 cell line and resistant HEPG2R cell line**.**

1.7 References

1. Wu, Z. R.; Galmiche, A.; Liu, J.; Stadler, N.; Wendum, D.; Segal-Bendirdjian, E.; Paradis, V.; Forgez, P., Neurotensin regulation induces overexpression and activation of EGFR in HCC and restores response to erlotinib and sorafenib. *Cancer Letters* **2017,** *388*, 73-84.
2. Breast Cancer Awareness Month in October (World Health Organization 2012). http://www.who.int/cancer/events/breast_cancer_month/en.
3. Dutta, R.; Mahato, R. I., Recent advances in hepatocellular carcinoma therapy. *Pharmacology & Therapeutics* **2017,** *173*, 106-117.
4. Nagai, T.; Arao, T.; Furuta, K.; Sakai, K.; Kudo, K.; Kaneda, H.; Tamura, D.; Aomatsu, K.; Kimura, H.; Fujita, Y.; Matsumoto, K.; Saijo, N.; Kudo, M.; Nishio, K., Sorafenib Inhibits the Hepatocyte Growth Factor-Mediated Epithelial Mesenchymal Transition in Hepatocellular Carcinoma. *Molecular Cancer Therapeutics* **2011,** *10* (1), 169-177.
5. Fattovich, G.; Stroffolini, T.; Zagni, I.; Donato, F., Hepatocellular carcinoma in cirrhosis: Incidence and risk factors. *Gastroenterology* **2004,** *127* (5), S35-S50.
6. Lok, A. S. F., Perspective: Chronic hepatitis B. *New England Journal of Medicine* **2002,** *346* (22), 1682-1683.
7. Farazi, P. A.; DePinho, R. A., Hepatocellular carcinoma pathogenesis: from genes to environment. *Nature Reviews Cancer* **2006,** *6* (9), 674-687.
8. Bartosch, B., Hepatitis B and C Viruses and Hepatocellular Carcinoma. *Viruses-Basel* **2010,** *2* (8), 1504-1509.
9. McClain, C. J.; Hill, D. B.; Song, Z. Y.; Deaciuc, I.; Barve, S., Monocyte activation in alcoholic liver disease. *Alcohol* **2002,** *27* (1), 53-61.
10. Hoek, J. B.; Pastorino, J. G., Ethanol, oxidative stress, and cytokine-induced liver cell injury. *Alcohol* **2002,** *27* (1), 63-68.
11. Campbell, J. S.; Hughes, S. D.; Gilbertson, D. G.; Palmer, T. E.; Holdren, M. S.; Haran, A. C.; Odell, M. M.; Bauer, R. L.; Ren, H. P.; Haugen, H. S.; Yeh, M. M.; Fausto, N., Platelet-derived growth factor C induces liver fibrosis, steatosis, and hepatocellular carcinoma. *Proceedings of the National Academy of Sciences of the United States of America* **2005,** *102* (9), 3389-3394.

12. Osna, N. A.; Clemens, D. L.; Donohue, T. M., Ethanol metabolism alters interferon gamma signaling in recombinant HepG2 cells. *Hepatology* **2005,** *42* (5), 1109-1117.
13. Kurz, D. J.; Decary, S.; Hong, Y.; Trivier, E.; Akhmedov, A.; Erusalimsky, J. D., Chronic oxidative stress compromises telomere integrity and accelerates the onset of senescence in human endothelial cells. *Journal of Cell Science* **2004,** *117* (11), 2417-2426.
14. Recurrent Mutations May Be Linked to HCC Risk Factors. *Cancer Discovery* **2012,** *2* (7).
15. Sun, B. C.; Karin, M., Obesity, inflammation, and liver cancer. *Journal of Hepatology* **2012,** *56* (3), 704-713.
16. Sanyal, A. J.; Yoon, S. K.; Lencioni, R., The Etiology of Hepatocellular Carcinoma and Consequences for Treatment. *Oncologist* **2010,** *15*, 14-22.
17. Gurtsevitch, V. E., Human oncogenic viruses: Hepatitis B and hepatitis c viruses and their role in hepatocarcinogenesis. *Biochemistry-Moscow* **2008,** *73* (5), 504-513.
18. Caillot, F.; Derambure, C.; Bioulac-Sage, P.; Francois, A.; Scotte, M.; Goria, O.; Hiron, M.; Daveau, M.; Salier, J. P., Transient and etiology-related transcription regulation in cirrhosis prior to hepatocellular carcinoma occurrence. *World Journal of Gastroenterology* **2009,** *15* (3), 300-309.
19. El-Serag, H. B.; Rudolph, L., Hepatocellular carcinoma: Epidemiology and molecular carcinogenesis. *Gastroenterology* **2007,** *132* (7), 2557-2576.
20. Grandhi, M. S.; Kim, A. K.; Ronnekleiv-Kelly, S. M.; Kamel, I. R.; Ghasebeh, M. A.; Pawlik, T. M., Hepatocellular carcinoma: From diagnosis to treatment. *Surgical Oncology-Oxford* **2016,** *25* (2), 74-85.
21. Chen, J. G.; Parkin, D. M.; Chen, Q. G.; Lu, J. H.; Shen, Q. J.; Zhang, B. C.; Zhu, Y. R., Screening for liver cancer: results of a randomised controlled trial in Qidong, China. *Journal of Medical Screening* **2003,** *10* (4), 204-209.
22. Worns, M. A.; Galle, P. R., Future perspectives in hepatocellular carcinoma. *Digestive and Liver Disease* **2010,** *42*, S302-S309.
23. Marrero, J. A.; Feng, Z. D.; Wang, Y. H.; Nguyen, M. H.; Befeler, A. S.; Roberts, L. R.; Reddy, K. R.; Harnois, D.; Llovet, J. M.; Normolle, D.; Dalhgren, J.; Chia, D.; Lok, A. S.; Wagner, P. D.; Srivastava, S.; Schwartz, M., alpha-Fetoprotein, Des-gamma

Carboxyprothrombin, and Lectin-Bound alpha-Fetoprotein in Early Hepatocellular Carcinoma. *Gastroenterology* **2009,** *137* (1), 110-118.

24. Bruix, J.; Reig, M.; Sherman, M., Evidence-Based Diagnosis, Staging, and Treatment of Patients With Hepatocellular Carcinoma. *Gastroenterology* **2016,** *150* (4), 835-853.
25. Forner, A.; Diaz-Gonzalez, A.; Liccioni, A.; Vilana, R., Prognosis prediction and staging. *Best Pract Res Clin Gastroenterol* **2014,** *28* (5), 855-65.
26. Bruix, J.; Sherman, M., Management of Hepatocellular Carcinoma: An Update. *Hepatology* **2011,** *53* (3), 1020-1022.
27. Kojiro, M.; Roskams, T., Early hepatocellular carcinoma and dysplastic nodules. *Seminars in Liver Disease* **2005,** *25* (2), 133-142.
28. Dohmen, K.; Shigematsu, H.; Irie, K.; Ishibashi, H., Trends in clinical characteristics, treatment and prognosis of hepatocellular carcinoma. *Hepato-Gastroenterology* **2003,** *50* (54), 1872-1877.
29. Kaplan, D. E.; Chapko, M. K.; Mehta, R.; Dai, F.; Skanderson, M.; Aytaman, A.; Baytarian, M.; D'Addeo, K.; Fox, R.; Hunt, K.; Pocha, C.; Valderrama, A.; Taddei, T. H.; Grp, V. S., Healthcare Costs Related to Treatment of Hepatocellular Carcinoma Among Veterans With Cirrhosis in the United States. *Clinical Gastroenterology and Hepatology* **2018,** *16* (1), 106-+.
30. Heimbach, J. K.; Kulik, L. M.; Finn, R. S.; Sirlin, C. B.; Abecassis, M. M.; Roberts, L. R.; Zhu, A. X.; Murad, M. H.; Marrero, J. A., AASLD guidelines for the treatment of hepatocellular carcinoma. *Hepatology* **2018,** *67* (1), 358-380.
31. Kim, H.; Chae, K. J.; Yoon, S. H.; Kim, M.; Keam, B.; Kim, T. M.; Kim, D. W.; Goo, J. M.; Park, C. M., Repeat biopsy of patients with acquired resistance to EGFR TKIs: implications of biopsy-related factors on T790M mutation detection. *European Radiology* **2018,** *28* (2), 861-868.
32. Dhir, M.; Melin, A. A.; Douaiher, J.; Lin, C.; Zhen, W.; Hussain, S. M.; Geschwind, J. F. H.; Doyle, M. B. M.; Abou-Alfa, G. K.; Are, C., A Review and Update of Treatment Options and Controversies in the Management of Hepatocellular Carcinoma. *Annals of Surgery* **2016,** *263* (6), 1112-1125.
33. Bruix, J., Treatment of hepatocellular carcinoma. *Hepatology* **1997,** *25* (2), 259-262.

34. Zhang, J.; Zong, Y.; Xu, G. Z.; Xing, K., Erlotinib for advanced hepatocellular carcinoma A systematic review of phase II/III clinical trials. *Saudi Medical Journal* **2016,** *37* (11), 1184-1190.
35. Lin, G. L.; Xiao, H.; Zeng, Z. C.; Xu, Z. Y.; He, J.; Sun, T. W.; Liu, J.; Guo, G. W.; Ji, W. X.; Hu, Y., Constraints for symptomatic radiation pneumonitis of helical tomotherapy hypofractionated simultaneous multitarget radiotherapy for pulmonary metastasis from hepatocellular carcinoma. *Radiotherapy and Oncology* **2017,** *123* (2), 246-250.
36. Deng, G. L.; Zeng, S.; Shen, H., Chemotherapy and target therapy for hepatocellular carcinoma: New advances and challenges. *World J Hepatol* **2015,** *7* (5), 787-98.
37. Yeo, W.; Mok, T. S.; Zee, B.; Leung, T. W.; Lai, P. B.; Lau, W. Y.; Koh, J.; Mo, F. K.; Yu, S. C.; Chan, A. T.; Hui, P.; Ma, B.; Lam, K. C.; Ho, W. M.; Wong, H. T.; Tang, A.; Johnson, P. J., A randomized phase III study of doxorubicin versus cisplatin/interferon alpha-2b/doxorubicin/fluorouracil (PIAF) combination chemotherapy for unresectable hepatocellular carcinoma. *J Natl Cancer Inst* **2005,** *97* (20), 1532-8.
38. Cicchini, C.; Battistelli, C.; Tripodi, M., SETDB1 is a new promising target in HCC therapy. *Chin Clin Oncol* **2016,** *5* (6), 73.
39. Llovet, J. M.; Bruix, J., Molecular targeted therapies in hepatocellular carcinoma. *Hepatology* **2008,** *48* (4), 1312-27.
40. Chong, C. R.; Janne, P. A., The quest to overcome resistance to EGFR-targeted therapies in cancer. *Nat Med* **2013,** *19* (11), 1389-400.
41. Zhang, B.; Deng, C.; Wang, L.; Zhou, F.; Zhang, S.; Kang, W.; Zhan, P.; Chen, J.; Shen, S.; Guo, H.; Zhang, M.; Wang, Y.; Zhang, F.; Zhang, W.; Xiao, J.; Kong, B.; Friess, H.; Zhuge, Y.; Yan, H.; Zou, X., Upregulation of UBE2Q1 via gene copy number gain in hepatocellular carcinoma promotes cancer progression through beta-catenin-EGFR-PI3K-Akt-mTOR signaling pathway. *Mol Carcinog* **2018,** *57* (2), 201-215.
42. Calvisi, D. F.; Ladu, S.; Gorden, A.; Farina, M.; Conner, E. A.; Lee, J. S.; Factor, V. M.; Thorgeirsson, S. S., Ubiquitous activation of Ras and Jak/Stat pathways in human HCC. *Gastroenterology* **2006,** *130* (4), 1117-28.

43. Furuse, J., Growth factors as therapeutic targets in HCC. *Critical Reviews in Oncology Hematology* **2008,** *67* (1), 8-15.
44. Kudo, M., Signaling Pathway and Molecular-Targeted Therapy for Hepatocellular Carcinoma. *Digestive Diseases* **2011,** *29* (3), 289-302.
45. Thomas, M. B.; Chadha, R.; Glover, K.; Wang, X. M.; Morris, J.; Brown, T.; Rashid, A.; Dancey, J.; Abbruzzese, J. L., Phase 2 study of erlotinib in patients with unresectable hepatocellular carcinoma. *Cancer* **2007,** *110* (5), 1059-1067.
46. Dong, L. H.; Li, H.; Wang, F.; Li, F. Q.; Zhou, H. Y.; Yang, H. J., [Expression of liver-type fatty acid-binding protein and vascular endothelial growth factor and their correlation in human hepatocellular carcinoma]. *Nan Fang Yi Ke Da Xue Xue Bao* **2007,** *27* (3), 318-21.
47. Jain, R. K., Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* **2005,** *307* (5706), 58-62.
48. Gerber, H. P.; Ferrara, N., Pharmacology and pharmacodynamics of bevacizumab as monotherapy or in combination with cytotoxic therapy in preclinical studies. *Cancer Res* **2005,** *65* (3), 671-80.
49. Lee, D. W.; Jang, M. J.; Lee, K. H.; Cho, E. J.; Lee, J. H.; Yu, S. J.; Kim, Y. J.; Yoon, J. H.; Kim, T. Y.; Han, S. W.; Oh, D. Y.; Im, S. A.; Kim, T. Y., TTP as a surrogate endpoint in advanced hepatocellular carcinoma treated with molecular targeted therapy: meta-analysis of randomised controlled trials. *British Journal of Cancer* **2016,** *115* (10), 1201-1205.
50. Marks, E. I.; Yee, N. S., Molecular Genetics and Targeted Therapy in Hepatocellular Carcinoma. *Curr Cancer Drug Targets* **2016,** *16* (1), 53-70.
51. Thomas, M., Molecular targeted therapy for hepatocellular carcinoma. *J Gastroenterol* **2009,** *44 Suppl 19*, 136-41.
52. Kudo, M., Signaling pathway/molecular targets and new targeted agents under development in hepatocellular carcinoma. *World J Gastroenterol* **2012,** *18* (42), 6005-17.
53. Toyoda, M.; Hashimoto, N.; Tokita, K.; Goldstein, B. J.; Yokosuka, O.; Kanatsuka, A.; Suzuki, Y.; Saito, Y., Increased activity and expression of MAP kinase in HCC model

rats induced by 3'-methyl-4-dimethylamino-azobenzene. *J Hepatol* **1999,** *31* (4), 725-33.

54. Carloni, V.; Vizzutti, F.; Pantaleo, P., Farnesyltransferase inhibitor, ABT-100, is a potent liver cancer chemopreventive agent. *Clin Cancer Res* **2005,** *11* (11), 4266-74.
55. Hwang, Y. H.; Choi, J. Y.; Kim, S.; Chung, E. S.; Kim, T.; Koh, S. S.; Lee, B.; Bae, S. H.; Kim, J.; Park, Y. M., Over-expression of c-raf-1 proto-oncogene in liver cirrhosis and hepatocellular carcinoma. *Hepatol Res* **2004,** *29* (2), 113-121.
56. Chaparro, M.; Gonzalez Moreno, L.; Trapero-Marugan, M.; Medina, J.; Moreno-Otero, R., Review article: pharmacological therapy for hepatocellular carcinoma with sorafenib and other oral agents. *Aliment Pharmacol Ther* **2008,** *28* (11-12), 1269-77.
57. Huynh, H.; Nguyen, T. T.; Chow, K. H.; Tan, P. H.; Soo, K. C.; Tran, E., Over-expression of the mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK in hepatocellular carcinoma: its role in tumor progression and apoptosis. *BMC Gastroenterol* **2003,** *3*, 19.
58. Sabatini, D. M., mTOR and cancer: insights into a complex relationship. *Nat Rev Cancer* **2006,** *6* (9), 729-34.
59. Schmitz, K. J.; Wohlschlaeger, J.; Lang, H.; Sotiropoulos, G. C.; Malago, M.; Steveling, K.; Reis, H.; Cicinnati, V. R.; Schmid, K. W.; Baba, H. A., Activation of the ERK and AKT signalling pathway predicts poor prognosis in hepatocellular carcinoma and ERK activation in cancer tissue is associated with hepatitis C virus infection. *J Hepatol* **2008,** *48* (1), 83-90.
60. Su, R.; Nan, H.; Guo, H.; Ruan, Z.; Jiang, L.; Song, Y.; Nan, K., Associations of components of PTEN/AKT/mTOR pathway with cancer stem cell markers and prognostic value of these biomarkers in hepatocellular carcinoma. *Hepatol Res* **2016,** *46* (13), 1380-1391.
61. Prasad, V.; Goldstein, J. A., US News and World Report cancer hospital rankings: do they reflect measures of research productivity? *PLoS One* **2014,** *9* (9), e107803.
62. Miller, K. D.; Siegel, R. L.; Lin, C. C.; Mariotto, A. B.; Kramer, J. L.; Rowland, J. H.; Stein, K. D.; Alteri, R.; Jemal, A., Cancer treatment and survivorship statistics, 2016. *CA Cancer J Clin* **2016,** *66* (4), 271-89.

63. Sun, Y.; Luo, X.; Yang, K.; Sun, X.; Li, X.; Zhang, C.; Ma, S.; Liu, Y.; Yin, J., Neural overexpression of multidrug resistance-associated protein 1 and refractory epilepsy: a meta-analysis of nine studies. *Int J Neurosci* **2016,** *126* (4), 308-17.
64. Altieri, F.; Grillo, C.; Maceroni, M.; Chichiarelli, S., DNA damage and repair: from molecular mechanisms to health implications. *Antioxid Redox Signal* **2008,** *10* (5), 891-937.
65. Regev, R.; Katzir, H.; Yeheskely-Hayon, D.; Eytan, G. D., Modulation of P-glycoprotein-mediated multidrug resistance by acceleration of passive drug permeation across the plasma membrane. *FEBS J* **2007,** *274* (23), 6204-14.
66. Yang, M.; Li, H.; Li, Y.; Ruan, Y.; Quan, C., Identification of genes and pathways associated with MDR in MCF-7/MDR breast cancer cells by RNA-seq analysis. *Mol Med Rep* **2018**.
67. Baguley, B. C., Multiple drug resistance mechanisms in cancer. *Mol Biotechnol* **2010,** *46* (3), 308-16.
68. Shen, D.; Pastan, I.; Gottesman, M. M., Cross-resistance to methotrexate and metals in human cisplatin-resistant cell lines results from a pleiotropic defect in accumulation of these compounds associated with reduced plasma membrane binding proteins. *Cancer Res* **1998,** *58* (2), 268-75.
69. Shen, D. W.; Goldenberg, S.; Pastan, I.; Gottesman, M. M., Decreased accumulation of [14C] carboplatin in human cisplatin-resistant cells results from reduced energy-dependent uptake. *J Cell Physiol* **2000,** *183* (1), 108-16.
70. Jain, R. K., Delivery of molecular and cellular medicine to solid tumors. *Adv Drug Deliv Rev* **2012,** *64* (Suppl), 353-365.
71. Teodori, E.; Dei, S.; Martelli, C.; Scapecchi, S.; Gualtieri, F., The functions and structure of ABC transporters: implications for the design of new inhibitors of Pgp and MRP1 to control multidrug resistance (MDR). *Curr Drug Targets* **2006,** *7* (7), 893-909.
72. Guo, Q.; Sui, Z. G.; Xu, W.; Quan, X. H.; Sun, J. L.; Li, X.; Ji, H. Y.; Jing, F. B., Ubenimex suppresses Pim-3 kinase expression by targeting CD13 to reverse MDR in HCC cells. *Oncotarget* **2017,** *8* (42), 72652-72665.

73. Litman, T.; Druley, T. E.; Stein, W. D.; Bates, S. E., From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. *Cell Mol Life Sci* **2001,** *58* (7), 931-59.
74. Holohan, C.; Van Schaeybroeck, S.; Longley, D. B.; Johnston, P. G., Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer* **2013,** *13* (10), 714-26.
75. Correction to: "A Small Molecule with Anticancer and Antimetastatic Activities Induces Rapid Mitochondrial-Associated Necrosis in Breast Cancer. *J Pharmacol Exp Ther* **2017,** *360* (1), 225.
76. Ambudkar, S. V.; Dey, S.; Hrycyna, C. A.; Ramachandra, M.; Pastan, I.; Gottesman, M. M., Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu Rev Pharmacol Toxicol* **1999,** *39*, 361-98.
77. Keppler, D., Multidrug resistance proteins (MRPs, ABCCs): importance for pathophysiology and drug therapy. *Handb Exp Pharmacol* **2011,** (201), 299-323.
78. Dean, M.; Rzhetsky, A.; Allikmets, R., The human ATP-binding cassette (ABC) transporter superfamily. *Genome Res* **2001,** *11* (7), 1156-66.
79. Sun, Y. L.; Patel, A.; Kumar, P.; Chen, Z. S., Role of ABC transporters in cancer chemotherapy. *Chinese Journal of Cancer* **2012,** *31* (2), 51-7.
80. Dean, M.; Allikmets, R., Complete characterization of the human ABC gene family. *J Bioenerg Biomembr* **2001,** *33* (6), 475-9.
81. Sodani, K.; Patel, A.; Kathawala, R. J.; Chen, Z.-S., Multidrug resistance associated proteins in multidrug resistance. *Chinese Journal of Cancer* **2012,** *31* (2), 58.
82. Cole, S. P.; Bhardwaj, G.; Gerlach, J. H.; Mackie, J. E.; Grant, C. E.; Almquist, K. C.; Stewart, A. J.; Kurz, E. U.; Duncan, A. M.; Deeley, R. G., Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* **1992,** *258* (5088), 1650-4.
83. Newman, D. J.; Cragg, G. M., Natural Products as Sources of New Drugs from 1981 to 2014. *Journal of Natural Products* **2016,** *79* (3), 629-661.
84. Ahmed, M. S.; Halaweish, F. T., Cucurbitacins: potential candidates targeting mitogen-activated protein kinase pathway for treatment of melanoma. *Journal of Enzyme Inhibition and Medicinal Chemistry* **2014,** *29* (2), 162-167.

85. Abbas, S.; Vincourt, J. B.; Habib, L.; Netter, P.; Greige-Gerges, H.; Magdalou, J., The cucurbitacins E, D and I: Investigation of their cytotoxicity toward human chondrosarcoma SW 1353 cell line and their biotransformation in man liver. *Toxicology Letters* **2013,** *216* (2-3), 189-199.
86. Altmann, K. H.; Gertsch, J., Anticancer drugs from nature-natural products as a unique source of new microtubule-stabilizing agents (vol 24, pg 327, 2007). *Natural Product Reports* **2012,** *29* (12), 1481-1481.
87. Cai, Y.; Fang, X. F.; He, C. W.; Li, P.; Xiao, F.; Wang, Y. T.; Chen, M. W., Cucurbitacins: A Systematic Review of the Phytochemistry and Anticancer Activity. *American Journal of Chinese Medicine* **2015,** *43* (7), 1331-1350.
88. Jayaprakasam, B.; Seeram, N. P.; Nair, M. G., Anticancer and antiinflammatory activities of cucurbitacins from Cucurbita andreana. *Cancer Letters* **2003,** *189* (1), 11-16.
89. Fuller, R. W.; Cardellina, J. H.; Cragg, G. M.; Boyd, M. R., Cucurbitacins - Differential Cytotoxicity, Dereplication and First Isolation from Gonystylus-Keithii. *Journal of Natural Products* **1994,** *57* (10), 1442-1445.
90. Alghasham, A. A., Cucurbitacins - a promising target for cancer therapy. *Int J Health Sci (Qassim)* **2013,** *7* (1), 77-89.
91. Huang, Y.; De Bruyne, T.; Apers, S.; Ma, Y. L.; Claeys, M.; Vanden Berghe, D.; Pieters, L.; Vlietinck, A., Complement-inhibiting cucurbitacin glycosides from Picria fel-terrae. *Journal of Natural Products* **1998,** *61* (6), 757-761.
92. Zhang, M.; Bian, Z. G.; Zhang, Y.; Wang, J. H.; Kan, L.; Wang, X.; Niu, H. Y.; He, P., Cucurbitacin B inhibits proliferation and induces apoptosis via STAT3 pathway inhibition in A549 lung cancer cells. *Molecular Medicine Reports* **2014,** *10* (6), 2905-2911.
93. Jacquot, C.; Rousseau, B.; Carbonnelle, D.; Chinou, I.; Malleter, M.; Tomasoni, C.; Roussakis, C., Cucurbitacin-D-induced CDK1 mRNA Up-regulation Causes Proliferation Arrest of a Non-small Cell Lung Carcinoma Cell Line (NSCLC-N6). *Anticancer Research* **2014,** *34* (9), 4797-4806.

94. Bernard, S. A.; Olayinka, O. A., Search for a novel antioxidant, anti-inflammatory/analgesic or anti-proliferative drug: Cucurbitacins hold the ace. *Journal of Medicinal Plants Research* **2010,** *4* (25), 2821-2826.
95. Valente, L. M. M.; Gunatilaka, A. A. L.; Glass, T. E.; Kingston, D. G. I.; Pinto, A. C., New Norcucurbitacin and Heptanorcucurbitacin Glucosides from Fevillea-Trilobata. *Journal of Natural Products* **1993,** *56* (10), 1772-1778.
96. Matsuda, H.; Nakashima, S.; Abdel-Halim, O. B.; Morikawa, T.; Yoshikawa, M., Cucurbitane-Type Triterpenes with Anti-proliferative Effects on U937 Cells from an Egyptian Natural Medicine, Bryonia cretica: Structures of New Triterpene Glycosides, Bryoniaosides A and B. *Chemical & Pharmaceutical Bulletin* **2010,** *58* (5), 747-751.
97. Bodnar, B.; Mernyak, E.; Wolfling, J.; Schneider, G.; Herman, B. E.; Szecsi, M.; Sinka, I.; Zupko, I.; Kupihar, Z.; Kovacs, L., Synthesis and Biological Evaluation of Triazolyl 13alpha-Estrone-Nucleoside Bioconjugates. *Molecules* **2016,** *21* (9).
98. Duncan, K. L. K.; Duncan, M. D.; ALley, M. C.; Sausville, E. A., Cucurbitacin E-induced disruption of the actin and vimentin cytoskeleton in prostate carcinoma cells. *Biochemical Pharmacology* **1996,** *52* (10), 1553-1560.
99. Sorensen, P. M.; Iacob, R. E.; Fritzsche, M.; Engen, J. R.; Brieher, W. M.; Charras, G.; Eggert, U. S., The natural product cucurbitacin E inhibits depolymerization of actin filaments. *ACS Chem Biol* **2012,** *7* (9), 1502-8.
100. Chan, K. T.; Li, K.; Liu, S. L.; Chu, K. H.; Toh, M.; Xie, W. D., Cucurbitacin B inhibits STAT3 and the Raf/MEK/ERK pathway in leukemia cell line K562. *Cancer Letters* **2010,** *289* (1), 46-52.
101. Ahmed, M. S.; Halaweish, F. T., Cucurbitacins: potential candidates targeting mitogen-activated protein kinase pathway for treatment of melanoma. *J Enzyme Inhib Med Chem* **2014,** *29* (2), 162-7.
102. Bollee, G.; Flamant, M.; Schordan, S.; Fligny, C.; Rumpel, E.; Milon, M.; Schordan, E.; Sabaa, N.; Vandermeersch, S.; Galaup, A.; Rodenas, A.; Casal, I.; Sunnarborg, S. W.; Salant, D. J.; Kopp, J. B.; Threadgill, D. W.; Quaggin, S. E.; Dussaule, J. C.; Germain, S.; Mesnard, L.; Endlich, K.; Boucheix, C.; Belenfant, X.; Callard, P.; Endlich, N.; Tharaux, P. L., Epidermal growth factor receptor promotes

glomerular injury and renal failure in rapidly progressive crescentic glomerulonephritis (vol 17, pg 1242, 2011). *Nature Medicine* **2011,** *17* (11), 1521-1521.

103. Holbro, T.; Hynes, N. E., ErbB receptors: Directing key signaling networks throughout life. *Annual Review of Pharmacology and Toxicology* **2004,** *44*, 195-217.

104. Management of hepatocellular carcinoma: from prevention to molecular targeted therapy. Proceedings of the 3rd International Kobe Liver Symposium on HCC with an International Liver Cancer Association (ILCA) Scientific Session. Hyogo, Japan, June 6-7, 2009. *Oncology* **2010,** *78 Suppl 1*, 1-190.

105. Silva, I. T.; Carvalho, A.; Lang, K. L.; Dudek, S. E.; Masemann, D.; Duran, F. J.; Caro, M. S.; Rapp, U. R.; Wixler, V.; Schenkel, E. P.; Simoes, C. M.; Ludwig, S., In vitro and in vivo antitumor activity of a novel semisynthetic derivative of cucurbitacin B. *PLoS One* **2015,** *10* (2), e0117794.

106. Puri, R.; Sud, R.; Khaliq, A.; Kumar, M.; Jain, S., Gastrointestinal toxicity due to bitter bottle gourd (Lagenaria siceraria)-a report of 15 cases. *Indian J Gastroenterol* **2011,** *30* (5), 233-6.

107. Mertins, S. D.; Myers, T. G.; Hollingshead, M.; Dykes, D.; Bodde, E.; Tsai, P.; Jefferis, C. A.; Gupta, R.; Linehan, W. M.; Alley, M.; Bates, S. E., Screening for and identification of novel agents directed at renal cell carcinoma. *Clin Cancer Res* **2001,** *7* (3), 620-33.

108. Wang, X.; Tanaka, M.; Peixoto, H. S.; Wink, M., Cucurbitacins: elucidation of their interactions with the cytoskeleton. *PeerJ* **2017,** *5*, e3357.

109. Ahmed, M. S.; Kopel, L. C.; Halaweish, F. T., Structural optimization and biological screening of a steroidal scaffold possessing cucurbitacin-like functionalities as B-Raf inhibitors. *ChemMedChem* **2014,** *9* (7), 1361-7.

110. Matsuda, H.; Nakashima, S.; Abdel-Halim, O. B.; Morikawa, T.; Yoshikawa, M., Cucurbitane-type triterpenes with anti-proliferative effects on U937 cells from an egyptian natural medicine, Bryonia cretica: structures of new triterpene glycosides, bryoniaosides A and B. *Chem Pharm Bull (Tokyo)* **2010,** *58* (5), 747-51.

111. Bartalis, J.; Halaweish, F. T., In vitro and QSAR studies of cucurbitacins on HepG2 and HSC-T6 liver cell lines. *Bioorganic & Medicinal Chemistry* **2011,** *19* (8), 2757-2766.

112. Leese, M. P.; Hejaz, H. A.; Mahon, M. F.; Newman, S. P.; Purohit, A.; Reed, M. J.; Potter, B. V., A-ring-substituted estrogen-3-O-sulfamates: potent multitargeted anticancer agents. *J Med Chem* **2005,** *48* (16), 5243-56.

Chapter Two

Study the cytotoxicity of natural products or semi-synthesized natural products against hepatocellular carcinoma cell line

2.1 Introduction

Many natural products, biological compounds, total synthesis or vaccines are used as the main sources of small organic molecules which know as a drug drugs[1]. Since the ancient times, natural products play an important role as resource of medicines. For instance, some herbs used to be chewing to decrease the pain and some of them used to be wrapped around wounds to heal it. Using natural products to treat diseases and injuries known as folk medicine[2]. Recently, the large improve in developing materials to study the biological mechanism of all new chemical entities, encouraged researchers to investigate more in the pharmacological effects of natural compounds to clarify their synergistic impact and their clinical effects on the body. Natural compounds could be providing novel medicinal approaches toward a variety of diseases, cancer one of them[3].

Hepatocellular carcinoma (HCC) is one of the highest cause of mortality and the third causing of death worldwide[4, 5]. It has been reported that Asia and Africa have the highest report of HCC with high incidence among men. HCC was somewhat rare in the United States compare to the other countries; however, recently it became one of the main cause of death in the United States[6]. HCC is a continuous and slowly progressing disease that is generally associated with other factors such as cirrhosis, hepatitis C virus (HCV), and hepatitis B virus (HBV) and toxin/ environmental disorders (obesity, diabetes and alcoholic consumption)[6, 7]. Many therapeutic options are available now for HCC such as local ablation therapy, surgical resection and liver transplantation[8]. However, these options are not viable for late diagnosed patients. Chemotherapeutic drugs such as Erlotinib and Sorafenib are common treatments for HCC[9]. In addition, drugs resistance and undesirable side effects are the most challenge problems associated with these chemotherapy drugs[10]. Therefore, there is an urgent need to find a new drug candidate to overcome these problems.

HCC molecular pathogenesis is classified into two main complex mechanisms; 1)

mutation, which happen in some tumor suppress genes or oncogenes; 2) some diseases or metabolic disorder such as hepatitis infection, metabolic effects (such as obesity, insulin resistance, type-2 diabetes), toxin (such as alcohol) that cause tissue damage which lead to cirrhosis[11, 12]. Both of these mechanisms have been related with irregularity in different cell signaling pathways that continue the process of carcinogenic results. From a therapeutic view, all of these signaling pathways are very significant to treat HCC. Consequently, growth factors-mediated, angiogenic signaling, epidermal growth factor (EGFR), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), and the mitogen activated protein kinase (MAPK) pathways are the most distinguished targets for treating HCC due to their noticeable overexpression during the disease **Figure 2.1**[13,14].

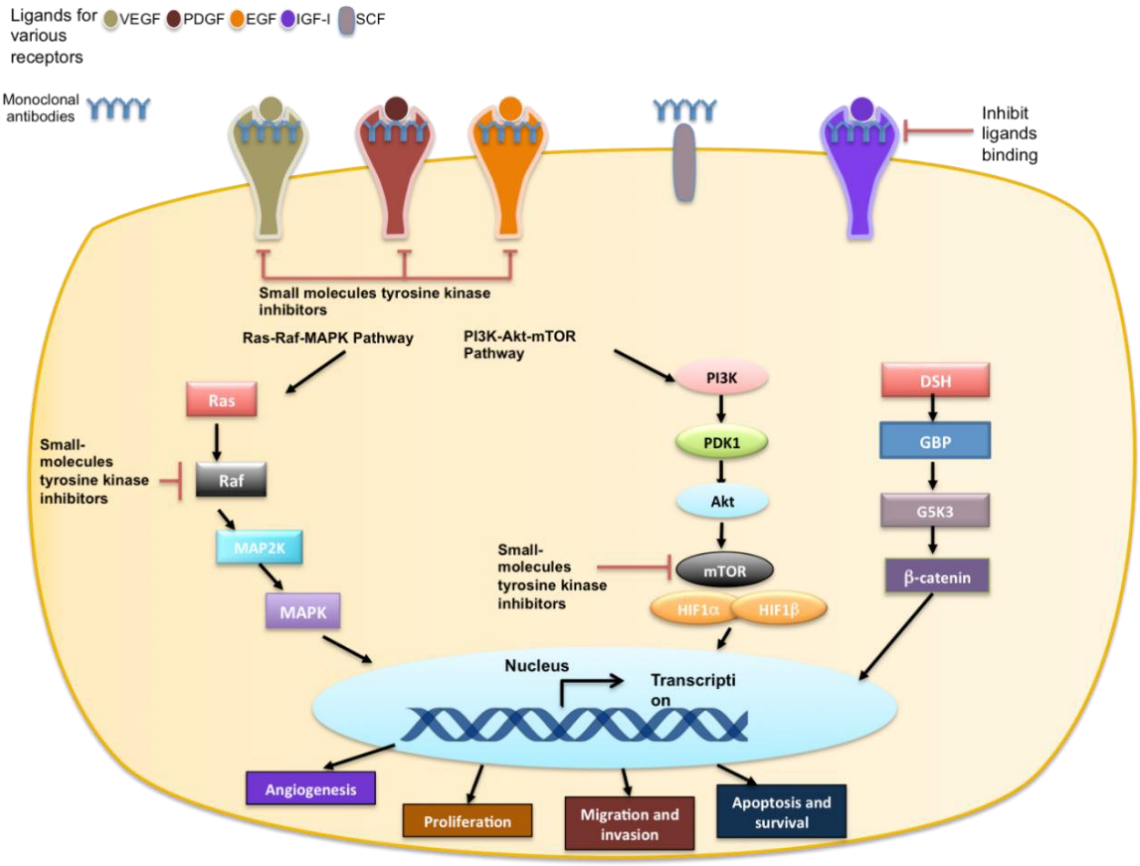

Figure 2.1 Signaling pathway/molecular targets and new targeted agents under development in hepatocellular carcinoma.

Epidermal growth factor receptor (EGFR) tyrosine kinase (TK) **figure 3.2** is one of the tyrosine kinases that has been confirmed in many studies as a promising target for the treatment of different carcinoma including HCC[15]. EGFR, which is also known as ErbB1, is a member of family of growth factor receptors including ErbB2, ErbB3 and ErbB4. Paracrine or juxtracrine extracellular ligand binding such as epidermal growth factor (EGF) and transforming growth factor (TGF)-α stimulate the EGFR, which lead to hetro- or homo dimerization and conformational change that activate the tyrosine kinase and allow autophosphorylation[16, 17] .The phosphorylation is the key role for this signaling pathway for cancer treatment. When the phosphorylation occurs, number of signaling pathways activated leading to cancer cell invasions, proliferation, metastasis, inhibitory of apoptosis and angiogenesis (**figure 2.2)**[18, 19]. Therefore, inhibition of EGFR-TK signaling cascades provides an approach for the treatment of hepatocellular carcinoma (HCC).

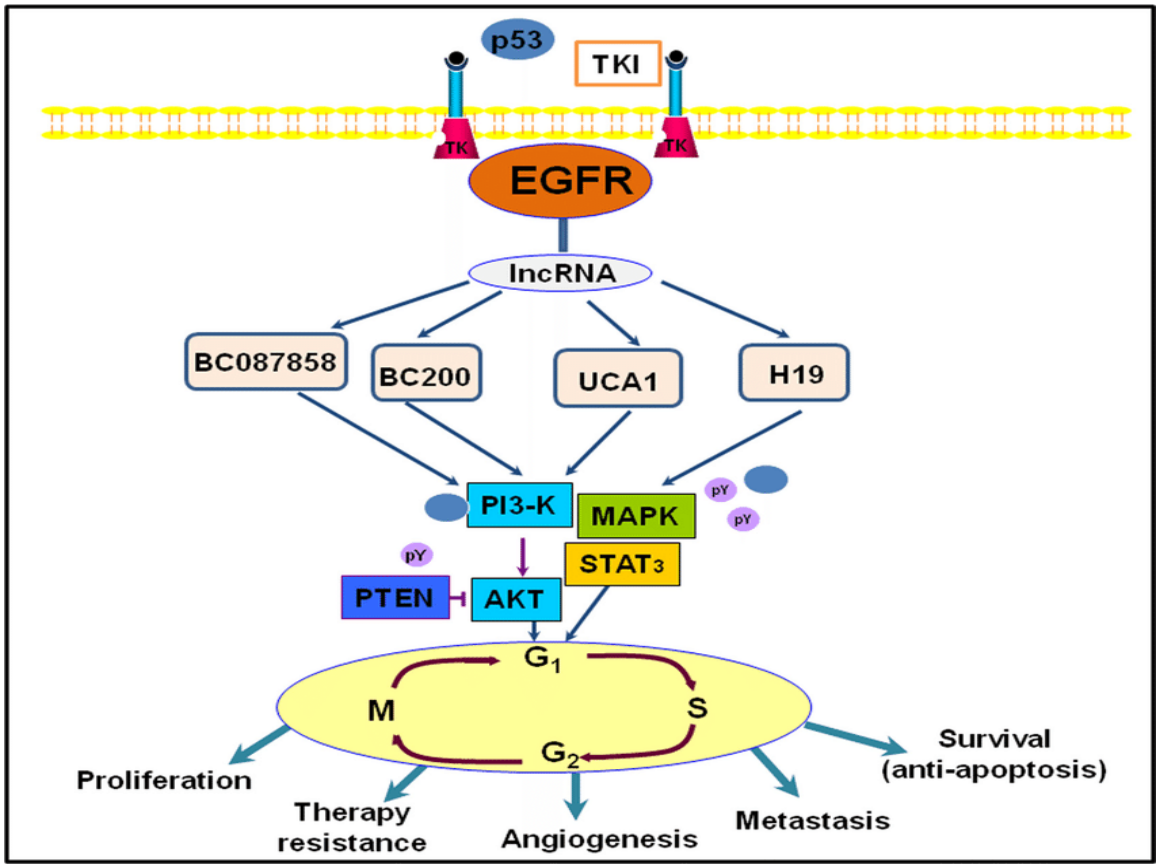

Figure 2.2 EGFR signaling pathway in HCC.

Cucurbitacins (CUCS) **(Figure 2.3)** are natural products extracted from plant of Cucurbitacea family such as *Gonystylus keithii*, *Cayaponia tayu*, and *Citrillus colcynte*. Cucurbitacins plant family first used in folk medicine due its biological significant and activity as anti-inflammatory agents. There are many types of cucurbitacins classified as following; A, B, C, D, and E, to T. In addition, hundreds of cucurbitacins derivatives have been isolated and identified some[20]. They have been used for treatment of different diseases such as chronic hepatitis, liver cirrhosis, jaundice, dyspepsia, inflammation and cancer[21]. Recently, many studies have shown significant activities of CUCS as potential candidates for treatment of hepatocellular carcinoma (HCC)[22]. Current knowledge of molecular targets and singling pathways of different types of cancers provide a clear understanding of tumor cell regulation, which in turn paved the way to synthesis promising potential drug candidates.

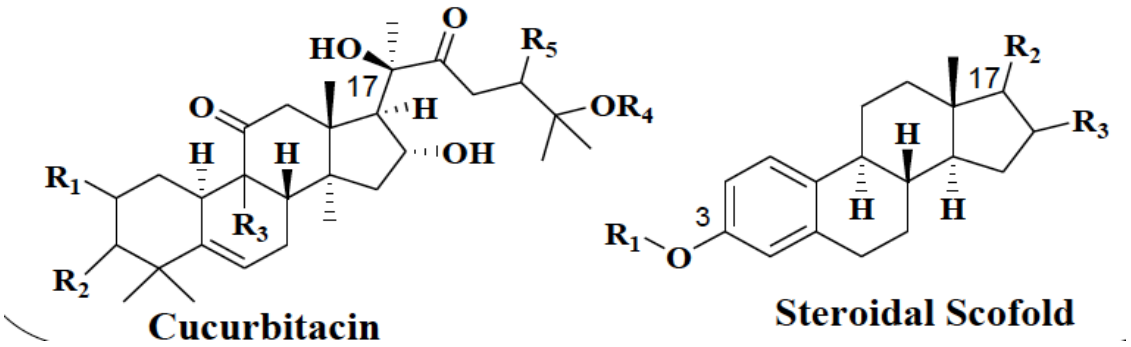

Figure 2.3 Structures of Cucurbitacins and Starting Material Estrone.

CUCS demonstrated a wide range of biological activities due to their cytotoxicity on cancer cells and their potency on different biological pathways. These biological activities nominate CUCS as a potential drug that targeting multiple types of cancer[23]. Cucurbitacin D, isolated from Cucurbita Texan, and 3-epi-isocucurbitacin D prevent client maturation without induction of the HSR. Cucurbitacin D also disrupted interactions between Hsp90 and two cochaperones, Cdc37 and p23[24].

To avoid the undesirable adverse effects of the natural products and increase their selectivity, many structural modifications to their structure causes the improvement in their efficiency[25]. Therefore, the identification of the most significant pharmacophore of the

natural products is essential to conduct structural modifications.

What make the CUCS interested in our group is that CUCS has tetracyclic moiety, they are very similar to that of steroids **(Fig. 2.3)**. However, they in fact that C-10 methyl is located at C-9, possess a gem-dimethyl group at C-4 and the configuration of ring B and ring C[26] . Due to this the similarity between the core structures of CUCS and steroids, the concept of hybrid drug design were used to install the essential pharmacophore of the CUCS into the steroid structure as promising alternative for the complicated functionalized structure of the CUCS[27, 28] . Specifically, using the estrone skeleton as a starting material to install different functional moieties including the CUCS side chain and other functionalities has been done by Kopel et al [27]. Furthermore, adding various moieties to the estrone skeleton structure at C-3, such as methoxy and hydroxyl groups, beside the CUCS side chain at C-17 in the presence of double bond at C16-C17 demonstrate an increase in the biological activity of these series of compounds toward several targets. The presence of the double bond at C16-C17 changed the conformation of estrone CUCS-like compounds which improves its binding affinity towards the EGFR-TK[29].

In our group novel CUCUS-inspired estrone analogs with aliphatic side chain such as MMA102, MMA132 were synthesized by installing the CUCS side chain at C-16 and C17 of estrone scaffold. Those compounds demonstrated a very promising binding affinity by making a hydrophobic interaction as in MMA102, which has the opposite stereochemistry of cucurbitacin D side chain with amino acids residues of the crystal structure of EGFR binding pocket; while analogue MMA132, which possess the exact stereochemistry of the side chain of cucurbitacin D, demonstrated an outstanding binding affinity through hydrophobic interaction with amino acids residues of the EGFR along H-bond with MET: 796:A, which is the same amino acid as that of Erlotinib make H-bond with and responsible for its anti-cancer activity **(Figure 2.4).**

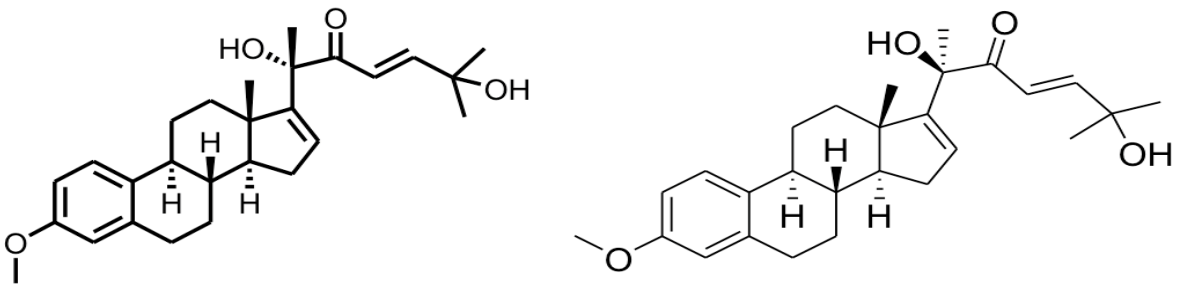

Figure 2.4 Chemical structures of MMA132 and MMA102.

Chemotherapeutic agents, such as erlotinib and lapatinib, have been shown as potent inhibitor against EGFR by inhibiting its phosphorylation; these are known chemotherapeutic agents for the treatment of HCC and other types of cancer. Erlotinib (Tarceva) is a very active and selective inhibitor of the EGRF-TK protein. It has an advantage over most of the anti-cancer agents in that it can be taken orally, inhibits cellular proliferation and causes cell cycle arrest at the G_1 phase. In addition, it has been approved as an active drug for pancreatic and lung cancer by the FDA although is still in Phase II clinical trials as an anti-cancer drug for HCC[30].

2.2 Induction of apoptosis by Erlotinib

Apoptosis plays important role in normal tissue development and maintaining the hemostasis[31]. Therefore any mutation or any defect in this pathways leads to different diseases such as degenerative and autoimmune diseases[32]. Carl Vogt 1842[33] discovered it and in 1965 Lockshin and Williams introduced the term programmed cell death[34]. Only after a decade, the term apoptosis was introduced by Kerr et al[35]. , Two pathways, namely intrinsic and extrinsic pathway regulate and induce apoptosis **(Figure 2.9)**[36]. Caspases regulates both pathways, which are synthesized as inactive enzymes and converted to active enzyme by cleavage. The active caspases cleaves different target proteins that are necessary in DNA repair and cytoskeleton assembly ultimately causing cell death[37]. The caspases are classified into two categories, initiators and executioners. Binding of the ligand such as Fas ligand (FasL) and tumor necrosis factor (TNF) to the death receptor (DR)[38] causes the induction of extrinsic pathway. DRs include TNF-R1, Fas-Apo1, DR3, TNF-related apoptosis-inducing ligand receptor-1 (TRAIL-1), TRAIL-2 and DR6. The

death receptors comprise of extracellular cysteine rich domain (CRD) and an intracellular death domain (DD)[39]. Upon ligand binding, the receptor undergoes trimerization leading to the recruitment of initiator caspases 8 and 10 and Fas-associated death domain (FADD) to the receptor to form the death inducing signal complex (DISC) to amplify the apoptotic signal. In addition, the initiator caspases undergo autocatalytic activation which activate the effector caspases 3, 6 and/or 7[32]. Erlotinib induces the apoptosis through the caspase-3 pathway. Erlotinib was confirmed that induced caspase-3 activity increased dose-dependently to 300%[40]. In the cytoplasm which forms apoptosome complex by binding to Apaf-1 and procaspase 9. This apopotosome complex activate caspase 9 by auto cleavage. The cleaved caspase 9 cleaves procaspase 3 which is the executioner caspase leading to the proteolytic cleavage of different cellular proteins inducing apoptosis[41].

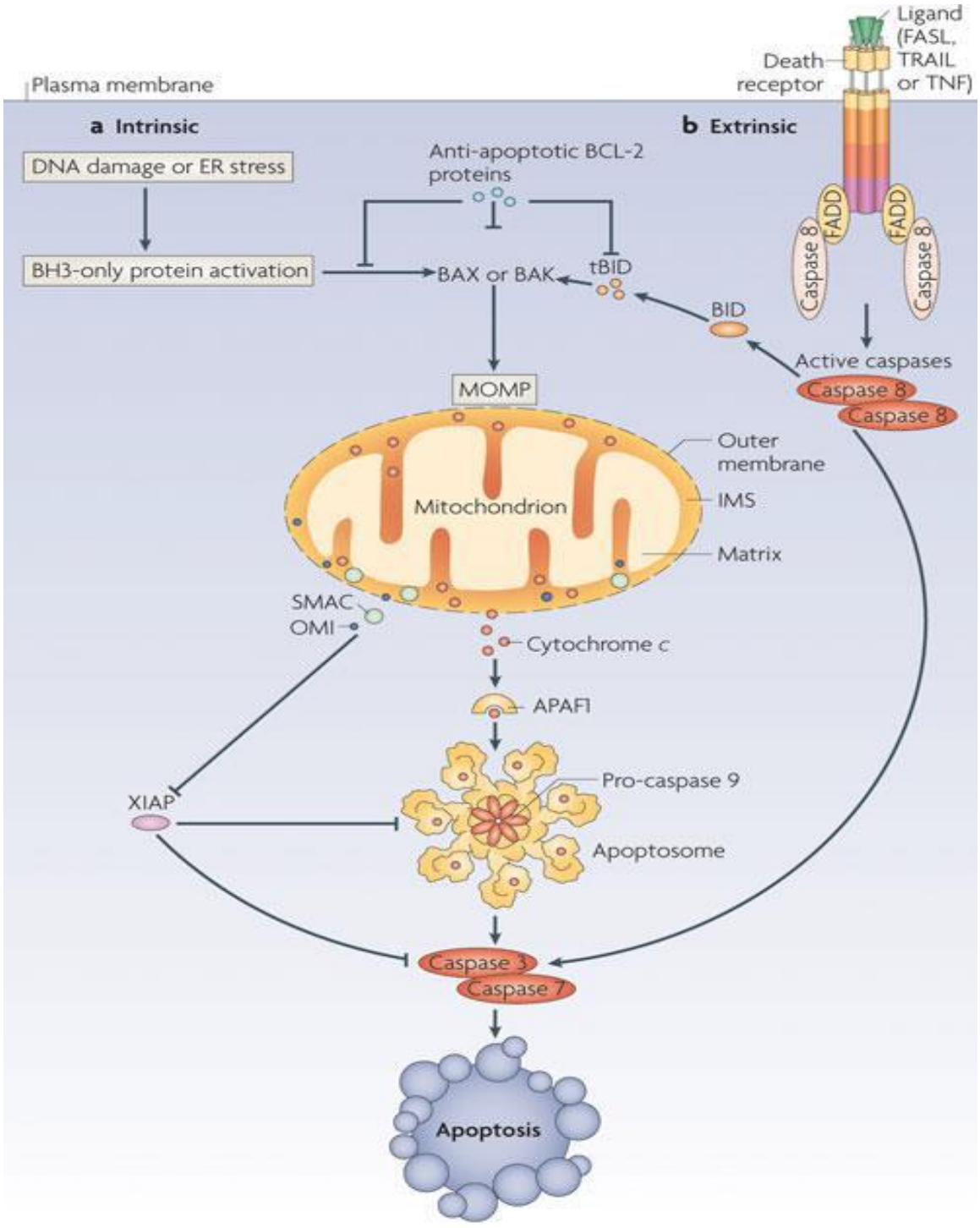

Figure 2.5 Intrinsic and extrinsic apoptotic pathway.

Aim of the Study: Therefore, understanding the antiproliferative activity of Erlotinib paved the way for studying the antiproliferative mechanism pf potential drug candidate. The aim of this study to investigate the potential anti-proliferative activity of CIEA as potential drug candidate for treatment of HCC.

2.3 Erlotinib and cell cycle regulation

Cell cycle is divided into 4 sequential stages **(Figure 2.5)**[42]. Cells need longer time to prepare for DNA replication in S phase rather than doubling their content from protein and organelles. The cell prepares itself for replication by monitoring the environment and building their proteins in the two gaps G_1 and G_2., Depending on the external conditions and the signals coming from other cells the length of the G1 phase varies from days, weeks or even years. Cells can enter a resting phase called G0 if the external environment is not favorable. The cells can enter the S phase once the condition is favorable again. G_2 phase plays important role by checking the quality of replicated DNA, and synthesizing the proteins required for commitment of mitosis (M phase)[43, 44].

DNA damage result in activation of the cell cycle checkpoints by cyclin-dependent kinases leading to the cell cycle arrest at G_1, S and G_2/M phase[45] . The antiproliferative potency of Erlotinib in hepatocellular carcinoma cells contributed to the induction of cell cycle arrest at G_1/G_0 phase of the cell cycle , that way decreasing the proportion of cells in S-phase[40].

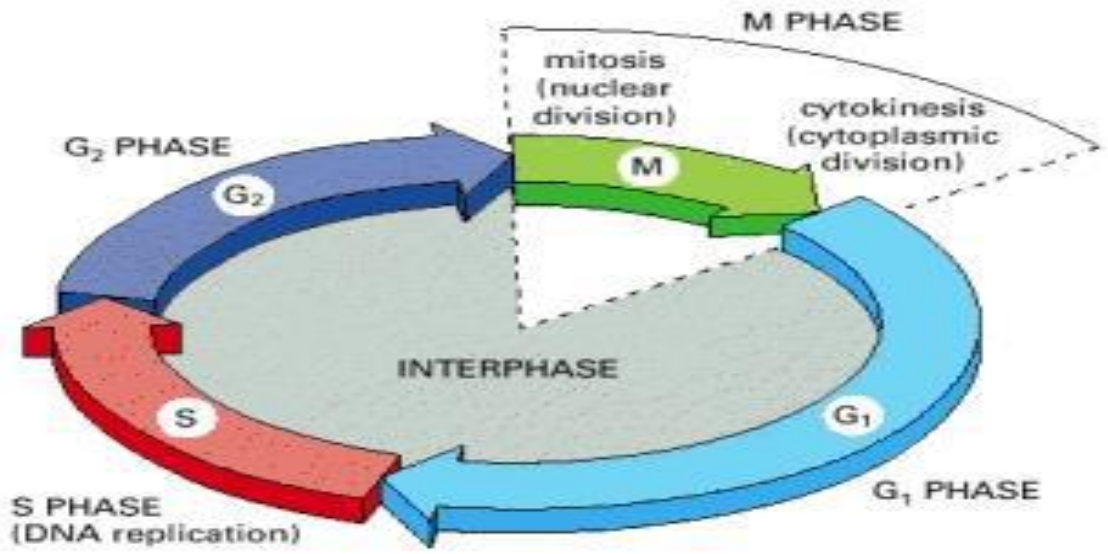

Figure 2.6 The cell cycle is divided into four phases.

2.4 ERK MAP kinase in G1 cell cycle progression and cancer

One of the key process that convey signals from the cell surface to the nucleus is the Ras/extracellular-signal-regulated kinase (ERK) mitogen activated protein (MAP) kinase signaling pathway. In the Ras/ERK signaling pathway a variety of extracellular stimuli induce sequential activation of mainly three protein kinases; Raf, MEK, and ERK **(Figure 2.6)**[46]**.** ERK is activated by MEK via phosphorylation on both threonine and tyrosine residues in the TEY sequence. Activated ERK, in turn phosphorylates both cytoplasmic and nuclear substrates, including many enzymes, cytoskeletal proteins and transcription factors. Recently, many studies have identified several scaffold proteins and inhibitors proteins that have potential role in Ras/ERK signaling pathway. These proteins can modulate the duration, magnitude, and subcellular location of ERK activity which provide variations in ERK signaling[47, 48]. There is multiple research which suggests that the differences in ERK activity generate variations in signaling outputs that will cause regulation of the cell fate decisions. In addition, interaction with other pathways could also be crucial for determining signaling specificity. The role of Ras/ERK signaling pathway in cell cycle progression in G_1 phase and cell proliferation is well established[49].

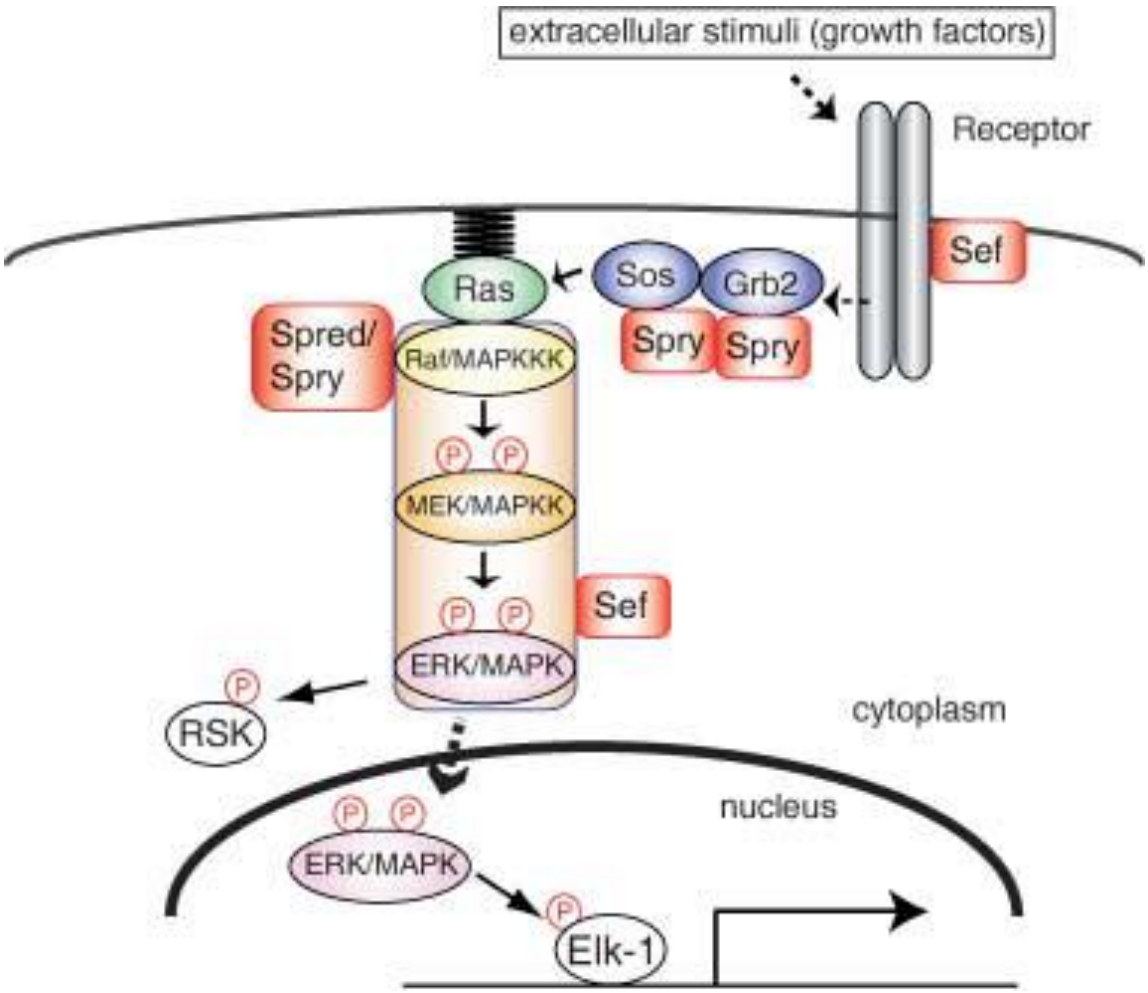

Figure 2.7 The Ras/extracellular-signal-regulated kinase (ERK) signaling pathway.

In Cell cycle progression from G_0/G_1 to S phase requires activation of the Ras/ERK signaling pathway which is related to cyclin D induction and consequent retinoblastoma (Rb) phosphorylation[50],[51]. ERK is phosphorylated and activated by many external stimuli like growth factors which then translocate from the cytoplasm later to the nucleus, where ERK phosphorylates and activates several nuclear ERK targets, including transcription factors such as Elk-1. Therefore, ERK is responsible for the expression of the immediate early genes, such as c-fos[52]. The expression of the immediate early genes in turn regulates subsequent induction of the delayed early genes, including a first class of G1 cyclins, cyclin D. Upregulation of cyclin D expression results in upregulation of the cyclin D–CDK4/6 complex. Activation of cyclin D–CDK4/6 kinase activity leads to phosphorylation and inactivation of Rb, which then activates the E2F family of transcription factors and induces

expression of target genes, including a second class of G1 cyclins, cyclin E[53]. As a result of activation cyclin E–CDK2 kinase, it will lead to further phosphorylation and inactivation of Rb, thus further increase the activity of the E2F family. This positive feedback leads to the synthesis of proteins required for S phase entry **(Figure 2.7)**[54],[55]**.** Therefore, in response to growth factor stimulation, ERK triggers these sequential events, including sequential induction of a number of genes, and thereby causes S phase entry.

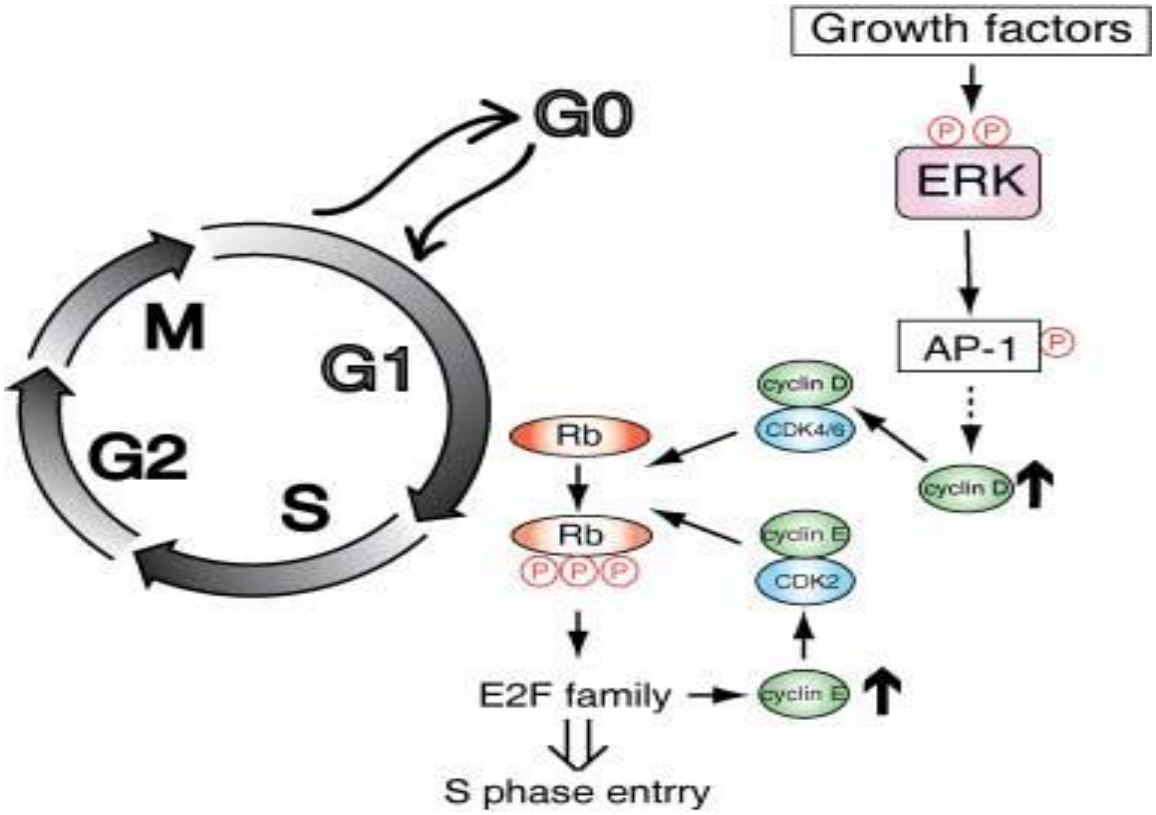

Figure 2.8 Regulation of G1 cell cycle progression through ERK signaling.

Recently it has been suggested that for inducing S phase entry of quiescent fibroblastic cells sustained ERK activation, but not transient activation[56, 57]. It is necessary to sustain the ERK activity for approximately 2 or 3 h before the onset of S phase[58].Thus, through ERK activity is a key factor for keeping G1 phase progression. ERK activation can induce cyclin D expression several hours after growth factor stimulation[59]. More recently, Yamamoto et al. from a Genome-wide analyses of transcriptional programs in cell cycle progression from G_0/G_1 to S phase have shown that in addition to ERK-dependent upregulated genes, there are also ERK-dependent downregulated genes[60]. However, the

expression level of most of these ERK-dependent downregulated genes is maintained at a lower level throughout G_1 phase, and the decreased expression levels return to the original levels rapidly if ERK inactivation occurs. It is to be noted that these ERK-dependent downregulated genes are known antiproliferative genes until the onset of S phase to allow successful G1 phase progression **(Figure 2.8)**[46]. In addition to the mitogenic signals, the cells also receive varied stimuli such as environmental stresses that induce transient ERK activation. Transient ERK activation unable to induce sustained downregulation of antiproliferative genes, therefore these inappropriate stimuli do not cause cell proliferation. Thus, this mechanism ensures prevention of inappropriate stimuli from causing cell cycle progression[46].

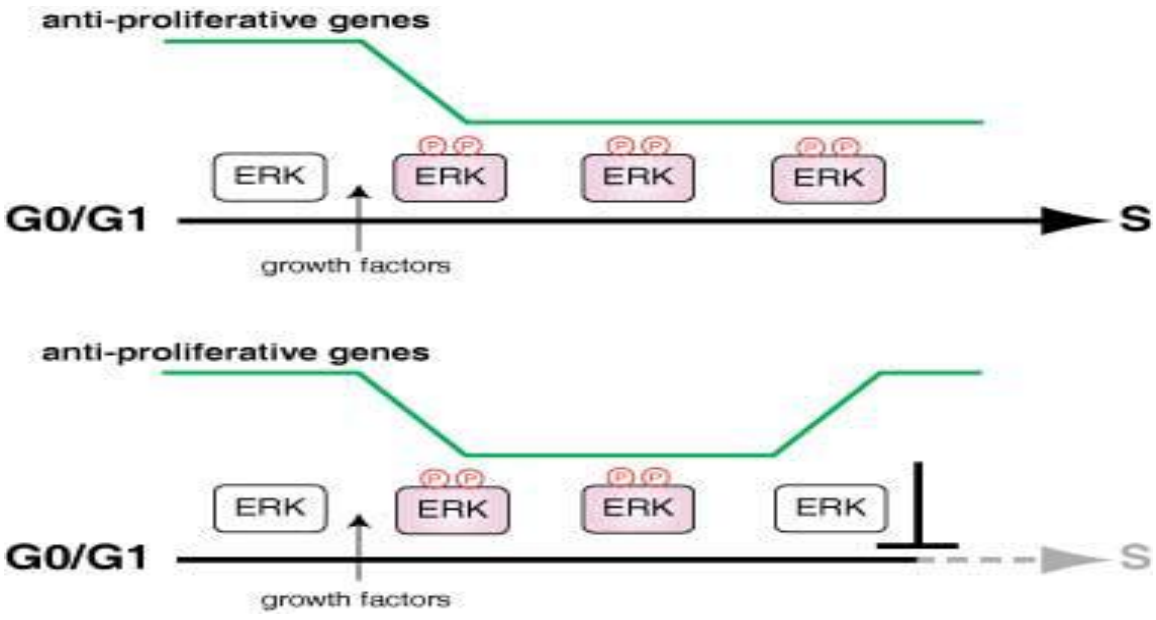

Figure 2.9 Role of ERK-dependent downregulation of antiproliferative genes in G1 phase progression.

2.5 Materials and Methods

2.5.1 Hepatocellular carcinoma cell lines and the drugs

The human hepatoma cells line, HepG2 were received from ATCC. The cells were maintained in EMEM (Eagle's Minimum Essential Medium) from (ATCC) with L-Glutamine supplemented with 10% fetal bovine serum (FBS) (Atlanta biologicals) and 1% penicillin (100 IU/mL)/streptomycin (100 µg/mL) (Corning) at 37°C, 5% CO2. The

passage number range for cells line maintained between 4-13. The cells were cultured in75 cm2 cell culture flask[61].

2.5.2 Cytotoxicity assay

The sensitive hepatoma cells (HepG2) were seeded in 96-well plate as 5*104 cells/mL (100 µL/well). A serial dilution of CIEA compounds were added after overnight incubation of the cells at 37°C and 5% CO2. DMSO (Acros Organics) was used as a control (0.1 %). The cells were incubated with the compounds for 48 hrs. After that 15 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Aldrich) (5 mg/mL PBS) were added to each well and the plate was incubated for another 4 hrs. The formazan crystals were solubilized by 100 µL acidified SDS solution (10% SDS/0.01 N HCl) (Fisher BioReagents). The absorbance was measured after 14 hrs of incubation at 37°C and 5% CO2 at 570 nm by Hidex Sense Microplate readers[62]

2.5.3 Cell cycle analysis

The study of cellular DNA content and cell cycle distribution are useful to detect variations of growth patterns due to a variety of physical, chemical, or biological means, to detect apoptosis, and to study tumor behavior and suppressor gene mechanisms[63]. In a given population, cells are distributed among three major phases of cell cycle: G0/G1 phase (one set of paired chromosomes per cell), S phase (DNA synthesis with variable amount of DNA), and G2/M phase (two sets of paired chromosomes per cell, prior to cell division). DNA content can be measured by using fluorescent technique , DNA-selective stains that exhibit emission signals proportional to DNA mass[64]. Flow cytometric analysis of these stained populations is then used to produce a frequency histogram that reveals the various cell cycle phases. This analysis is typically performed on permeabilized or fixed cells using a cell-impermeant nucleic acid stain. But it is also possible using live cells and a cell-permeant nucleic acid stain. While the choices for fixed cell staining are varied, there are only a few examples of useful cell-permeant nucleic acid stains. The Vybrant® DyeCycle™ Green and Orange stains are DNA-selective, cell membrane permeant, and nonfluorescent stains for DNA content analysis in living cells. The Vybrant® DyeCycle™ Green and Orange stains (green is the one that was used in this study) are fluorescent upon

binding to double-stranded DNA. These stains take advantage of the commonly available 488 nm excitation source, placing cell cycle studies on live cells within reach of all flow cytomatrices. Vybrant® DyeCycle™ Green stain is excited at 488 nm with emission ~520 nm[65].

Method A:

The cells were seeded as 2.5*105 cells/mL in a 6-well plate (2 mL/well) and allowed to adhere overnight at 37°C and 5% CO2. The cells were either incubated with different concentrations of MMA132 (1, 2, or 4 μM) and MMA102 (1.5, 3,and 6 μM)for 24 hrs .The cells were washed twice with ice-cold 1X PBS (Hyclone™ L aboratories , Inc)and collected after trypsinzatio[66]. The cell pellet was washed two times with ice-cold 1X PBS and fixed with ice-cold 70% ethanol overnight at -20°C. After that, the cells were washed once with ice-cold PBS and the second wash was done with ice-cold PBS-2% FBS. The cell pellet was re-suspended in 500 μL propidium iodide (PI)/RNase staining solution(BD Biosciences) for 15 min at room temperature (RT) in the dark and analyzed within 1 hr by (BD Accuri C6; Becton-Dickinson, Mountain View,CA)[67].

Method B:

According manufacturer protocol[6817617667676767174], Cells were seeded into six-well plates at a concentration of 300,000 cells/ well and allowed to attach in culture overnight, then treated with IC50 values of compounds or positive control (Erlotinib) for 48 h. Afterwards, cells were washed with PBS and harvested. Cell cycle analysis was investigated in accordance with the manufacturers protocol with slight modification. Briefly, Vybrant® DyeCycle™ Green Stain (Thermo Fisher Scientific) was added to 1 ml of cell suspension at a final concentration of 0.0625 μM. After 45 minutes of incubation at 37˚C, the samples were analyzed by flow cytometry and compared to DMSO-treated cells. All these experiments were performed on BD Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA, USA) using BD Accuri™ C6 software, version 1.0.

2.5.4 Caspases 3/7 detection

Effect of MMA132 treatment on the expression level of caspase 3 and 9. Cells were treated with 2 μM of MMA132 at different time points (1hr, 24hr, 48hr). β actin (ThermoFisher Scientific) was used as a positive control. The apoptotic effect of MMA132 was confirmed by measuring the level of activated caspase 3 and 9 (Santa Cruz Biotechnology) and PARP-1[69].

2.5.5 Cell Migration (Wound Healing Assay)

The cells were seeded into 24-well tissue culture plate at a density that after 24 h of growth, they should reach ~70-80% confluence as a monolayer. By a new 1 ml pipette tip the well was gently and slowly scratched across the center. While scratching across the surface of the well, the long-axial of the tip was always perpendicular to the bottom of the well. The resulting gap distance therefore equals to the outer diameter of the end of the tip. After scratching, gently the wells were washed twice with cold 1X PBS to remove the detached cells. The cells were either incubated with of MMA132 (2μM) and MMA102 (3μM) for different time points (zero time, 1hr, 24hrs, and 48 hrs). The photos for the monolayer and cell movement were taken by a microscope at different time points. The gap distance can be quantitatively evaluated using ImageJ software[70].

2.5.6 Western blot analysis

The HepG2 protein lysate and western blot method was prepared and done according to El-senduny et al[71]. HepG2 cells were treated with 2 μM and 3 μM of MMA132 and MMA102 respectively, and incubated for 1, 2, 4, 8, 24, 48 hrs. Briefly, cells were lysed with 1X RIPA buffer containing protease and phosphatase inhibitors. The BCA protein assay was used to quantify total protein concentration. An amount of 20 μg was loaded onto 10% SDSPAGE (ERK, pERK, MEK, pMEK, RAF, pRAF) and 8% SDSPAGE (EGFR, pEGFR) per well. The protein was transferred onto 0.45micron nitrocellulose membrane and blocked with 5% BSA, and subsequently incubated with ERK, pERK, MEK, pMEK, RAF, pRAF and EGFR, pEGFR primary antibodies overnight at 4°C. The membrane was subjected to the corresponding IR-conjugated secondary antibodies. The membrane was developed by using LiCOR odyssy imager. β-actin was used as a positive control.

2.6 Results

2.6.1 Anti-proliferation and Cytotoxicity Effect of CIEA to HepG2 Cell Line

To determine the anti-proliferation activity of CIEA on the HepG2 cell line, the cells were treated with tested compounds for 48 hrs and viability percentage was determined by MTT assay. Figure 1-10a and b show the cytotoxic effects of the CIEA on HepG2 cell lines. Table 1-3 summarizes the IC50 values of each CIEA as the most active compound against HepG2 cell lines. The anti-proliferation activity of the synthesized CIEA using MTT cell viability assay starting with compounds that have R-configuration showed that six CIEA have potent inhibitory activities on HepG2 cell line such as MMA132, MMA102, MMA290, MMA245, MMA265, and MMA240 compare to the standard Erlotinib with 25 µM. **(Figure 2.10).**

In this study, MMA132 and MMA102 were chosen for further investigation because they are the most cytotoxic CIEA with 12 times activity more than that of Erlotinib **(Figure 2.11).**

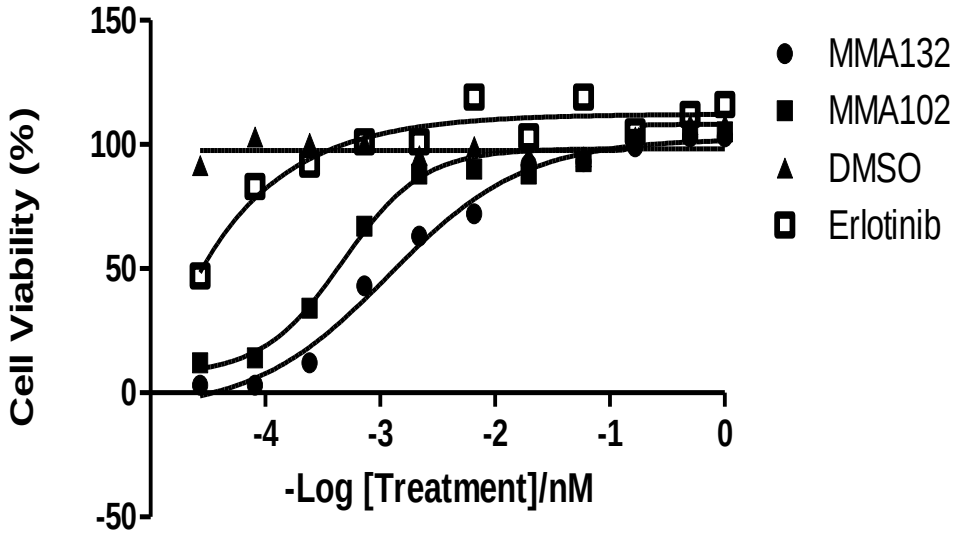

Figure 2.10 IC_{50} curves for MMA132, MMA102 compounds and Erlotinib on HepG2 cell line compared to DMSO control.

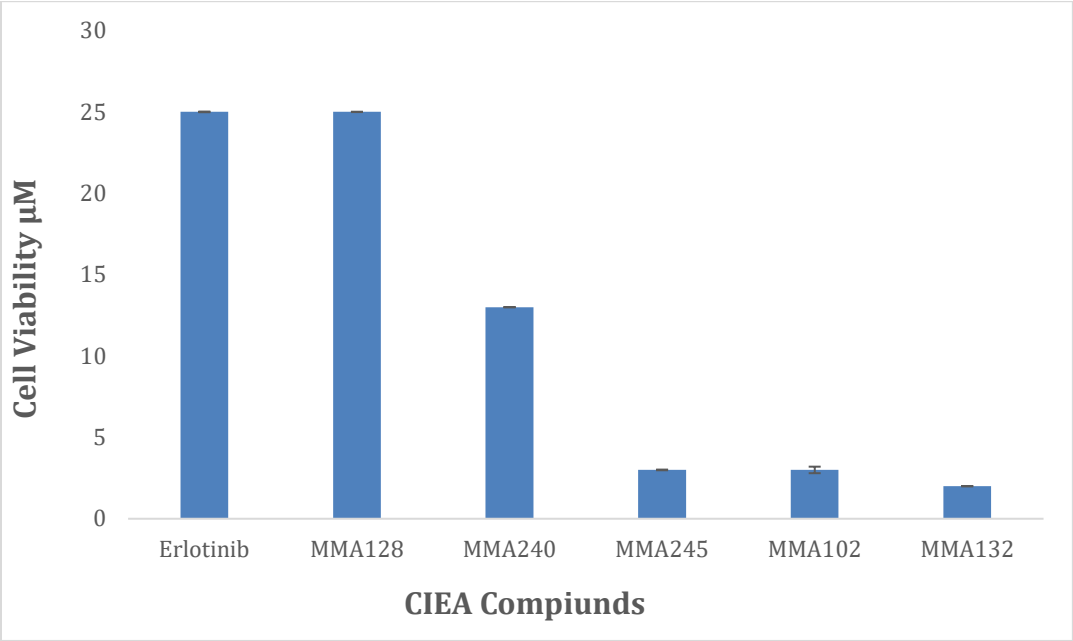

Figure 2.11 Cytotoxicity of CIEA on the HepG2 cell.

Table 2.1 Anti-proliferation results against HepG2 cell line.

CIEA	IC_{50} µM
Erlotinib*	25±0.02
MMA132	2±0.01
MMA128	25±0.2
MMA265	16±0.05
MMA240	13±0.01
MMA305	29±0.4
MMA102	3±0.2
MMA287	25±0.4
MMA290	6±0.01
MMA245	**3**±0.02

* = Reference drug

2.6.2 MMA132 and MMA102 effect on cell cycle distribution

The HepG2 cells were incubated with different concentrations of MMA132 (1 µM, 2 µM and 4 µM) and MMA102 (1.5 µM, 3 µM, and 6 µM) for 24 hrs then the DNA content was quantified in each phase of the cell cycle. The cells treated with MMA132 were arrested permanently at G1 phase, even at 1/2 its IC_{50} value **(Figure 2.12).** Cells treated with different concentration of MMA102 were stopped at G1 phase, after increase the concentration to 6 µM, the cells arrested at G2 phase **(Figure 2.14**). Cycle arrested at G1 phase was confirmed by detection of ELK/pELK level in the cell lysate by western blot. The level of pElk was slightly decreased **(Figure 2.16).** Elk one of ERK signaling pathway transcription factors, which plays a vital role to G1 regulation[53].

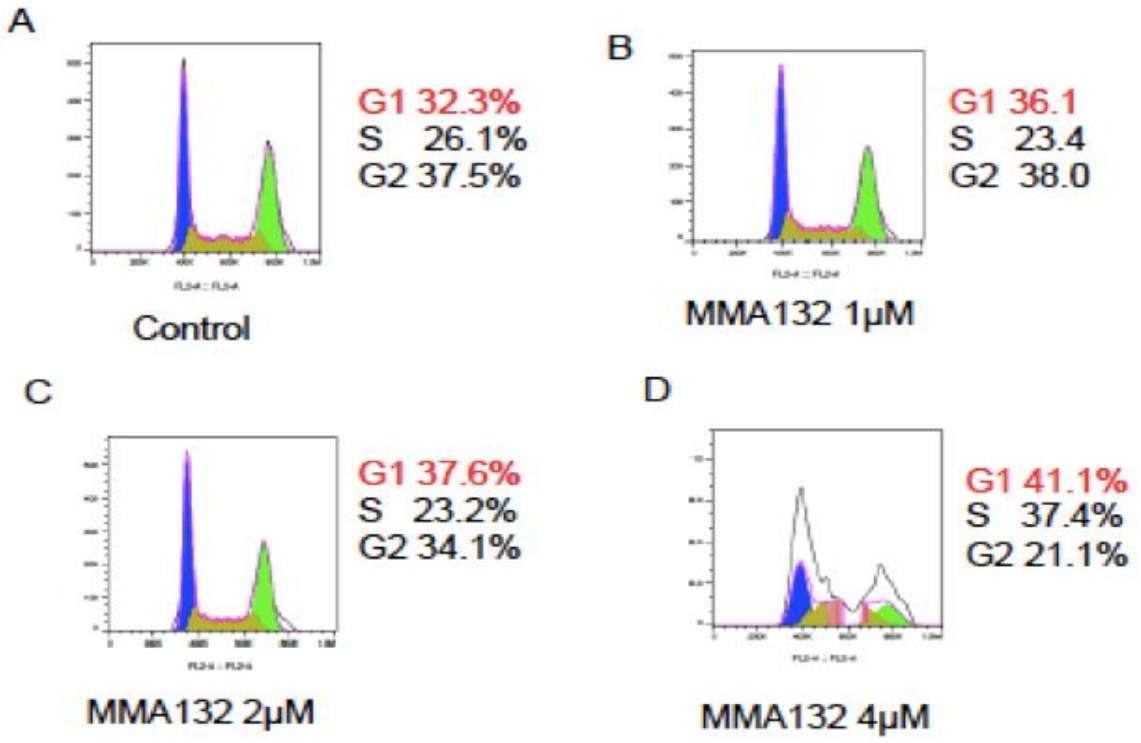

Figure 2.12 Flow cytometric analysis of the cell cycle of HepG2 cancer cells. (A) Control DMSO. (B) 1µM MMA132. (C) 2 µM MMA132. (D) 4µM h and cell cycle analysis was performed as described in Materials and methods. The data are shown the percentages of cells in the G1, G2 and S phase. The histograms were analyzed to determine the percentage of cells in each phase of the cell.

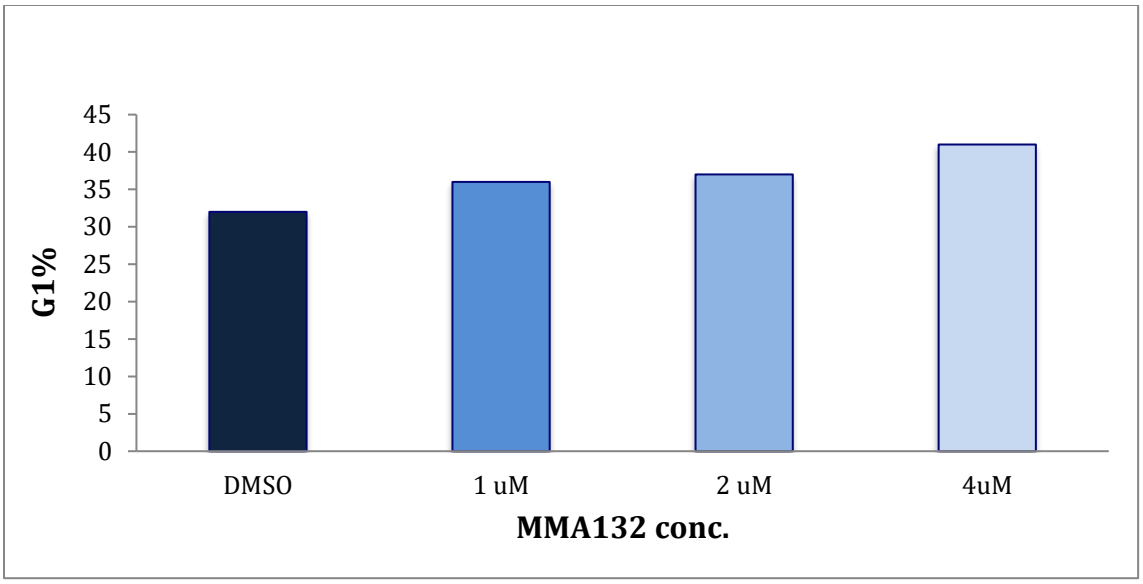

Figure 2.13 Histogram showing the G1 percentage on HepG2 cell line treated with MMA132

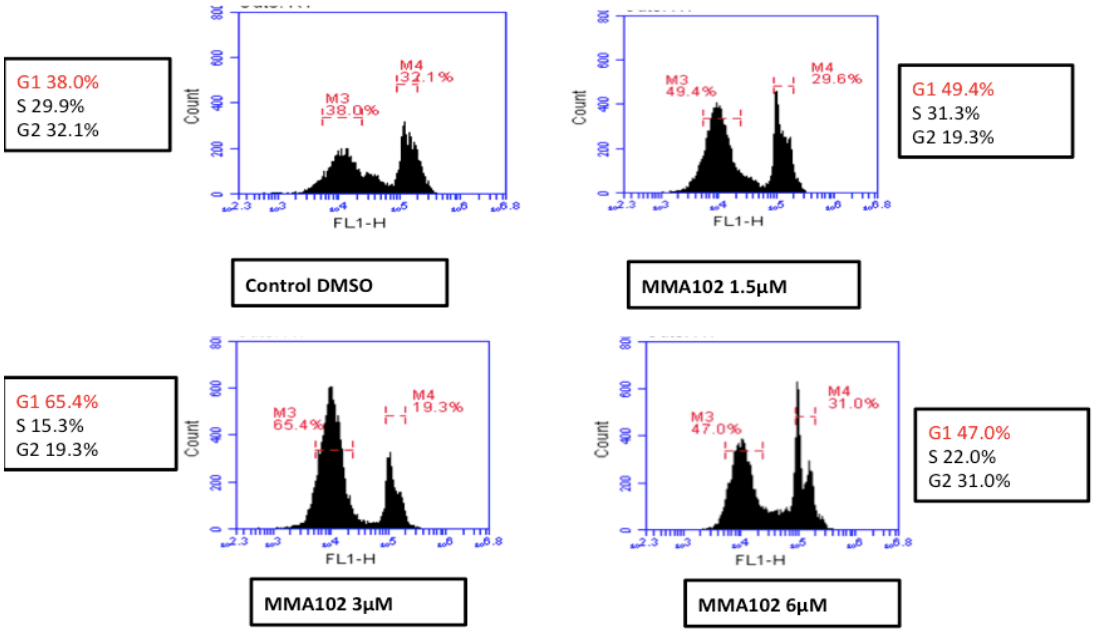

Figure 2.14 Flow cytometric analysis of the cell cycle of HepG2 cancer cells. Cells were treated with different concentration of MMA102 (1.5 µM, 3 µM and 6 µM) respectively compared to DMSO control. The data are shown the percentages of cells in the G_1, G_2 and S phase. The histograms were analyzed to determine the percentage of cells in each phase of the cell cycle.

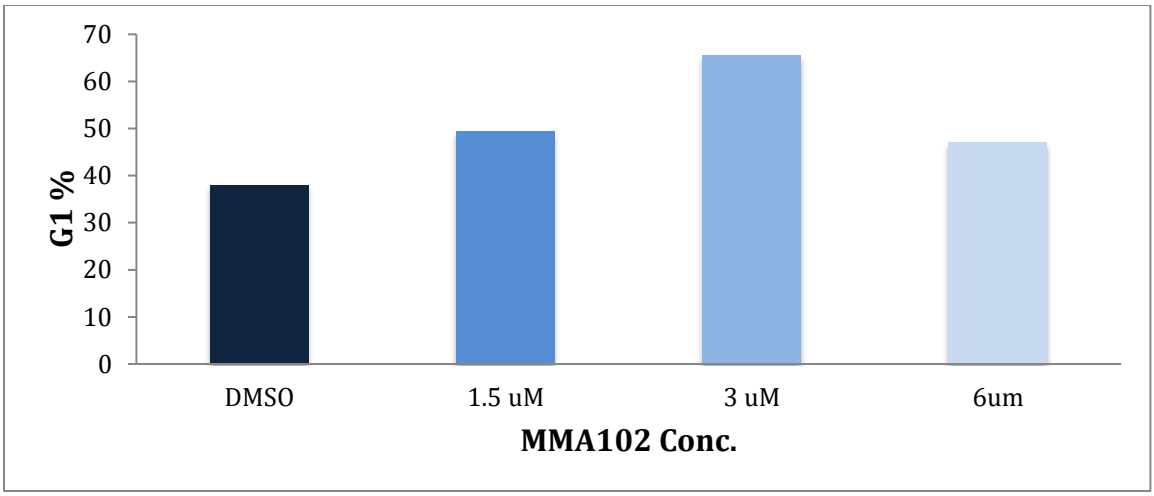

Figure2.15 Histogram showing the G1 percentage on HePG2 cell line treated with MMA102.

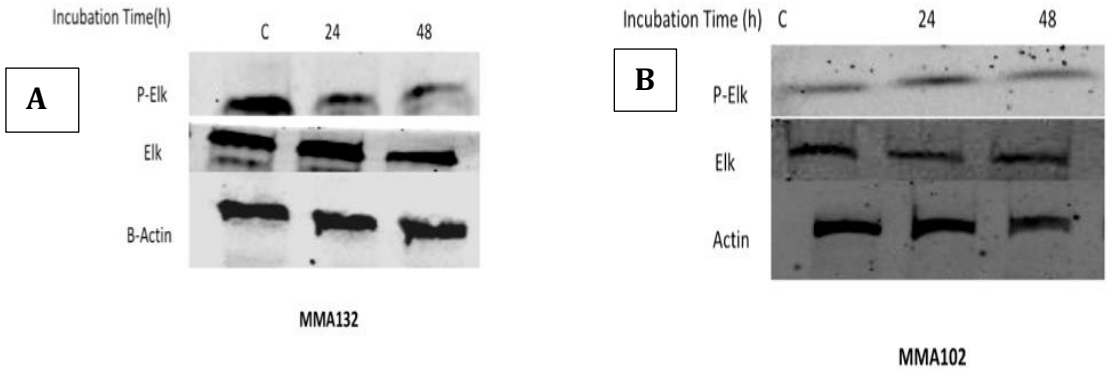

Figure 2.16 Effects of MMA132 and MMA102 on ELK activation. Western blot analysis of the ELK1; (A) ELK phosphorylation inhibited and change the expression after 24 hrs which is related to G1 cell-cycle arrested by MMA132. (B) Effect of MMA102 on ELK/pElk.

2.6.3 Effect of MMA132 and MMA102 on Cell Migration

HepG2 cells were incubated with 2 µm MMA132, 3 µM MMA102, 25 µM Erlotinib, 0.01% DMSO as a control. Cells were incubated with different treatment. Pictures were taken for the monolayer on microscope (LEICA DMI 400 B) at different time points (0, 1 hrs, 24 hrs, 48 hrs). The images showed that HepG2 cell can migrate into the scratched space in ~24 hours **(Figure 2.17).** With 2 µm MMA132, 3 µM MMA102 and 25 µm Erlotinib treatment, the wound was still open after 24 hours indicating that inhibit cell migration or invasion compared to DMSO (control) by MMA132, MMA102 and Erlotinib. Consistent with these results, the 'wound-healing' assays show that parental cells

fill the gap completely by 48 hrs with DMSO and the wound was completely healed. By contrast, gap filling MMA102 and Erlotinib cells was much slower after 48 hrs, in addition MMA132 was inhibited cell migration 100%.

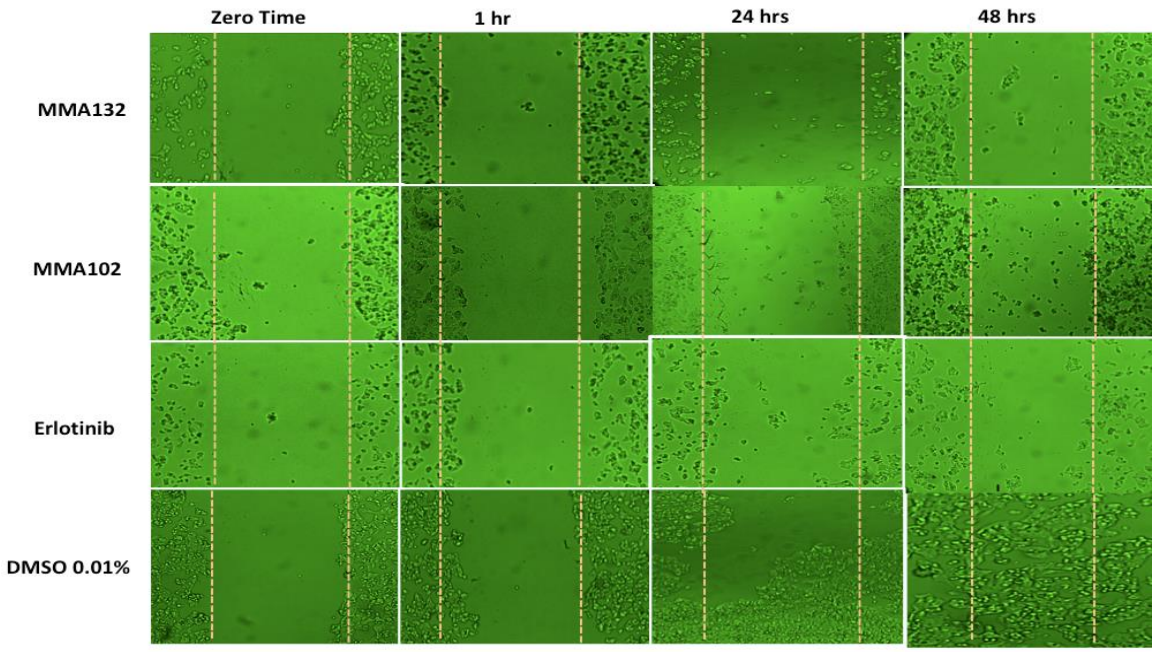

Figure 2.17 MMA132, MMA102 and Erlotinib inhibit cell migration in wound-healing assays and the migration of cells in the open space was observed under a phase-contrast microscope (200) at the indicated times.

2.6.4 Study the effect of MMA132 on apoptosis

Effect of MMA132 treatment on the expression level of caspase 3 and 9 was investigated. HepG2 were treated with 2 µM of MMA132 at different time points (1 hrs, 24 hrs, 48 hrs) and β-actin was used as a positive control. The apoptotic effect of MMA132 was demonstrated by measuring the level of activated caspase 3 and 9. Cells treated with MMA132 induced the caspase 9 level after 24 hrs compared to loading control β-Actin. Also, there is an increase in the caspase 3 level which was confirmed by inhibition of PARP. Moreover, it was a significant increase in the levels of both caspases 3 and 9 compared to cleaved ones and inhibited the PARP level which is considered as an indicator for stopping the DNA damage repair which controls the apoptosis **(Figure 2.18).**

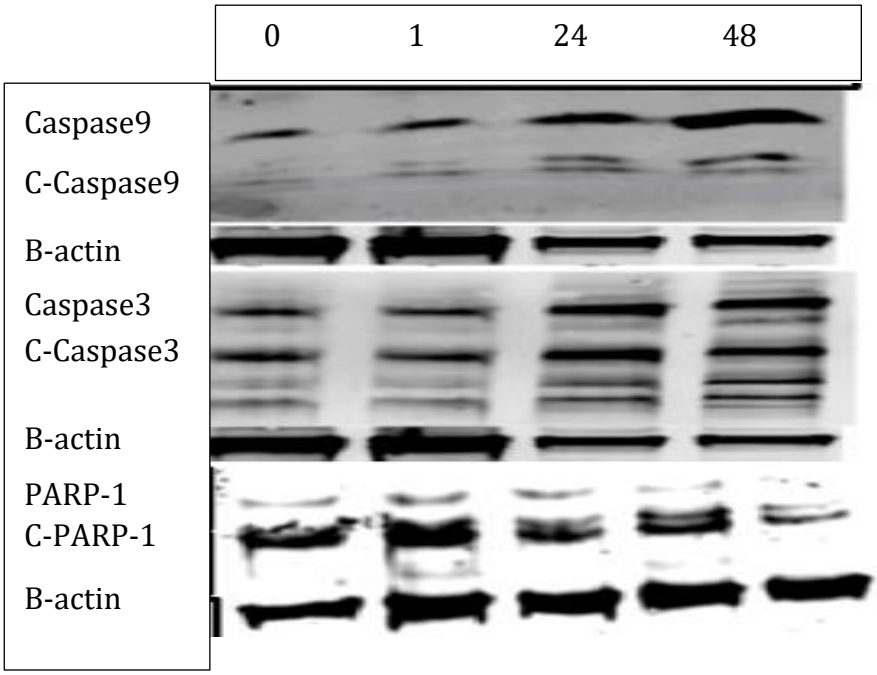

Figure 2.18 Effect of MMA132 treatment on the expression level of pro- and anti-apoptotic proteins. The cells were treated with 2 µM concentrations for 1, 24 and 48 hrs. β-Actin was used as a loading control. PARP; poly ADP ribose polymerase, Caspase 3 and Caspase 9.

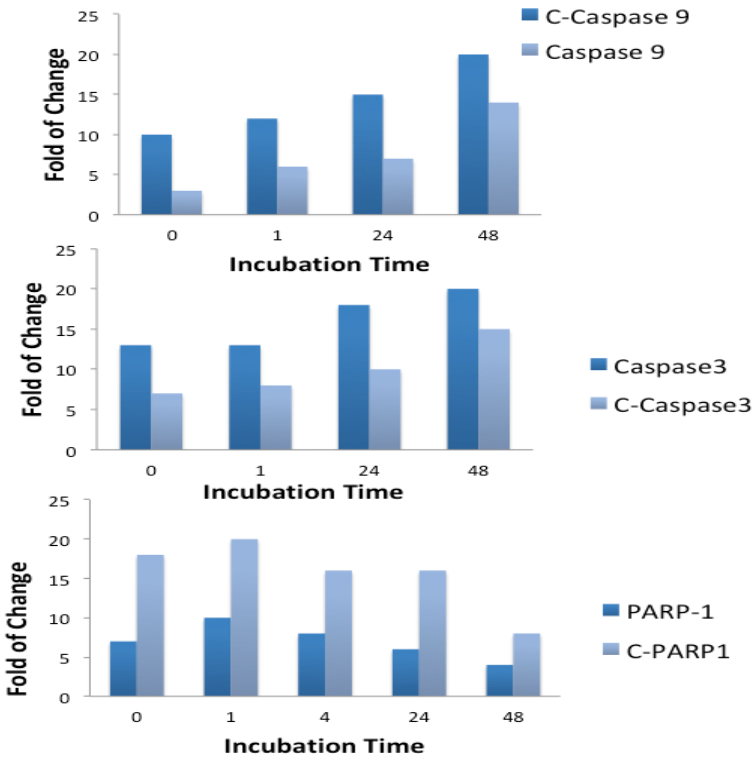

Figure 2.19 Histogram showing the inducing of apoptosis by MMA132

2.6.5 Mechanism of cytotoxic and synergetic effects

The vital role of (EGFR) in tumor proliferation and its overexpression in HCCs have provided the rationale for targeting and interrupting this key signaling network. EGFR blockade through monoclonal antibodies and tyrosine kinase inhibitors has translated into promising evidence of clinical benefit in many types of cancer HCC one of them. EGFR is expressed in a high proportion of HCCs, and EGFR-inhibitors, such as Erlotinib which has been shown to inhibit HCC growth and metastasis formation *in vitro* and *in vivo*[72]. So, that's why is urgently needed to understand the mechanism of RGFR signaling pathway.

The level EGFR/pEGFR, B-RAF/pB-RAF, MEK/pMEK, and ERK/pERK were detected after treatment of compounds MMA132 and MMA102 with 2 μM and 3 μM, respectively. MMA132 and MMA102 showed effect phosphorylation forms of RGFR signaling pathway. It was found that the phosphorylation level of EGFR was decreased after 48 hrs treatment with MMA132 by 90%. MMA102 inhibited p-EGFR level after 2 hrs of treatment. In contrast, MMA132 showed only slightly effect at pB-RAF after 24 hrs while MMA102 decreased phosphorylation level of B-RAF after 8 hrs compared to loading control β-Actin. Moreover, the level of ERK, MEK and their phosphorylated forms were detected. Both MMA132 and MMA102 Leads to decrease in the level of phosphorylated ERK1/2 and MEK **(Figure 2.20)** and **(Figure 2.22).**

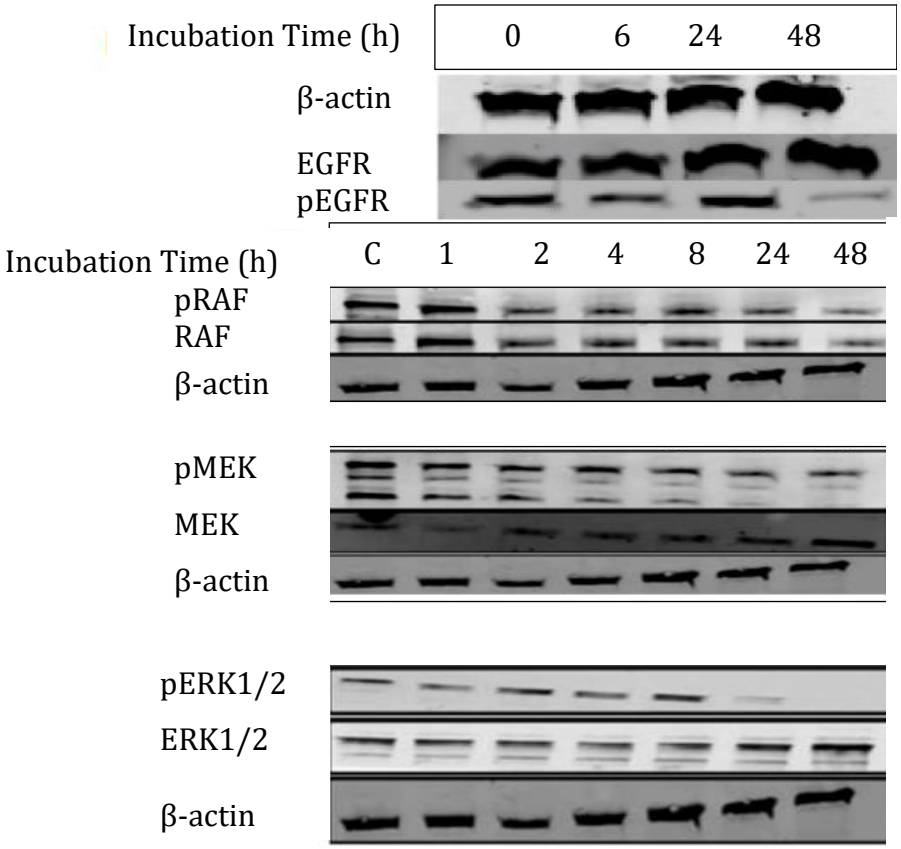

Figure 2.20 Changes in the expression level of EGFR signaling proteins by MMA132. HepG2 cells were treated with the indicated time points of 2 μM of MMA132. β-Actin was used as loading control.

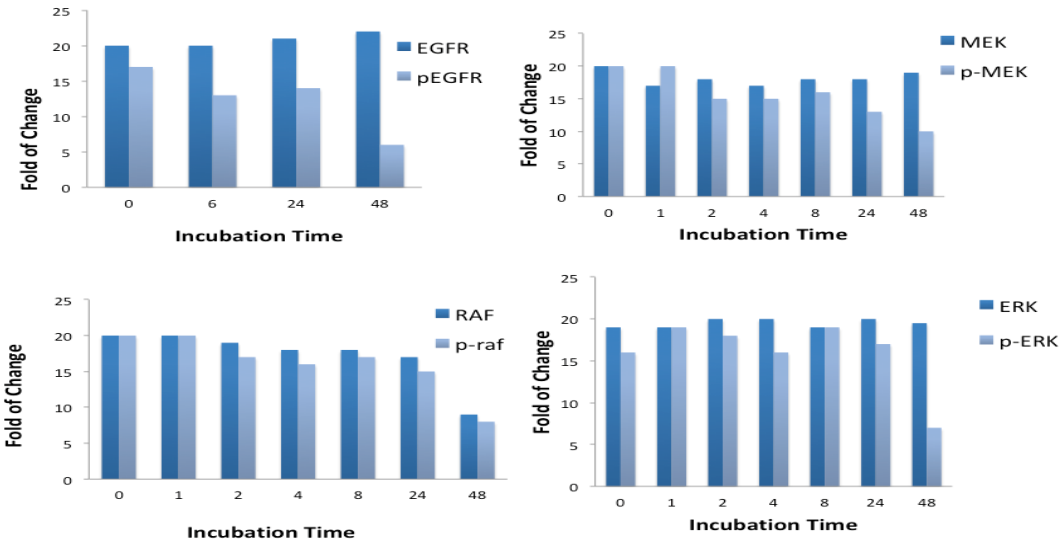

Figure 2.21 Histogram presented the inhibition of EGFRE by MMA132 on HepG2 cell line.

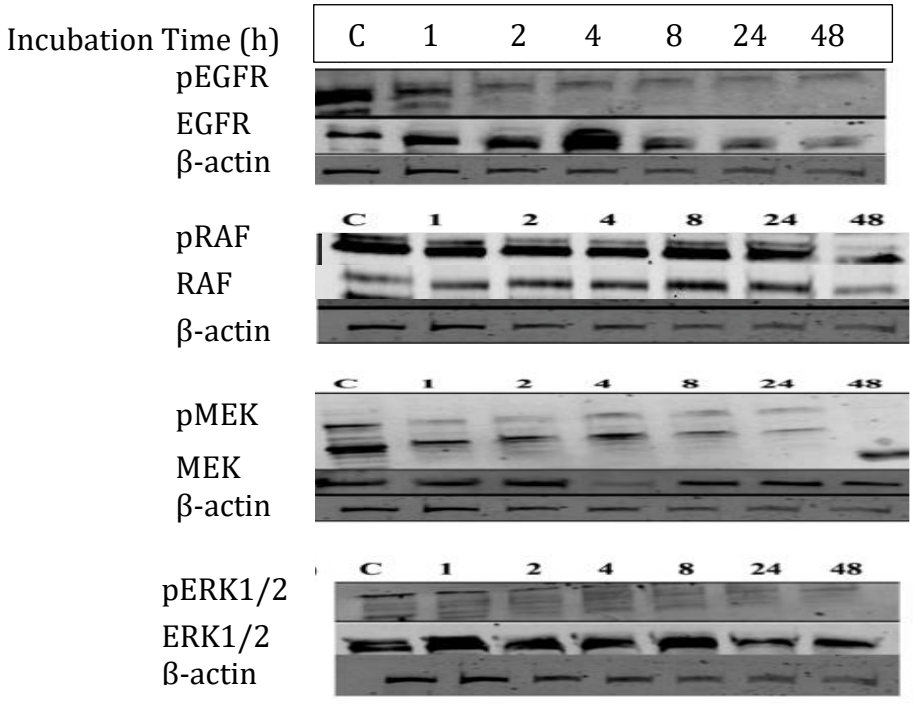

Figure 2.22 Changes in the expression level of EGFR proteins pathway by MMA102. (HepG2) cell lines were treated with the indicated time points of 3µM of MM 132. β-Actin was using as loading control.

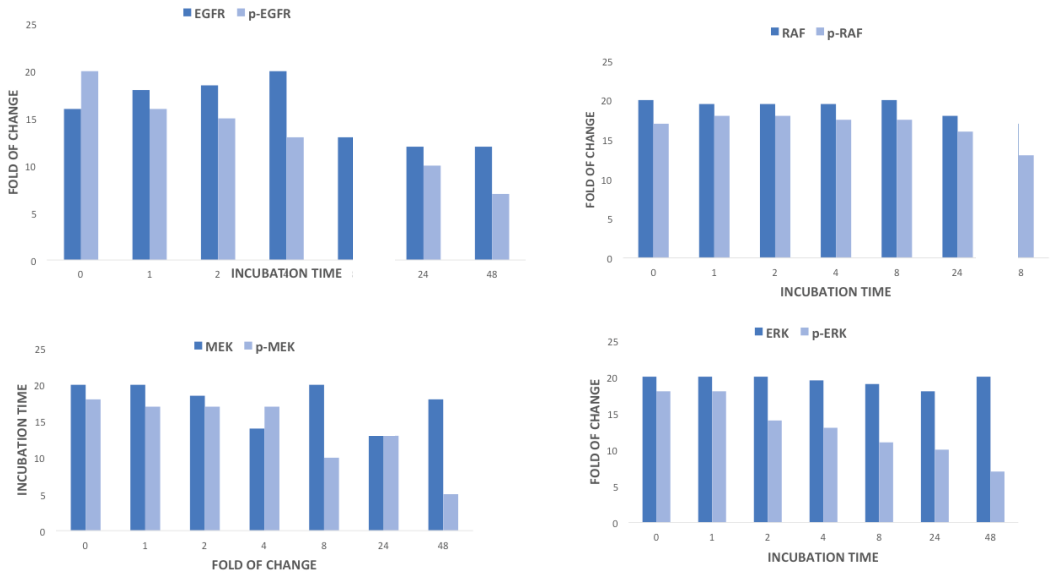

Figure 2.23 Histogram presented the inhibition of EGFRE by MMA102 on HepG2 cell line.

2.7 Discussion

Hepatocellular carcinoma is the most prevalent type of cancer worldwide. Recently, many evidences have been confirmed that epidermal growth factor receptor (EGFR) is overexpressed in HCC. Erlotinib (N-(3-ethynylphenyl)-6,7-bis (2-methoxyethoxy)-4-quinazolinamine) is a standard drug that acts as a potent and reversible inhibitor of EGFR-TK activity[40]. In the present study, MMA132 and MMA102 showed an antiproliferative effect against HepG2 cell line at a concentration 2 μM and 3 μM, respectively, compared to the standard drug Erlotinib, EGFR inhibitor, which is 25 μM after 48 hrs incubation. Furthermore, MMA132 showed the greatest activity (2 μM) which is 12 times more than Erlotinib 25 μM (Standard drug).

The cell cycle provides a critical understanding for coordination between proliferation and cell death. HCC cancer cells line cells undergo replication and division by traversing the tightly regulated cell cycle. Growth factors play a critical role in initiating signaling events stimulating cell cycle progression, which is crucial for their mitogenic and tumorigenic effects. Epidermal growth factor receptor (EGFR) and its ligands are frequently upregulated in human cancers. The oncogenic effects of EGFR include initiation of DNA synthesis, increase cell growth, invasion, and metastasis[48]. Specific upregulation of EGFR results in cell cycle arrest, apoptosis, or dedifferentiation of cancer cells while downregulation of EGFR signaling has therapeutic benefit clinical studies. Therefore, better understanding of the mechanisms of regulation and coordination between the cell cycle and EGFR inhibitor will lead to the development of novel cancer therapies[46]. The activity of MMA132 and MMA102 against Hepatocellular carcinoma cells were demonstrated through cell cycle arrest at G1 phase.

To confirm cell cycle arrest at G1 arrested by MMA132 and MMA102, the understanding mechanism by which inhibition of EGFR results in apoptosis in HepG2 cells is needed. In this work, is needed to link the inhibition of the EGFR to the multiple downstream processes, such as DNA fragmentation, and cleavage of PARP by caspase 3. These pathways are linking surface receptors to apoptosis[39]. ELK1 and pELK were investigated because they have been shown to be involved in the extracellular-signal-

regulated kinase (ERK) signaling pathway, inhibition of the ERK signaling pathway in cell cycle progression from G1 to S, inhibit phosphorylation of nuclear ERK substrate, such as ELK1/pELK[46]. In addition, EGFR inhibitor such as Erlotinib which is the stander drug in this study was reported as G1 arrested after incubation of the HepG2 cell line with (1-50 μM) for 24 hrs and led to decrease the proportion of cells in the S phase[40] which is similar to our results. The proportion of the cells in G2/M phase of the cell cycle during incubation with MMA132 and MMA102 remained nearly unaffected. MMA132 arrested the cell cycle at G1 phase and inhibited pELK1 after 24 hrs of treatment while MMA102 stopped the cycle at G1 phase at concentrations 1.5 and 3 μM. Furthermore, increasing the concentration of MMA102 to 6 μM led to arresting the cells at G2 phase.

In apoptosis, caspase 3 cleaves 116-KDa, PARP-1 into 85 and 24 KDa PARP-1 which are required for DNA repair[73]. The single DNA strand damage and binds to DNA leading to the induction of proteins required for base excision repair. Overexpression of PARP-1 was correlated to the poor prognosis and survival of HCC[74]. Inhibition of PARP-1 is proposed in increasing the sensitivity to DNA-damaging drugs such as Erlotinib[75]. MMA132 induced the cleavage of caspase 9 which activates caspase 3 which leads to inhibition of PARP-1. On the other hand, Huether et al, 2003, investigated that Erlotinib (EGFR inhibitor) induced activation of caspase 3 after 6 hrs at 50 μm in HepG2 cell line, while one of our compounds (MMA132) induced caspase 3 at 2μm concentration[74]. This clearly demonstrated that our lead novel drug candidate produced the apoptotic effect through caspase 3 and caspase 9.

MMA132, MMA102 and Erlotinib inhibited the invasive and migration potential of HepG2 cells at different time points. Inhibition of the hepatocellular carcinoma cells migration (HepG2) by MMA132, MMA102 and Erlotinib. The wounds were allowed to heal with DMSO treatment control and the migration was inhibited after 48 hrs with MMA132[76]. The data revealed that MMA132 inhibited the cell migration by 100% after 48 hrs treatment which is better than Erlotinib that inhabited the cell migration by 93% after 48 hrs of treatment.

EGFR signaling pathway involved in progression and initiation of HCC, in

addition, EGFR plays other roles in HCC including RAS, RAF, MEK, and ERK activation cascade which leads to induction of transcription of cell proliferation[77]. To understand the mechanism of MMA132 and MMA102 in inhibition of EGFR pathway, western blot analysis was conducted. Both MMA132 and MMA102 showed that the phosphorylation level of EGFR decreased after 48 hrs. Inhibition of pEGFR lead to downstream signaling pathway which lead to tumor proliferation[78]. Also, the significant increase in EGFR expression after 4 hrs of treatment is supports the idea that the receptor's expression is being upregulated in response to the treatment.

RAF in EGFR pathway is involved in regulation of many pathological process, in addition any overexpression of RAF or activation of phosphorylation RAF is a common indicator in proliferation of cancer cells[78]. Both MMA132 and MMA102 inhibited RAF in phosphorylation form, which will cause inhibition of cell proliferation. Moreover, activation of MEK and its phosphorylation plays role in the inducing proliferation and drug resistance[79]. MMA132 and MMA102 caused inhibition of pMEK in EGFR pathway against HepG2 cell line. Furthermore, ERK/pERK are related to map kinase, which regulates cell growth, differentiation, and survival[46]. CIEA MMA132 and MMA102 inhibited the pERK level while total ERK remains unchanged in both treatment. In general, this clearly demonstrated that MMA132 and MMA102 significantly inhibit EGFR and other key proteins that control several cell functions including cell resistance to chemotherapy.

2.8 Summary and conclusions

This work highlights the anti-proliferative activity of novel CIEA compounds MMA132 and MMA102 on sensitive HCC cells (HepG2) and the mechanism behind this effect. Moreover, CIEA increase the apoptotic rate, by inhibition of ERK1/2 signaling pathways, and induced caspase 3 and caspase 9 and inhibit PARP-1. CIEA compounds MMA132, MMA102, MMA265, MMA240, MMA290 and MMA245 showed significantly anti-proliferation activity against HepG2 cell line compared to Erlotinib. In addition, MMA132 and MMA102 arrested cells at G1 phase which was confirmed by ERK signaling pathway and inhibited pELK1pathway. Also, MMA132 and MMA102 inhibited

the whole EGFR pathway as demonstrated through inhibition of the phosphorylation of key proteins in EGFR pathway. Moreover, it was investigated that MMA132 induced apoptosis significantly as indicated from induction of caspase 3 (67%) and caspase 9 (87%) and inhibition of PARP.

Briefly, this study established the antiproliferation mechanism of potential drug candidate for treatment of hepatocellular carcinoma.

2.9 References

1. Newman, D. J.; Cragg, G. M., Natural products as sources of new drugs over the last 25 years. *J Nat Prod* **2007,** *70* (3), 461-77.
2. Zhang, M. M.; Qiao, Y.; Ang, E. L.; Zhao, H., Using natural products for drug discovery: the impact of the genomics era. *Expert Opin Drug Discov* **2017,** *12* (5), 475-487.
3. Mishra, B. B.; Tiwari, V. K., Natural products: an evolving role in future drug discovery. *Eur J Med Chem* **2011,** *46* (10), 4769-807.
4. Jou, J. H.; Muir, A. J., Hepatocellular Carcinoma Surveillance. *Clin Gastroenterol Hepatol* **2018,** *16* (1), 19-20.
5. Fisichella, R.; Berretta, S., Hepatitis C-related hepatocellular carcinoma: diagnostic and therapeutic management in HIV-patients. *Eur Rev Med Pharmacol Sci* **2018,** *22* (4), 860-862.
6. D'Aleo, F.; Ceccarelli, M.; Venanzi Rullo, E.; Facciola, A.; Di Rosa, M.; Pinzone, M. R.; Condorelli, F.; Visalli, G.; Picerno, I.; Berretta, M.; Pellicano, G. F.; Nunnari, G., Hepatitis C-related hepatocellular carcinoma: diagnostic and therapeutic management in HIV-patients. *Eur Rev Med Pharmacol Sci* **2017,** *21* (24), 5859-5867.
7. Qian, L.; Liu, Y.; Xu, Y.; Ji, W.; Wu, Q.; Liu, Y.; Gao, Q.; Su, C., Matrine derivative WM130 inhibits hepatocellular carcinoma by suppressing EGFR/ERK/MMP-2 and PTEN/AKT signaling pathways. *Cancer Lett* **2015,** *368* (1), 126-34.
8. Patidar, Y.; Singhal, P.; Gupta, S.; Mukund, A.; Sarin, S. K., Radiofrequency ablation of surface v/s intraparenchymal hepatocellular carcinoma in cirrhotic patients. *Indian J Radiol Imaging* **2017,** *27* (4), 496-502.
9. Argyrou, C.; Moris, D.; Vernadakis, S., Hepatocellular carcinoma development in non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. Is it going to be the "Plague" of the 21st century? A literature review focusing on pathogenesis, prevention and treatment. *J BUON* **2017,** *22* (1), 6-20.
10. Lipinska, N.; Romaniuk, A.; Paszel-Jaworska, A.; Toton, E.; Kopczynski, P.; Rubis, B., Telomerase and drug resistance in cancer. *Cell Mol Life Sci* **2017,** *74* (22), 4121-4132.

11. Whittaker, S.; Marais, R.; Zhu, A. X., The role of signaling pathways in the development and treatment of hepatocellular carcinoma. *Oncogene* **2010,** *29* (36), 4989-5005.
12. Xiang, X.; You, X. M.; Zhong, J. H.; Li, L. Q., Hepatocellular carcinoma in the absence of cirrhosis in patients with chronic hepatitis B virus infection. *J Hepatol* **2017,** *67* (4), 885-886.
13. Kim, M. N.; Kim, B. K.; Han, K. H., Hepatocellular carcinoma in patients with chronic hepatitis C virus infection in the Asia-Pacific region. *J Gastroenterol* **2013,** *48* (6), 681-8.
14. Lin, W.; Zhong, M.; Yin, H.; Chen, Y.; Cao, Q.; Wang, C.; Ling, C., Emodin induces hepatocellular carcinoma cell apoptosis through MAPK and PI3K/AKT signaling pathways in vitro and in vivo. *Oncol Rep* **2016,** *36* (2), 961-7.
15. Chattopadhyay, D.; Manas, D. M.; Reeves, H. L., The development of targeted therapies for hepatocellular cancer. *Curr Pharm Des* **2007,** *13* (32), 3292-300.
16. Liu, Y.; Poon, R. T.; Shao, W.; Sun, X.; Chen, H.; Kok, T. W.; Fan, S. T., Blockage of epidermal growth factor receptor by quinazoline tyrosine kinase inhibitors suppresses growth of human hepatocellular carcinoma. *Cancer Lett* **2007,** *248* (1), 32-40.
17. Kudo, M., Signaling Pathway and Molecular-Targeted Therapy for Hepatocellular Carcinoma. *Digestive Diseases* **2011,** *29* (3), 289-302.
18. Yarden, Y.; Sliwkowski, M. X., Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* **2001,** *2* (2), 127-37.
19. Furuse, J., Growth factors as therapeutic targets in HCC. *Critical Reviews in Oncology Hematology* **2008,** *67* (1), 8-15.
20. Chen, X.; Bao, J.; Guo, J.; Ding, Q.; Lu, J.; Huang, M.; Wang, Y., Biological activities and potential molecular targets of cucurbitacins: a focus on cancer. *Anticancer Drugs* **2012,** *23* (8), 777-87.
21. Ahmed, M. S.; Kopel, L. C.; Halaweish, F. T., Structural optimization and biological screening of a steroidal scaffold possessing cucurbitacin-like functionalities as B-Raf inhibitors. *ChemMedChem* **2014,** *9* (7), 1361-7.

22. Ahmed, M. S.; Halaweish, F. T., Cucurbitacins: potential candidates targeting mitogen-activated protein kinase pathway for treatment of melanoma. *J Enzyme Inhib Med Chem* **2014,** *29* (2), 162-7.
23. Gabrielsen, M.; Schuldt, M.; Munro, J.; Borucka, D.; Cameron, J.; Baugh, M.; Mleczak, A.; Lilla, S.; Morrice, N.; Olson, M. F., Cucurbitacin covalent bonding to cysteine thiols: the filamentous-actin severing protein Cofilin1 as an exemplary target. *Cell Commun Signal* **2013,** *11*, 58.
24. Hall, J. A.; Seedarala, S.; Rice, N.; Kopel, L.; Halaweish, F.; Blagg, B. S. J., Cucurbitacin D Is a Disruptor of the HSP90 Chaperone Machinery. *Journal of Natural Products* **2015,** *78* (4), 873-879.
25. Radjasa, O. K.; Vaske, Y. M.; Navarro, G.; Vervoort, H. C.; Tenney, K.; Linington, R. G.; Crews, P., Highlights of marine invertebrate-derived biosynthetic products: their biomedical potential and possible production by microbial associants. *Bioorg Med Chem* **2011,** *19* (22), 6658-74.
26. Fuller, R. W.; Cardellina, J. H., 2nd; Cragg, G. M.; Boyd, M. R., Cucurbitacins: differential cytotoxicity, dereplication and first isolation from Gonystylus keithii. *J Nat Prod* **1994,** *57* (10), 1442-5.
27. Kopel, L. C.; Ahmed, M. S.; Halaweish, F. T., Synthesis of novel estrone analogs by incorporation of thiophenols via conjugate addition to an enone side chain. *Steroids* **2013,** *78* (11), 1119-25.
28. Parihar, S.; Gupta, A.; Chaturvedi, A. K.; Agarwal, J.; Luqman, S.; Changkija, B.; Manohar, M.; Chanda, D.; Chanotiya, C. S.; Shanker, K.; Dwivedi, A.; Konwar, R.; Negi, A. S., Gallic acid based steroidal phenstatin analogues for selective targeting of breast cancer cells through inhibiting tubulin polymerization. *Steroids* **2012,** *77* (8-9), 878-86.
29. Bunyathaworn, P.; Boonananwong, S.; Kongkathip, B.; Kongkathip, N., Further study on synthesis and evaluation of 3,16,20-polyoxygenated steroids of marine origin and their analogs as potent cytotoxic agents. *Steroids* **2010,** *75* (6), 432-44.
30. Thomas, M. B.; Chadha, R.; Glover, K.; Wang, X.; Morris, J.; Brown, T.; Rashid, A.; Dancey, J.; Abbruzzese, J. L., Phase 2 study of erlotinib in patients with unresectable hepatocellular carcinoma. *Cancer* **2007,** *110* (5), 1059-67.

31. Sankari, S. L.; Masthan, K. M.; Babu, N. A.; Bhattacharjee, T.; Elumalai, M., Apoptosis in cancer--an update. *Asian Pac J Cancer Prev* **2012,** *13* (10), 4873-8.
32. Al-Sadoon, M. K.; Abdel-Maksoud, M. A.; Rabah, D. M.; Badr, G., Induction of apoptosis and growth arrest in human breast carcinoma cells by a snake (Walterinnesia aegyptia) venom combined with silica nanoparticles: crosstalk between Bcl2 and caspase 3. *Cell Physiol Biochem* **2012,** *30* (3), 653-65.
33. Sutherland, R. M.; MacDonald, H. R.; Howell, R. L., Multicellular spheroids: a new model target for in vitro studies of immunity to solid tumor allografts. *J Natl Cancer Inst* **1977,** *58* (6), 1849-53.
34. Sutherland, R. M.; McCredie, J. A.; Inch, W. R., Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. *J Natl Cancer Inst* **1971,** *46* (1), 113-20.
35. Don, M. M.; Ablett, G.; Bishop, C. J.; Bundesen, P. G.; Donald, K. J.; Searle, J.; Kerr, J. F., Death of cells by apoptosis following attachment of specifically allergized lymphocytes in vitro. *Aust J Exp Biol Med Sci* **1977,** *55* (4), 407-17.
36. Sears, R. C.; Nevins, J. R., Signaling networks that link cell proliferation and cell fate. *Journal of Biological Chemistry* **2002,** *277* (14), 11617-11620.
37. Creeley, C. E.; Olney, J. W., Drug-Induced Apoptosis: Mechanism by which Alcohol and Many Other Drugs Can Disrupt Brain Development. *Brain Sci* **2013,** *3* (3), 1153-81.
38. van Delft, M. F.; Smith, D. P.; Lahoud, M. H.; Huang, D. C.; Adams, J. M., Apoptosis and non-inflammatory phagocytosis can be induced by mitochondrial damage without caspases. *Cell Death Differ* **2010,** *17* (5), 821-32.
39. Ahn, H. J.; Kim, Y. S.; Kim, J. U.; Han, S. M.; Shin, J. W.; Yang, H. O., Mechanism of taxol-induced apoptosis in human SKOV3 ovarian carcinoma cells. *J Cell Biochem* **2004,** *91* (5), 1043-52.
40. Huether, A.; Hopfner, M.; Sutter, A. P.; Schuppan, D.; Scherubl, H., Erlotinib induces cell cycle arrest and apoptosis in hepatocellular cancer cells and enhances chemosensitivity towards cytostatics. *J Hepatol* **2005,** *43* (4), 661-9.
41. Wei, H.; Li, Z.; Hu, S.; Chen, X.; Cong, X., Apoptosis of mesenchymal stem cells induced by hydrogen peroxide concerns both endoplasmic reticulum stress and

mitochondrial death pathway through regulation of caspases, p38 and JNK. *J Cell Biochem* **2010,** *111* (4), 967-78.

42. Sugimura, K.; Ohno, T.; Wada, Y.; Ueda, Y.; Kimura, T.; Azuma, I., A lymphocyte blastogenesis inhibitory factor (LBIF) reversibly arrests a human melanoma cell line, A375, at G1 and G2 phases of cell cycle. *Exp Cell Res* **1990,** *188* (2), 272-8.
43. Kung, A. L.; Zetterberg, A.; Sherwood, S. W.; Schimke, R. T., Cytotoxic effects of cell cycle phase specific agents: result of cell cycle perturbation. *Cancer Res* **1990,** *50* (22), 7307-17.
44. Ducommun, B., [The cell cycle and its regulation]. *Ann Pathol* **2000,** *20 Suppl*, S1-2.
45. Filippovich, I. V.; Sorokina, N. I.; Robillard, N.; Chatal, J. F., Radiation-induced apoptosis in human ovarian carcinoma cells growing as a monolayer and as multicell spheroids. *Int J Cancer* **1997,** *72* (5), 851-9.
46. Torii, S.; Yamamoto, T.; Tsuchiya, Y.; Nishida, E., ERK MAP kinase in G cell cycle progression and cancer. *Cancer Sci* **2006,** *97* (8), 697-702.
47. Marshall, C. J., Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **1995,** *80* (2), 179-85.
48. Ebisuya, M.; Kondoh, K.; Nishida, E., The duration, magnitude and compartmentalization of ERK MAP kinase activity: mechanisms for providing signaling specificity. *J Cell Sci* **2005,** *118* (Pt 14), 2997-3002.
49. Sears, R. C.; Nevins, J. R., Signaling networks that link cell proliferation and cell fate. *J Biol Chem* **2002,** *277* (14), 11617-20.
50. Cao, A. L.; Tang, Q. F.; Zhou, W. C.; Qiu, Y. Y.; Hu, S. J.; Yin, P. H., Ras/ERK signaling pathway is involved in curcumin-induced cell cycle arrest and apoptosis in human gastric carcinoma AGS cells. *J Asian Nat Prod Res* **2015,** *17* (1), 56-63.
51. Lee, B.; Lee, S. J.; Park, S. S.; Kim, S. K.; Kim, S. R.; Jung, J. H.; Kim, W. J.; Moon, S. K., Sanguinarine-induced G1-phase arrest of the cell cycle results from increased p27KIP1 expression mediated via activation of the Ras/ERK signaling pathway in vascular smooth muscle cells. *Arch Biochem Biophys* **2008,** *471* (2), 224-31.
52. Toschi, E.; Bacigalupo, I.; Strippoli, R.; Chiozzini, C.; Cereseto, A.; Falchi, M.; Nappi, F.; Sgadari, C.; Barillari, G.; Mainiero, F.; Ensoli, B., HIV-1 Tat regulates endothelial

cell cycle progression via activation of the Ras/ERK MAPK signaling pathway. *Mol Biol Cell* **2006,** *17* (4), 1985-94.

53. Wittenberg, C.; Sugimoto, K.; Reed, S. I., G1-specific cyclins of S. cerevisiae: cell cycle periodicity, regulation by mating pheromone, and association with the p34CDC28 protein kinase. *Cell* **1990,** *62* (2), 225-37.
54. Dyson, N., The regulation of E2F by pRB-family proteins. *Genes Dev* **1998,** *12* (15), 2245-62.
55. Sherr, C. J.; Roberts, J. M., CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* **1999,** *13* (12), 1501-12.
56. Meloche, S.; Seuwen, K.; Pages, G.; Pouyssegur, J., Biphasic and synergistic activation of p44mapk (ERK1) by growth factors: correlation between late phase activation and mitogenicity. *Mol Endocrinol* **1992,** *6* (5), 845-54.
57. Roovers, K.; Assoian, R. K., Integrating the MAP kinase signal into the G1 phase cell cycle machinery. *Bioessays* **2000,** *22* (9), 818-26.
58. Yamamoto, T.; Ebisuya, M.; Ashida, F.; Okamoto, K.; Yonehara, S.; Nishida, E., Continuous ERK activation downregulates antiproliferative genes throughout G1 phase to allow cell-cycle progression. *Curr Biol* **2006,** *16* (12), 1171-82.
59. Balmanno, K.; Cook, S. J., Sustained MAP kinase activation is required for the expression of cyclin D1, p21Cip1 and a subset of AP-1 proteins in CCL39 cells. *Oncogene* **1999,** *18* (20), 3085-97.
60. Murphy, L. O.; Smith, S.; Chen, R. H.; Fingar, D. C.; Blenis, J., Molecular interpretation of ERK signal duration by immediate early gene products. *Nat Cell Biol* **2002,** *4* (8), 556-64.
61. Fotakis, G.; Timbrell, J. A., In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol Lett* **2006,** *160* (2), 171-7.
62. El-Senduny, F. F.; Badria, F. A.; EL-Waseef, A. M.; Chauhan, S. C.; Halaweish, F., Approach for chemosensitization of cisplatin-resistant ovarian cancer by cucurbitacin B. *Tumor Biology* **2016,** *37* (1), 685-698.

63. Madan, E.; Gogna, R.; Kuppusamy, P.; Bhatt, M.; Pati, U.; Mahdi, A. A., TIGAR induces p53-mediated cell-cycle arrest by regulation of RB–E2F1 complex. *British Journal of Cancer* **2012,** *107* (3), 516-26.
64. Zemans, R. L.; Briones, N.; Young, S. K.; Malcolm, K. C.; Refaeli, Y.; Downey, G. P.; Worthen, G. S., A novel method for long term bone marrow culture and genetic modification of murine neutrophils via retroviral transduction. *Journal of Immunological Methods* **2009,** *340* (2), 102-115.
65. Bailon-Moscoso, N.; Cevallos-Solorzano, G.; Romero-Benavides, J. C.; Orellana, M. I. R., Natural Compounds as Modulators of Cell Cycle Arrest: Application for Anticancer Chemotherapies. *Current Genomics* **2017,** *18* (2), 106-31.
66. Huether, A.; Höpfner, M.; Sutter, A. P.; Schuppan, D.; Scherübl, H., Erlotinib induces cell cycle arrest and apoptosis in hepatocellular cancer cells and enhances chemosensitivity towards cytostatics. *Journal of Hepatology* **2005,** *43* (4), 661-669.
67. Mohammed, F. A.; Elkady, A. I.; Syed, F. Q.; Mirza, M. B.; Hakeem, K. R.; Alkarim, S., Anethum graveolens (dill) – A medicinal herb induces apoptosis and cell cycle arrest in HepG2 cell line. *Journal of Ethnopharmacology* **2018,** *219*, 15-22.
68. Belfrage, A. K.; Abdurakhmanov, E.; Åkerblom, E.; Brandt, P.; Oshalim, A.; Gising, J.; Skogh, A.; Neyts, J.; Danielson, U. H.; Sandström, A., Discovery of pyrazinone based compounds that potently inhibit the drug-resistant enzyme variant R155K of the hepatitis C virus NS3 protease. *Bioorganic & Medicinal Chemistry* **2016,** *24* (12), 2603-2620.
69. Qian, L.; Bradford, A. M.; Cooke, P. H.; Lyons, B. A., Grb7 and Hax1 may colocalize partially to mitochondria in EGF treated SKBR3 cells and their interaction can affect Caspase3 cleavage of Hax1. *J Mol Recognit* **2016,** *29* (7), 318-33.
70. Trepat, X.; Chen, Z.; Jacobson, K., Cell Migration. *Compr Physiol* **2012,** *2* (4), 2369-92.
71. El-Senduny, F. F.; Badria, F. A.; El-Waseef, A. M.; Chauhan, S. C.; Halaweish, F., Approach for chemosensitization of cisplatin-resistant ovarian cancer by cucurbitacin B. *Tumour Biol* **2016,** *37* (1), 685-98.

72. Höpfner, M.; Schuppan, D.; Scherübl, H., Growth factor receptors and related signalling pathways as targets for novel treatment strategies of hepatocellular cancer. *World journal of gastroenterology: WJG* **2008,** *14* (1), 1.
73. Choi, Y. H.; Kong, K. R.; Kim, Y. A.; Jung, K. O.; Kil, J. H.; Rhee, S. H.; Park, K. Y., Induction of Bax and activation of caspases during beta-sitosterol-mediated apoptosis in human colon cancer cells. *Int J Oncol* **2003,** *23* (6), 1657-62.
74. Nozaki, T.; Fujihara, H.; Watanabe, M.; Tsutsumi, M.; Nakamoto, K.; Kusuoka, O.; Kamada, N.; Suzuki, H.; Nakagama, H.; Sugimura, T.; Masutani, M., Parp-1 deficiency implicated in colon and liver tumorigenesis induced by azoxymethane. *Cancer Sci* **2003,** *94* (6), 497-500.
75. Plummer, E. R.; Calvert, H., Targeting poly(ADP-ribose) polymerase: a two-armed strategy for cancer therapy. *Clin Cancer Res* **2007,** *13* (21), 6252-6.
76. Saxena, N. K.; Sharma, D.; Ding, X.; Lin, S.; Marra, F.; Merlin, D.; Anania, F. A., Concomitant activation of the JAK/STAT, PI3K/AKT, and ERK signaling is involved in leptin-mediated promotion of invasion and migration of hepatocellular carcinoma cells. *Cancer Res* **2007,** *67* (6), 2497-507.
77. Li, R.; Yanjiao, G.; Wubin, H.; Yue, W.; Jianhua, H.; Huachuan, Z.; Rongjian, S.; Zhidong, L., Secreted GRP78 activates EGFR-SRC-STAT3 signaling and confers the resistance to sorafeinib in HCC cells. *Oncotarget* **2017,** *8* (12), 19354-19364.
78. Wu, Z.; Galmiche, A.; Liu, J.; Stadler, N.; Wendum, D.; Segal-Bendirdjian, E.; Paradis, V.; Forgez, P., Neurotensin regulation induces overexpression and activation of EGFR in HCC and restores response to erlotinib and sorafenib. *Cancer Lett* **2017,** *388*, 73-84.
79. Giannelli, G.; Sgarra, C.; Porcelli, L.; Azzariti, A.; Antonaci, S.; Paradiso, A., EGFR and VEGFR as potential target for biological therapies in HCC cells. *Cancer Lett* **2008,** *262* (2), 257-64.

Chapter Three

Study the effect of CIEA chemosensitization of HCC/HepG2 resistance cell line to Erlotinib

3.1 Introduction

Cancer is known as a one of serious genetic disorder when cells become abnormal and divided very fast. Therefore, if the abnormal cell division is untreated, it will affect the other tissues of the body and lead to death[1]. The major available treatments of cancer are chemotherapeutic agents, which can either stop or slow the abnormal fast division of the cells. But, cancer cells have the ability to develop resistance to traditional therapies, and the increasing spread of these drug resistant cancers require further research and treatment development[2]. The major challenge associated with the available chemotherapeutic agents is the drug resistance which involved 30- 80% of cancer patients Therefore, there is an urgent need to find a new agent to overcome the resistance to chemotherapeutic agents[3]. For this reason, it becomes an urgent need to find a new agent to overcome the chemotherapeutic resistance.

The phenomenon in which disease become tolerant to pharmaceutical treatments is known as drug resistances[4]. This concept originated when the resistance was observed in bacteria against antibiotics, but since then similar mechanisms have been found to occur in other diseases, cancer is one of them. Some methods of drug resistance are disease-specific, while others, such as drug efflux, which is observed in microbes and human drug-resistant cancers, are evolutionarily conserved[2].

Cancer resistance to chemotherapeutic agents is a common reason in patient mortality and due to poor/early prognosis of cancer[5]. At present, the approved drugs for advanced HCC is the multi-kinase inhibitor Sorafenib and Erlotinib, which improve overall survival of three months in the presence of relevant adverse events[6]. The high molecular heterogeneity of HCC participates in adjustment of the effectiveness of targeted therapies[7].

Many studies found that there are two main mechanisms involving in the process of resistance in EGFR signaling pathway. Firstly, the genetically secondary EGFR mutations could get rid of the inhibition of respective TKIs. Secondly, activation of bypass survival tracks via other RTKs or alternative downstream compounds also account for the acquired resistance[8] **.(Figure 3.1)** and **(Figure 3.2**) [9].

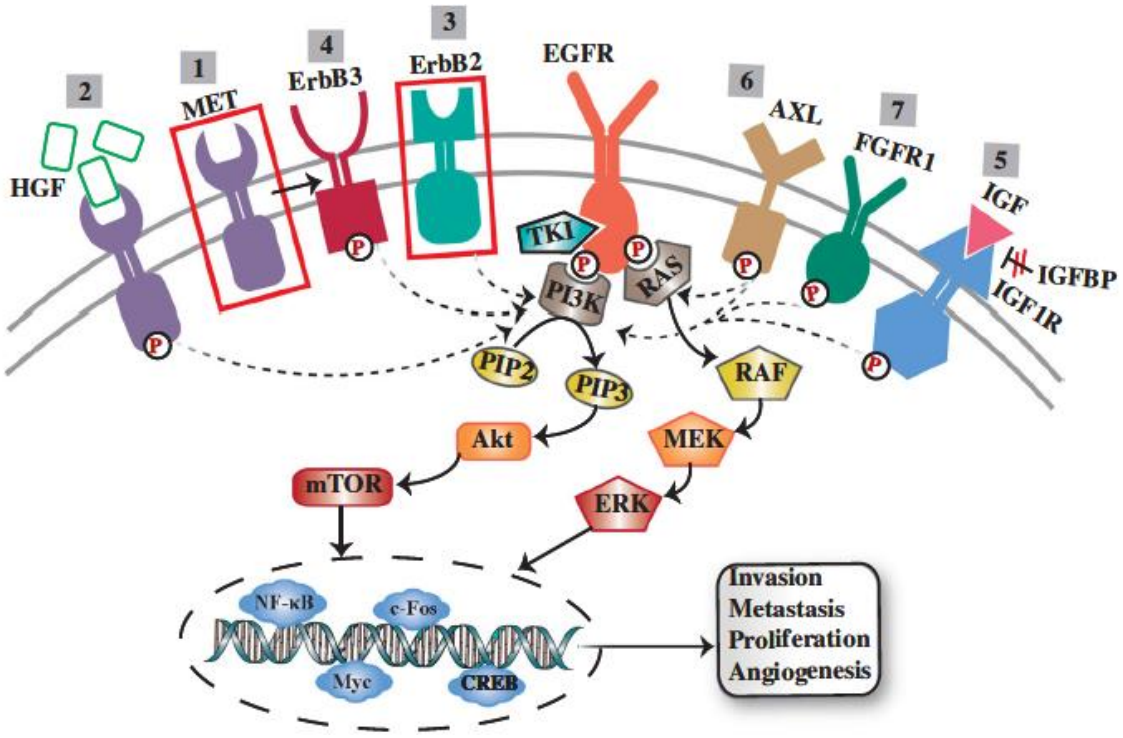

Figure 3.1 Secondary RTKs-induced EGFR-TKIs resistance. EGFR could trigger downstream PI3K/Akt and MAPK signaling axes which in turn stimulate the transcription factors to drive the associated genes expression which are related with proliferation, angiogenesis, invasion and metastasis. TKIs inhibit EGFR-drive signal transduction by interacting with the tyrosine kinase domain of EGFR. Other RTKs are involved in the development of TKIs resistance via EGFR-independent way: 1. Amplification of MET activates PI3K through transactivating ErbB3; 2. HGF overexpression; 3. ErbB2 amplification; 4. ErbB3 activation; 5. IGF1R activation by IGF binding or IGFBP reduction; 6. AXL activation; 7. FGFR1 activation.

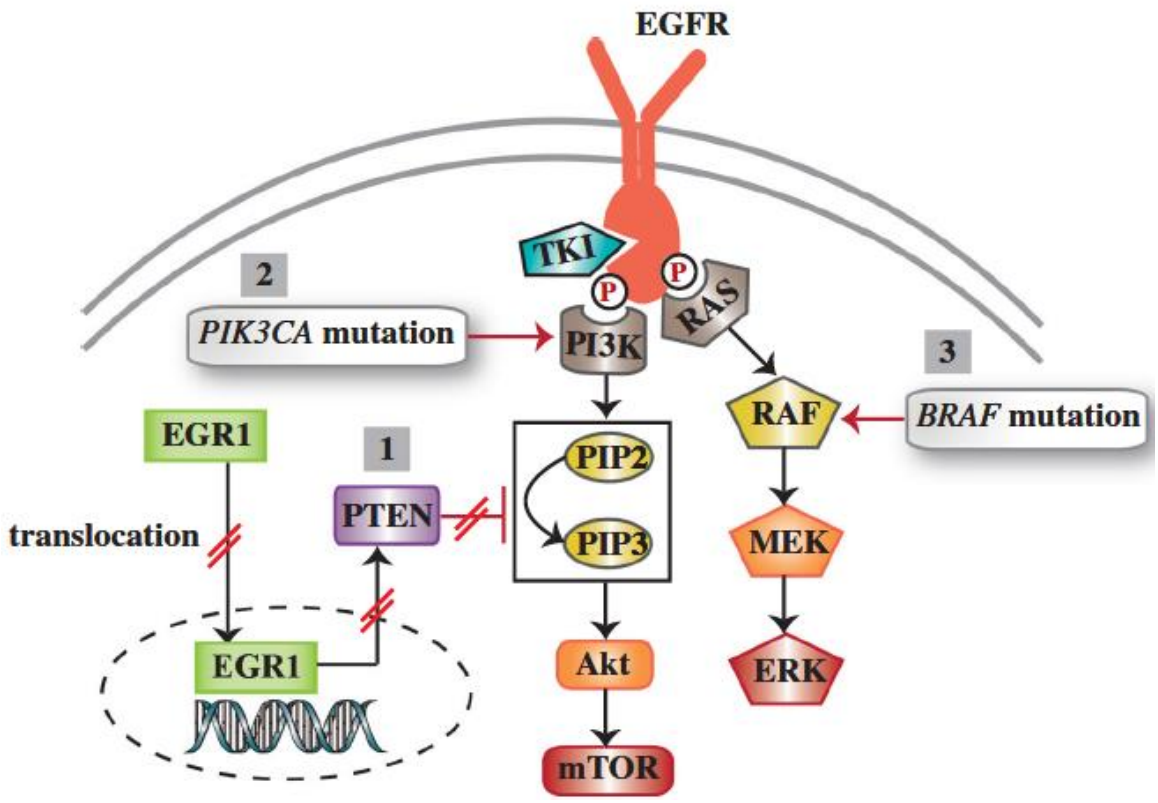

Figure 3.2 Alternative downstream compounds-induced EGFR-TKIs resistance. 1. PTEN loss: suppressed HGR1 downregulates PTEN expression which in general inhibits the PI3K/Akt activation. 2. PIK3CA mutation-derived abnormal activation of PI3K pathway. 3. BRAF mutation-drive abnormal activation of MAPK signaling axis

3.2 EGFR-independent signaling pathways involved in TKIs resistance

3.2.1 Secondary RTKs-induced TKIs resistance MET amplification:

MET, one of RTKs members family, is increased and correlation the TKIs resistance in EGFR-dependent cancers, especially in lung cancer and liver. In a gefitinib-sensitive lung cancer cells and liver cancer cells, focal amplification of MET was found to stimulate ErbB3 phosphorylation which in turn activates downstream PI3K/Akt signaling axis recovering the inhibitory effect of gefitinib on EGFR[10]. At the same time, ErbB3-specific shRNA inhibited the phosphorylation of Akt and controlled the progression of cell cycle in resistant cells. In addition, gefitinib/erlotinib–resistant lung cancer patients, (22%) of them were had elevated level of MET and hepatocellular carcinoma patients with classic

EGFR-activating mutations were reported to have concomitant MET amplification leading to de novo clinical resistance[11]. Besides lung and liver cancer, MET amplification-derived therapeutic resistance was also confirmed in other ErbB-dependent cancers, such as colorectal cancer, esophagogastric cancer, and ovarian cancer[12,13]. Nevertheless, the reason why this mechanism has not been reported in other EGFR resistant cells lines and cancers is not clear so far especially for HCC patients. Recently , several clinical trials are conducted to estimate the activity of combining the MET-targeted drugs (MET-TKIs or MET-MAbs) with EGFR TKIs in the treatment of EGFR-mutant tumor with MET-amplification[14] .

3.2.2 Hepatocyte growth factor (HGF) overexpression:

HGF, is the ligand of MET[15]. The binding between HGF and MET induced various biological effects, for instance mitogenic, morphogenic, and antiapoptotic activities [16].Several studies shown that the activation of PI3K/Akt pathway behind the TKI resistance and contribute to the carcinogenesis, proliferation, and metastasis in EGFR-mutant lung cancer[17]. The research implied that HGF could play a crucial role in resistance to EGFR-TKI[18].

3.2.3 ErbB2/HER2 amplification:

In recent studies, there are many inconsistent views concerning the influence of ErbB2 dysregulation on the sensitivity of tumor cells to EGFR-TKIs[19,20]. Many recent studies have confirmed that ErbB2 amplification was recognized as an unacknowledged mechanism mediating the acquired TKIs resistance of many types of cancer with the absence of the EGFR T790 M mutation[21]. Moreover, in cases under the treatment with Erlotinib, inhibition of ErbB2 with small interfering RNAs (siRNAs) impeded the growth of PC-9, HCC827, and H3255 cell lines without EGFR T790 M[22]. Afatinib, a TKI targeting both EGFR and ErbB2, combined with anti-EGFR antibody could remarkably attenuate the ErbB2 signaling and in turn resumed the sensitivity of lung cancer and liver cancer to TKIs in vitro and in vivo[22-23].

3.2.4 ErbB3/HER3 activation:

Resistances to EGFR or ErbB2-TKIs during the treatment of several malignancies were initiated by ErbB3[24 ,25]**.**ErbB3 can be transactivated and transphosphorylated by forming a heterodimers with other ErbB members[26]. Functionally, ErbB3 plays an essential role in the removal of the TKIs-inhibited EGFR or ErbB2 to move and sustain the activation of typical PI3K/Akt signaling pathway in vitro and in vivo[24]. Unlike the EGFR and ErbB2 motivating the PI3K during the adaptor proteins, ErbB3 could bind the p85 subunit of PI3K to activate PI3K directly, involving the priority and spread of the ErbB3-drived resistance in TKIs-treated tumors[27].

ErbB3 is considered a drug resistance inducer because it is primarily mediated by three methods. At first, MET amplification was known to endow ErbB3 signaling with persistent activation and contribute to the resistance to gefitinib in lung cancer cell lines [10]. Second, many researches demonstrated that the ErbB2-ErbB3 heterodimer has an important role in the stimulation of downstream oncogenic signaling in ErbB2+ breast cancer cells[28]. The role of ErbB2 to the resistance to gefitinib and erlotinib was undermined significantly by TKIs, signaling activities buffering the inhibitory effects of TKIs on ErbB2 were recovered through up regulating the production of ErbB3 and weakening the activity of ErbB3 phosphatase [24]. Third, when EebB3 binding with its ligand heregulin (HRG) or neuregulin 1 (NRG1), it forms a heterodimer with another ErbB receptor. As a result , the ligand-receptor complex strongly triggered PI3K/Akt axis mediating the resistance to anticancer kinase inhibitors in various cancers [29, 30]. In result of this mechanism the role of ErbB3 in drug resistance mechanisms, is identified as an encouraging approach to resist drug resistance[31].

3.2.5 IGF1R activation

Activation of IGF1R is another mechanism allowing the gained resistance against gefitinib and Erlotinib to EGFR- amplified and EGFR-mutant cancer cell lines[31].In addition, the signaling mediated by IGF1R participated in the early stage of TKIs-resistance[32].

3.2.6 PIK3CA and BRAF mutations:

Mutational activation of the downstream signaling components, such as PI3K/Akt or MEK/ERK, which was independent on the EGFR was identified as a novel mechanism of TKIs resistance[33,34]. Additionally, BRAF, known as a member of RAS signaling pathway genes, BRAF was reported to be involved in promitogenic activity and acquired resistance to EGFR TKIs in lung cancer, colorectal cancer and liver cancer through activating the MAPK signaling axis[35, 36].

3.3 Mechanisms of resistance to third generation EGFR-TKIs

Recently , the third generation EGFR-TKIs, including osimertinib, rociletinib (CO-1686), HM61713 (BI 1482694), ASP8273, EGF816, and PF-06747775, are widely known to replace the first generation EGFR- TKIs to overcome the status of drug resistance[36, 37]. Subsequently, patients were also resistant to these TKIs after 10 months of treatment, suggesting that additional mechanisms may reduce the efficacy of these inhibitors[37].

In conclusion in EGFR inhibitors treatment such as Erlotinib, in approximately 50% of cases resistance is associated with further mutation of the EGFR, most commonly mutation leading to the substitution of methionine for the gatekeeper residue threonine at position 790 in the kinase domain (T790M). This mutation in turn increases the affinity of the EGFR kinase domain for ATP, which decrease the competitively to the binding of EGFR TKIs. Second-generation inhibitors such as afatinib are similarly outcompeted, but because they bind irreversibly to EGFR, they nevertheless exhibit some activity against EGFR T790M[38]. Third-generation EGFR TKIs such as rociletinib (CO-1686) and osimertinib bind to EFGR T790M with high affinity and have confirmed good activity in patients with T790M-mediated resistance to first generation EGFR TKIs[39]. But until this time, targeted anticancer therapies using small molecules provide significant benefits in patients with HCC who do not respond well to traditional treatment still challenged because of drug resistance cancer[40].

Erlotinib is consider as the unique drug treatment for advanced (HCC). It appeared as a silver lining in combating HCC after decades of search. However, it need many improvements before any satisfactory outcomes. One of the explanations is the genetic heterogeneity of HCC, which has led to confirming predictive biomarkers for primary resistance to erlotinib and sorafenib. This concept is utilized for personalized medicine or seeking therapeutic strategies such as combining erlotinib with other anticancer agents. Some of the combinations have already shown better effectiveness than Erlotinib or sorafenib alone, with good tolerance. In other hand the gained resistance to Erlotinib has become an interesting topic for many researchers. Since Erlotinib is a multikinase inhibitor, it targets several cellular signaling pathways but simultaneously or sequentially the addiction switches and compensatory pathways are activated. Based on the investigated mechanisms for acquired resistance to Erlotinib, some other molecular targeted drugs have been applied as second line treatment to treat HCC after the failure of sorafenib and Erlotinib therapy and more are under evaluation in clinical trials. However, the exact mechanisms accounting for Erlotinib resistance remains unclear. Further investigation on the crosstalk and relationship of associated pathways will better our understanding of the mechanisms and effective strategies for overcoming Erlotinib resistance in HCC[41]. The biggest worry about drug resistance to Erlotinib is increasing as the OS (overall survival)of HCC patients after Erlotinib treatment was only 2-3 months longer than placebo and Erlotinib was shown to result in a limited increase in median time to symptomatic progression and a low partial response rate due to drug the mechanisms of Erlotinib resistance[42]. Blocking alternative pathways may provide a promising strategy for improving the drug sensitivity and overcoming the resistance to EGFR inhibitors.

3.4 Mechanism of resistance to Chemotherapeutic agents

Different biological reactions represent the defense drug resistance mechanism include: Activation of cellular elimination process, cellular uptake process and metabolic reactions to inactivate the drug process inside the cell, all of these processes will cause decrease of chemotherapy concentration inside the cell **(Figure 1.6)**[2,43] and **(Figure 3.4)**[2]

.

3.4.1 Drug Inactivation

Drug activation in vivo includes a series of different proteins interacts with substances and these interactions can modify, by part of drug partially degrade, or complex the drug with other molecules or proteins, at the end leading to its activation. For most of the anticancer drugs metabolic activation is essential to get clinical efficacy. However, decrease in drug activation can be a worthy cause of resistance development in cancer cells[44]. In addition, another source of decrease in the activation of AraC can be down-regulation or mutation in this pathway, and this can lead to AraC drug resistance. Other important examples of drug activation and inactivation include the cytochrome P450 (CYP) system, glutathione-S-transferase (GST) superfamily, and uridine diphospho-glucuronosyltransferase (UGT) superfamily[45]. Because cancer cells can develop resistance through decreased drug activation, many anticancer drugs require metabolic activation[46].

Another cause of drug resistance can be apoptosis-related proteins. For instance, apoptosis can be promoted by the tumor suppressor protein p53 (TP53), in response to chemotherapy. TP53 is mutated in 50% of cancers[47]. Otherwise, inactivation of P53 regulators, such as caspase-9 and its cofactor, apoptotic protease activating factor 1 (Apaf-1), can also lead to drug resistance[48]. One more important mechanism of drug activation and inactivation is through direct detoxification and mitogen-activated protein kinase (MAPK) pathway[49]. This is observed in the GST superfamily, which is a group of detoxifying enzymes that function to protect cellular macromolecules from electrophilic compounds. Increase of GST expression in cancer cells induce detoxification of the anticancer drugs, as a result will cause less efficient cytotoxic damage of the cells. This increase is also associated with resistance to apoptosis initiated by a variety of stimuli[50].

3.4.2 Alteration of Drug Targets

Another method of development of resistance is through alteration of the molecular target like mutations or modifications for instance, topoisomerase II is a target for some anticancer drugs. It is an enzyme that prevents DNA from becoming super under-coiled. The complex between DNA and topoisomerase II is usually transient, but these drugs

stabilize it, leading to DNA damage, inhibition of DNA synthesis, and a halting of mitotic processes. Cancer cells can develop resistance to topoisomerase II-inhibiting drugs through mutations in the topoisomerase II gene[51]. One more type of anticancer drug for which resistance can develop are the one which targets signaling kinases, such as members of the epidermal growth factor receptor (EGFR) family and down-stream signaling partners such as Ras, Src, Raf, and MEK. These kinases are constitutively active in certain cancers, and this promotes uncontrolled cell growth. Mutations is the main cause of over-activation of these kinases; however, the same effect sometimes results from gene over-expression. In addition, the long term use of inhibitors targeting this kinase will result drug resistance[52].

The increased response rates to EGFR inhibitors in certain liver cancer with EGFR tyrosine kinase domain mutations are reported with acquired resistance within one year. An EGFR-T790M gatekeeper mutation was reported in half of all cases[53].

3.4.3 Drug Efflux

Drug efflux is considered one of the most studied mechanisms of cancer drug resistance which involves reducing drug accumulation by enhancing efflux. An important transmembrane protein belonging to the ATP-binding cassette (ABC) transporter family are responsible for this efflux in addition to transport of a variety of substances across cellular membranes. These proteins are present in human cells and all extant phyla. Although their structure varies from protein to protein (e.g., there are 49 known members of the ABC family in humans), they can be classified on the basis of presence of two distinct domains, first is a highly conserved nucleotide binding domain and another is more variable transmembrane domain[2]. When a given substrate binds to the transmembrane domain, ATP hydrolysis occurs at the nucleotide binding site and causes a change in conformation that pushes the substrate out of the cell. This mechanism plays important role for preventing the accumulation of toxins in cell[54]. Such ABC transporters are highly abundant in liver and intestinal cells, where they protect the cells by pumping all the harmful chemicals into the bile duct and intestinal lumen. These transporters also function in maintaining blood-brain barrier[55, 2].

The constitutive activation of signaling molecules like kinases drives the cell cycle out of control and results in cancer. Conversely, over-expression of proteins involved in the MAPK pathway, such HRas, c-Raf, MEK1/2, ERK1/2, which act downstream of receptor tyrosine kinases, increases the expression of Pgp. While inhibitors of the extracellular signal-regulated kinases (ERK) pathway down-regulate Pgp expression, growth factors like EGF and FGF increase it. Interestingly, inhibition of HSP90, a chaperone protein that stabilizes many signaling proteins, also down-regulates Pgp[2].

3.4.4 DNA Damage Repair

Some group of chemotherapy drugs target DNA and directly or indirectly damage DNA of cancerous cells. In this type of drugs, anticancer drug resistance can develop, in which cancer cells repair the damaged DNA by DNA damage response (DDR) mechanism which can reverse the drug-induced damage. For example, platinum-containing chemotherapy drugs such as Cisplatin cause harmful DNA crosslinks, which can lead to apoptosis. However, resistance to platinum-based drugs often arises due to nucleotide excision repair and homologous recombination, the primary DNA repair mechanisms involved in reversing platinum damage. Therefore, for the DNA-damaging cytotoxic drugs to be effective there should be the failure of the cancer cell's DDR mechanisms. Inhibition of repair pathways used in conjunction with DNA damaging chemotherapy could sensitize cancer cells and therefore increase efficacy of the therapy[56].

3.4.5 Cell Death Inhibition

Cell death by apoptosis and autophagy are two important regulatory events. Although these processes are antagonistic to one another, they both have a role to cell death. Apoptosis has two established pathways: an intrinsic pathway mediated by the mitochondria that involves B-cell lymphoma 2 (BCL-2) family proteins, caspase-9 and Akt, and an extrinsic pathway that involves death receptors on the cell surface. The intrinsic and extrinsic pathways merge through the activation of down-stream caspase-3, which ultimately causes apoptosis[2]. In several types of cancers, BCL-2 family proteins, Akt, and other antiapoptotic proteins are highly expressed and down-stream transcription

modulators like NF-κB and STAT are highly active, making these good targets for drug development. Because of activation of c-Jun N-terminal kinases (JNK) many cancer drugs also induce apoptosis, which is downstream of the MAPK pathway[57]. All of these mechanisms are contribute in drug resistant, can be effectively treated by using one drug that makes the cells susceptible to death through the altered expression or regulation of cell death pathway members in combination with another cytotoxic drug that kills the cells in their vulnerable states, as a result the apoptosis and autophagy in cancer have a role in drug resistance have which makes it especially important in the field of drug-resistant cancers[58].

3.4.6 Epithelial-Mesenchymal Transition and Metastasis

The epithelial to mesenchymal transition (EMT) is one of the solid tumor become metastasis mechanism. Metastasis itself is a complex phenomenon that includes changes in a cancer cell and the stromal cells that change its environment(cell migration**) (Figure 3.3)**[59]**.** It also includes angiogenesis, which is the process responsible to form a new blood vessel around metastatic tumors. The mechanism of EMT, starts when the cells within a tumor reduce the expression of cell adhesion receptors, including integrins and cadherins, which help in cell-cell attachment, and increase the expression of cell adhesion receptors that induce cell motility. Cell motility is also based on cytokines and chemokines, which may be released by cells in the microenvironment of tumors or by the tumors themselves. Additionally, higher expression of metalloproteases on the surface of tumors it is contribute in clear the road for the cells to move outward, promoting metastasis. The role of EMT in cancer drug resistance is an emerging area of research[60].

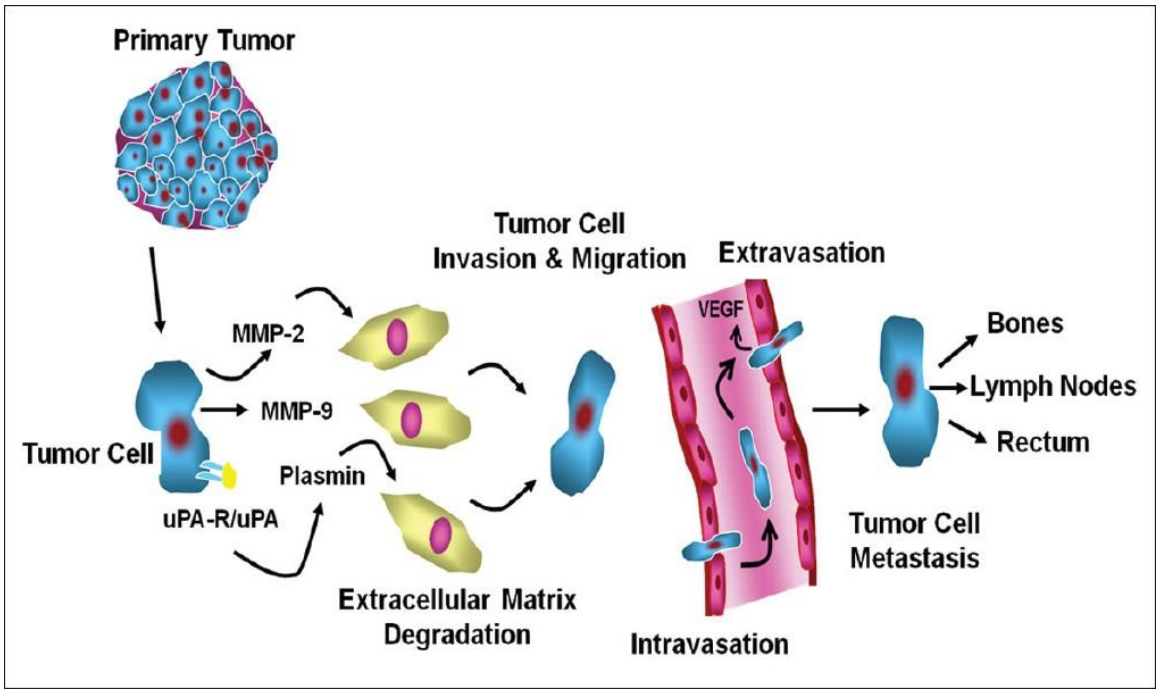

Figure 3.3 Multistep metastatic process of cancer cells. The molecular basis of tumor progression depends on local invasion, intravasation, survival in the circulation, extravasation and colonization.

3.5 Role of Epigenetics in Cancer Drug Resistance

Epigenetic modifications are an important set of mechanisms that cause resistance to cancer treatment and that have not been readily discussed and can also influence carcinogenesis. Epigenetic classified to two types changes are DNA methylation and histone modification via acetylation or methylation. DNA methylation consists of methyl groups binding to cytosines at CG-dinucleotides within regions known as CpG islands, primarily found in upstream gene promoter regions. But, methylation can occur at other loci throughout the genome. on the contrary, histone modifications alter chromatin conformation. For example, histone acetylation opens the chromatin, while deacetylation closes it. These mechanisms ultimately regulate the expression of genes throughout the chromosome, and in cancer, this normal regulation is broken. For example, tumor suppressor genes are often silenced via hypermethylation, and oncogenes are over-expressed via hypomethylation. However, epigenetic mechanisms are usually reversible,

and researchers may be able to take advantage of this opportunity to develop treatments that can counteract drug resistant cancers[2,61].

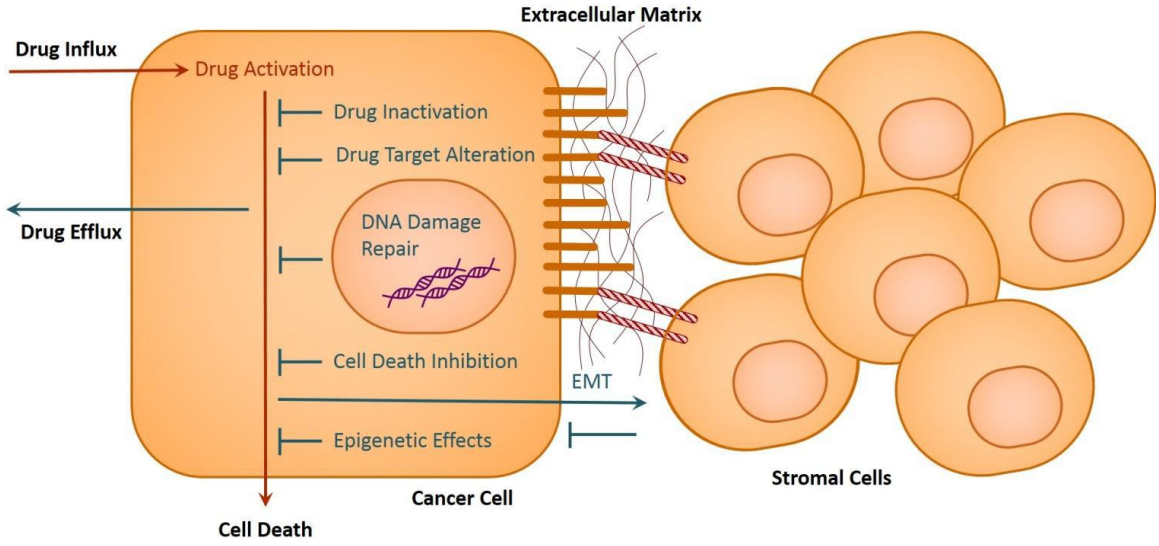

Figure 3.4 Depiction of the primary mechanisms that enable cancer cells to become drug resistant.

3.6 Properties of resistant cells generated from HCC cell lines treated with EGFR inhibitors (Erlotinib)

Epidermal growth factor receptor (EGFR) signaling plays an important role in HCC and therapeutics targeted against EGFR have been effective in treating a subset of patients bearing somatic EFGR mutations. Because, the cancer eventually progresses during treatment with EGFR inhibitors, even in the patients who respond to these drugs initially. Recently, many studies have identified that the conquest of resistance in approximately 50% of cases is due to generation of a secondary mutation (T790M) in the EGFR kinase domain. In about 20% of the cases, resistance is associated with the amplification of MET kinase. In the remaining 30-40% of the cases, the mechanism underpinning the therapeutic resistance is unknown[61].

In this study We want to determine whether CIEA compounds have Anti-proliferation effect against Erlotinib Resistant HepG2 cell line and study the mechanism of

theses CIEA compounds on EGFR signaling pathway on resistance to Erlotinib by establishing Erlotinib-resistant cell sub- line from HepG2 cells.

Aim of the study: There for understanding the mechanism and antiproliferative activity of CIEA against HEpG2-R for studying the effect of CIEA chemosensitization of HCC/HepG2 resistance cell line to chemotherapy drugs (Erlotinib)

3.7 Materials and Methods

3.7.1 Development of HepG2 resistant cell line

Human sensitive liver cancer cell line HepG2 (ATCC, ManassaVA) was used in this study to develop the resistance. The cells were maintained in EMEM (Eagle's Minimum Essential Medium) (ATCC, ManassaVA)) supplemented with 10% FBS (Atlanta biologicals) and 1% penicillin (100 IU/mL)/streptomycin (100 µg/mL) (Corning) at 37°C, 5% CO2. During culture, the medium was changed every other day. The cells were passaged every 5-6 days using Trypsin-EDTA (0.25% trypsin, 1 mM EDTA) (Hyclone™ L aboratories, Inc). The protocol to develop resistance cell line against Erlotinib(Selleckchem) was presented by Ghosh et al., 2012[62], starting with an Erlotinib (Selleckchem) concentration of 2.5 µM, the exposure dose was doubled every15 days until a final concentration of 25 µM was achieved , which is the IC50 of Erlotinib that has been reported against HepG2 cell line . The cells were maintained in continuous culture at 25 µM Erlotinib for 30 days. Then the resistance phenotype of the cells was characterized by a cell proliferation assay. Cell viability was then measured following exposure to varying concentrations of Erlotinib.

3.7.2 Cytotoxicity assay

The resistant hepatoma cells (HepG2-R) were seeded in 96-well plate as 5*104 cells/mL (100 µL/well). A serial dilution of MMA132 and MMA102 compounds were added after overnight incubation of the cells at 37°C and 5% CO2. DMSO (Acros Organics) was used as a control (0.1 %). The cells were incubated with the compounds for 48 hrs. After that 15 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

(MTT) (Sigma Aldrich) (5 mg/mL PBS) were added to each well and the plate was incubated for another 4 hrs. The formazan crystals were solubilized by 100 µL acidified SDS solution (10% SDS/0.01 N HCl) (Fisher BioReagents). The absorbance was measured after 14 hrs of incubation at 37°C and 5% CO2 at 570 nm by Hidex Sense Microplate readers[63].

3.7.3 Cell Cycle Analysis

According manufacturer protocol[64], HepG2-R cells were seeded into six-well plates at a concentration of 300,000 cells/ well and allowed to attach in culture overnight, then treated with IC50 values of compounds (MMA132 13 µM and MMA102 20 µM) for 48 h. Afterwards, cells were washed with PBS and harvested. Cell cycle analysis was investigated in accordance with the manufacturers protocol with slight modification. Briefly, Vybrant® DyeCycle™ Green Stain (Thermo Fisher Scientific) was added to 1 ml of cell suspension at a final concentration of 0.0625 µM. After 45 minutes of incubation at 37˚C, the samples were analyzed by flow cytometry and compared to DMSO-treated cells. All these experiments were performed on BD Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA, USA) using BD Accuri™ C6 software, version 1.0.

3.7.4 Cell Migration (Wound Healing Assay)

The HepG2-R cells were seeded into 24-well tissue culture plate at a density that after 24 h of growth, they should reach ~70-80% confluence as a monolayer. By a new 1 ml pipette tip the well was gently and slowly scratched across the center. While scratching across the surface of the well, the long-axial of the tip was always perpendicular to the bottom of the well. The resulting gap distance therefore equals to the outer diameter of the end of the tip. After scratching, gently the wells were washed twice with cold 1X PBS to remove the detached cells. The cells were either incubated with of MMA132 (13µM) and MMA102 (20µM) for different time points (zero time, 1hr, 24hrs, and 48 hrs). The photos for the monolayer and cell movement were taken by a microscope at different time points. The gap distance can be quantitatively evaluated using ImageJ software[65]

3.7.5 Western blot analysis

The HepG2-R protein lysate and western blot method was prepared and done according to El-senduny et al[66]. HepG2-R cells were treated with 13 µM and 20 µM of MMA132 and MMA102 respectively, and incubated for 1, 24, 48 hrs. Briefly, cells were lysed with 1X RIPA buffer(Thermoscientific™Pierce™) containing protease and phosphatase inhibitors. The BCA protein assay was used to quantify total protein concentration. An amount of 20 µg was loaded onto 10% SDSPAGE [ERK, PERK (Cell Signaling Technology), MEK, pMEK (ThermoFisher Scientific), RAF, pRAF (Cell Signaling Technology)] and 8% SDSPAGE [EGFR, pEGFR (Cell Signaling Technology)] per well. The protein was transferred onto 0.45micron nitrocellulose membrane and blocked with 5% BSA (Sigma Aldrich), and subsequently incubated with ERK, pERK, MEK, pMEK, RAF, pRAF and EGFR, pEGFR primary antibodies overnight at 4°C. The membrane was subjected to the corresponding IR-conjugated secondary antibodies. The membrane was developed by using LiCOR odyssy imager. β-actin was used as a positive control.

3.8 Results and Discussion:

3.8.1 Characterization of an Erlotinib resistant cell line

Human sensitive liver cancer cell line HepG2 from ATCC (Manassas, VA), was used in this study to develop the resistance properties. The cells were maintained in EMEM (Eagle's Minimum Essential Medium) supplemented with 10% FBS and glutamine. During culture, the medium was changed every other day. The cells were passaged every 5-6 days using Trypsin-EDTA (0.25% trypsin, 1 mM EDTA). The protocol to develop resistance cell line against Erlotinib was presented by Ghosh et al., 2012[62], starting with an Erlotinib (Selleckchem) concentration of 2.5 µM, the exposure dose was doubled every 15 days until a final concentration of 25 µM was achieved, which is the IC_{50} of Erlotinib that has been reported against HepG2 cell line . The cells were maintained in continuous culture at 25 µM Erlotinib for 30 days. Then the resistance phenotype of the cells was characterized by a cell proliferation assay. Cell viability was then measured following exposure to varying concentrations of Erlotinib.

After erlotinib resistant HepG2 cell line (HepG2-R) was generated by progressively exposing the cells to gradually increased concentrations of Erlotinib according to the protocol presented, the cells were passed every 5-6 days and developed resistant HepG2 cell line when the exposure dose was doubled every 15 days. Cell treated with 25 uM showed a full resistant properties to chemotherapy maintained for 30 days in the presence of erlotinib. Resistant cells to 25 μM erlotinib concentration were used for studying the mechanism of resistant cells to chemotherapy.

3.8.2 Study Anti-proliferation Activity of Erlotinib and CIEA Compounds (MMA132 and MMA102) to Resistance HepG2 Cell line

To confirm the resistant, phenotype was characterized by determining the ant-proliferation activity of Erlotinib resistant HepG2 cancer cells, the cells were treated for 48 hrs and its viability was determined by MTT assay. Figure 2-5 shows the cytotoxic effects of the Erlotinib, MMA132, and MMA102 on resistant cell lines. For the Erlotinib the resistant cells were characterized by quantifying cell viability at different concentrations of Erlotinib. Erlotinib showed no cytotoxic activity against HEpG2-R after 48 hrs incubation with 25 μM, which confirms the resistance properties of HepG2 to Erlotinib. The IC_{50} values of MMA132 and MMA102, as the most active compounds against sensitive HepG2 cell line, were 13 μM and 20 μM respectively against HepG2-R compared to erlotinib as a standard drug for hepatocellular carcinoma that has no cytotoxic activity on hepatoma resistant cell line. In addition, both CIEA compound MMA132 and MMA102 have high antiproliferative activity on resistance cells more than Erlotinib on sensitive cells as candidate compounds. The sensitization activity of each CIEA MMA132 and MMA102 was tested by incubating the cells for 48 hrs **(Figure 3.5).**

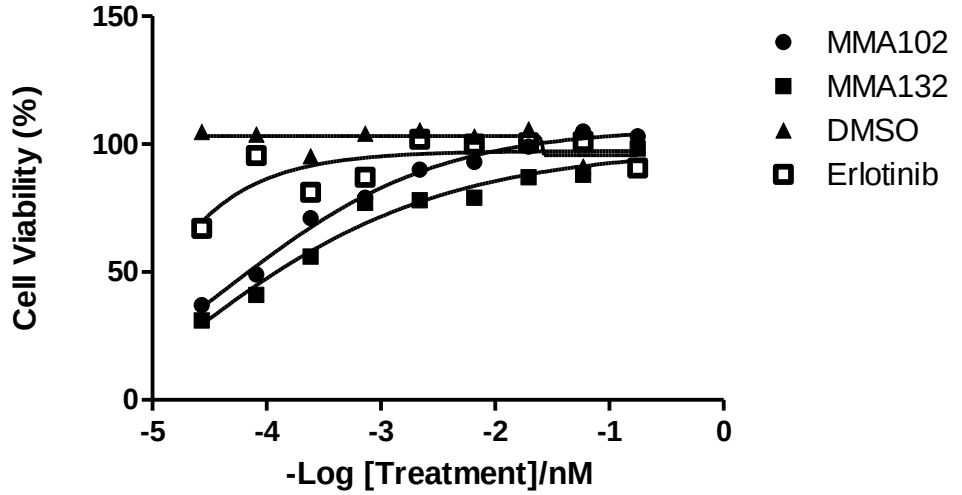

Figure 3.5 IC_{50} curves for MMA132, MMA102 compounds and Erlotinib on HepG2-R cell line compared to DMSO control.

3.8.3 Inhibition of Cell Migration (Wound Healing Assay) by MMA132 and MMA102 against HepG2-R

To investigate whether MMA132 and MMA102 have a role in cell migration and in vitro invasion on HepG2-R cell line and to determine the affected migration and in vitro invasion after development of resistant HepG2 cell line, we measured the cell migration in HepG2-R cells treated with 13 μM of MMA132 and 20 μM of MMA102. In these experiments, cells are grown to confluency and an incision was made in the center of the wells. Cells were initially seeded in 6-well culture plates with an artificial ‘‘wound’’ carefully created at 0 h, using a P-200 pipette tip to scratch on the confluent cell. The relative movement of cells to cover the ‘‘wound’’ was measured after 48 hours. Monolayer microphotographs were taken at 0 and 48 h. As shown in **Figure 3.6**, MMA132 inhibited cell migration and in vitro invasion of HepG2-R cells compared with controls. In addition, MMA102 still has activity to inhibit cell migration against HepG2-R cell line but less than the MMA102 activity against sensitive HepG2 cell line.

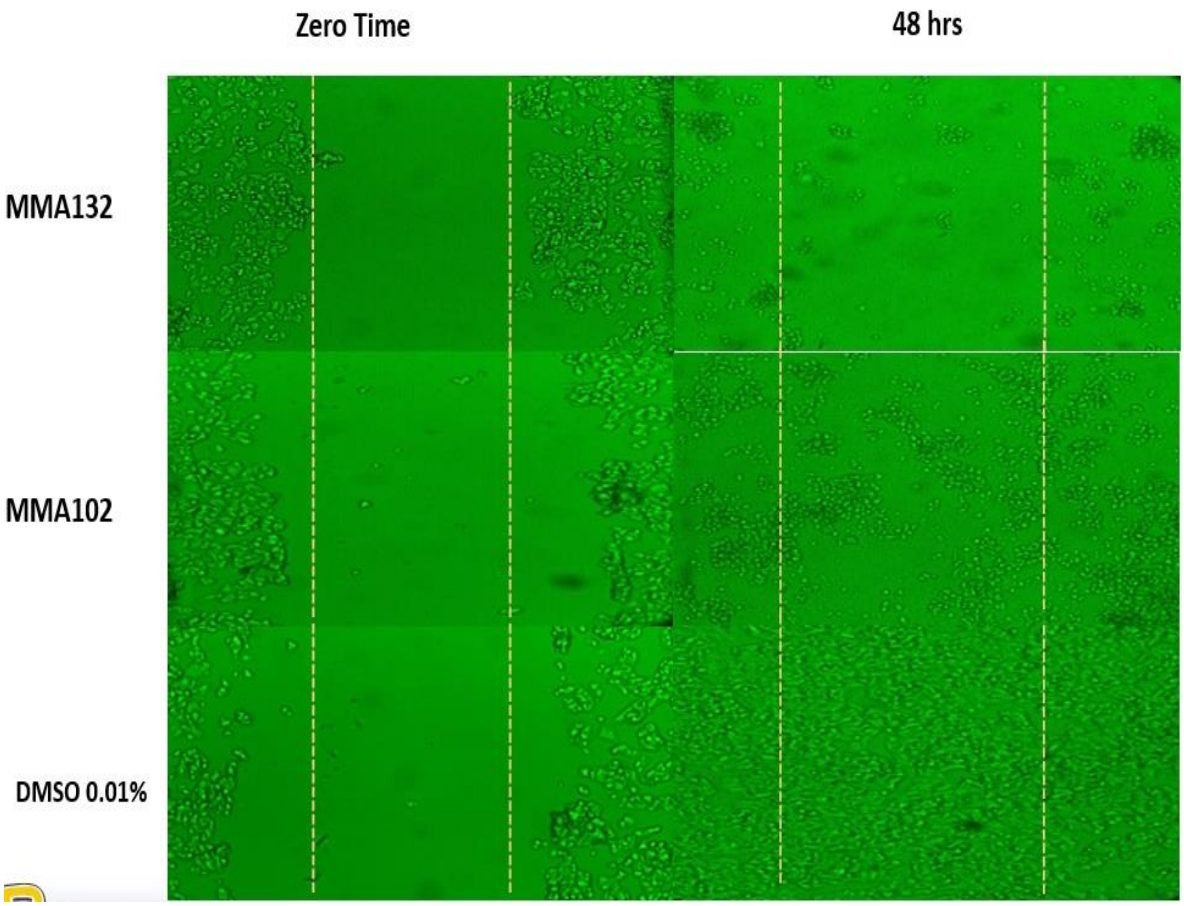

Figure 3.6 Wound-healing assay. HepG2-R cells were grown to confluency and a linear ‘‘wound’’ was made with a pipette tip. After 48 hours of incubation in the presence of DMSO (control) or with 13 μM of MMA132 and 20 μM of MMA102 a microscopic photograph was taken at zero time and after 48 hrs.

3.8.4 Effects on cell cycle distribution against HepG2-R cell line

In order to better understand the mechanism of CIEA against resistant HepG2 cell line, we analyzed the distribution of cells in different phases of the cell cycle by flow cytometry following treatment of cells with different compounds (with 13 μM of MMA132, 20 μM of MMA102 and DMSO as a control). We found that the resistant cells started to behave differently from sensitive cells in cell cycle arrest with different treatment caused cell accumulation in the G2 phase **(Figure 3.7).** For resistant cells, treatment of HepG2-R cells with 13 μM of MMA132 led to arrest 44.8% of cells in the G2 phase while treating with 20 μM of MMA102 led to arrest the cells in the G2 phase by 41.7% compared

to 38.0% in control. In general, these results indicated that cell cycle progression was significantly blocked in the G2 phase when resistant cells were treated with MMA132 and MMA102 respectively comparing to DMSO control.

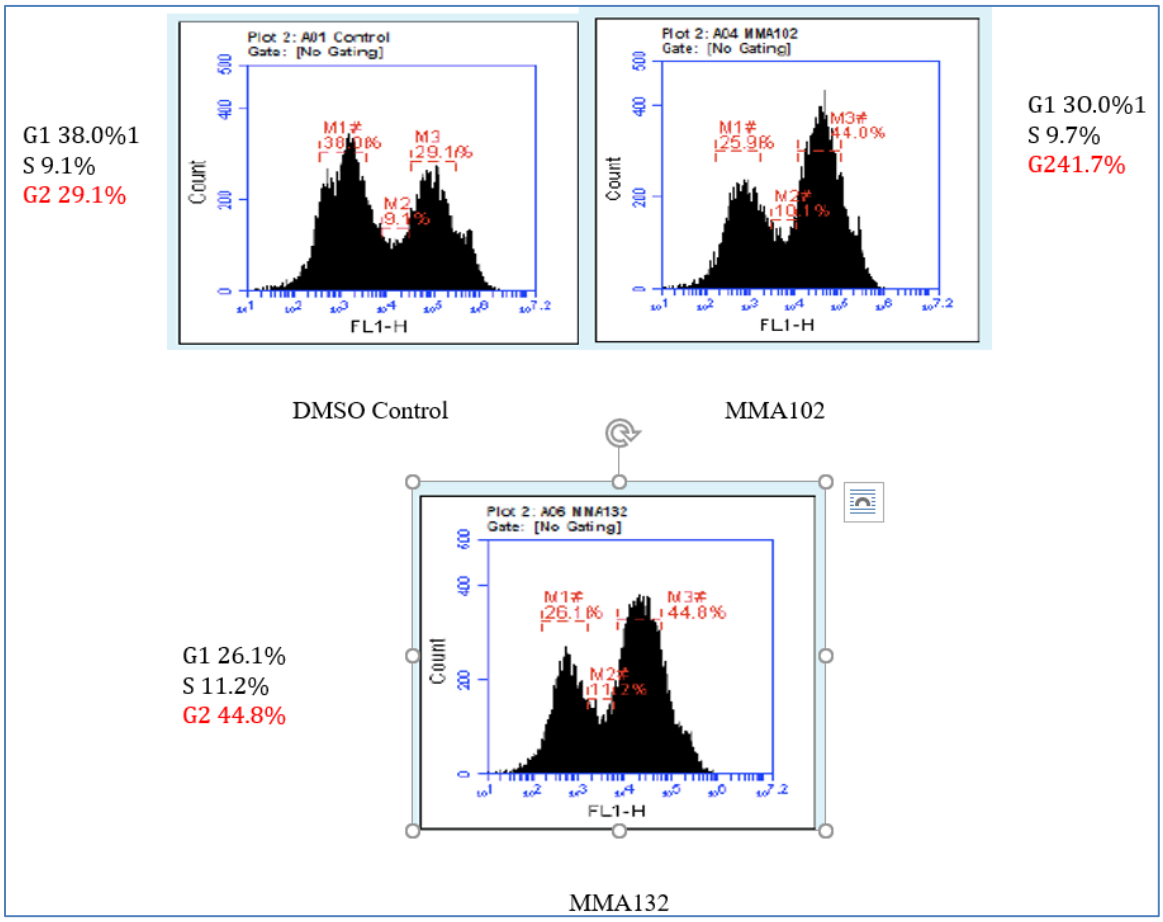

Figure 3.7 Flow cytometric analysis of the cell cycle of HepG2 cancer cells. Cells were treated with different concentration of MMA102 (1.5 µM, 3 µM and 6 µM) respectively compared to DMSO control.

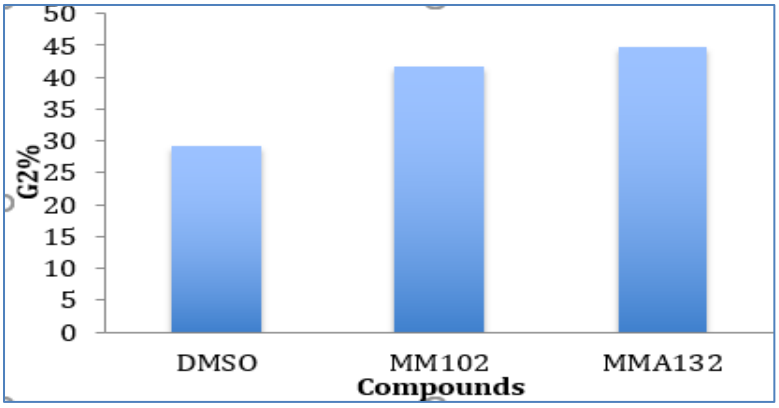

Figure 3.8 Histogram showing the percentage of cells in the G2 phase.

Table 3.1 cell cycle phases on HepG2 and HepG2-R with different treatment of MMA132 and MMA102

Cell Cycle Phases	HepG2	HepG2-R
MMA132		
G1	**41.0%**	26.1%
S	37.4%	11.2%
G2	21.0%	**44.8%**
MMA102		
G1	**47.0%**	30.0%
S	22.0%	9.7%
G2	31.0%	**41.7%**

3.8.5 MMA132 and MMA102 treatment against HepG2-R lead to an alteration in the EGFR signaling pathways

The level of EGFR proteins pathway was detected after 13 μM of MMA132 and 20 μM of MMA102 drug treatment. MMA132 showed an effect on the whole EGFR pathway, and this effect of inhibition started after 24 hrs of treatment for the most proteins pathway. It was found that the phosphorylation level of EGFR, RAF and MEK were decreased in the resistant cells **(Figure 3.9)**, which is similar to the inhibition caused by MMA132 in sensitive HepG2cell line **(Figure 2.20).** In contrast to, the phosphorylation level of ERK in resistant cells didn't show the same inhibition of MMA132 in sensitive HepG2 cell line. In addition, MMA132 showed a unique effect in EGFR pathway by reduced total EGFR.

Also, MMA102 **(Figure 3.11),** leads to a decrease in p-ERK and p-MEK after 24 hrs treatment in HepG2-R cell line **(Figure 3.11**).

The Raf/MEK/ERK pathway has different effects on growth, prevention of apoptosis, cell cycle arrest and induction of drug resistance in cells of hepatocellular carcinoma. The data revealed that MMA132 and MMA102 inhibited the p-RAF which is considered as an indicator for inhibition of cell proliferation. Because p-RAF regulates gene expression, it prevents apoptosis and induces cell proliferation[67].

MEK/ERK induced proliferation, drug resistance. MEK also influences chemotherapeutic drug resistance. Moreover, it has been observed that overexpression of MEK is associated with a worse prognosis of drug resistance. Both MMA132 and MMA102 decrease the phosphorylation level of MEK after 24 hrs. also, MMA102 inhibited the p-MEK by 100% after 48, which makes our CIEA as a promising drug candidate for drug resistance. In addition, ERK in EGFR signaling pathway in caner plays a vital role in inhibition of apoptosis and increases cell growth. ERK also has an essential role in regulation of the cell cycle arrest in cancer cell and induction of drug resistance through inhibition of apoptosis[67]. From **Figure 3.9** and **figure 3.11,** the data presented that MMA132 and MMA102 inhibited the phosphorylation form of ERK which means decreasing the cancer cell growth and inhibiting the drug resistance.

Moreover, targeted inhibition of the central components of this pathway appears to be an excellent choice for future therapeutic approaches. It has been observed that overexpression of both the Raf/MEK/ERK in EGFR pathway is associated Drug resistance[74].

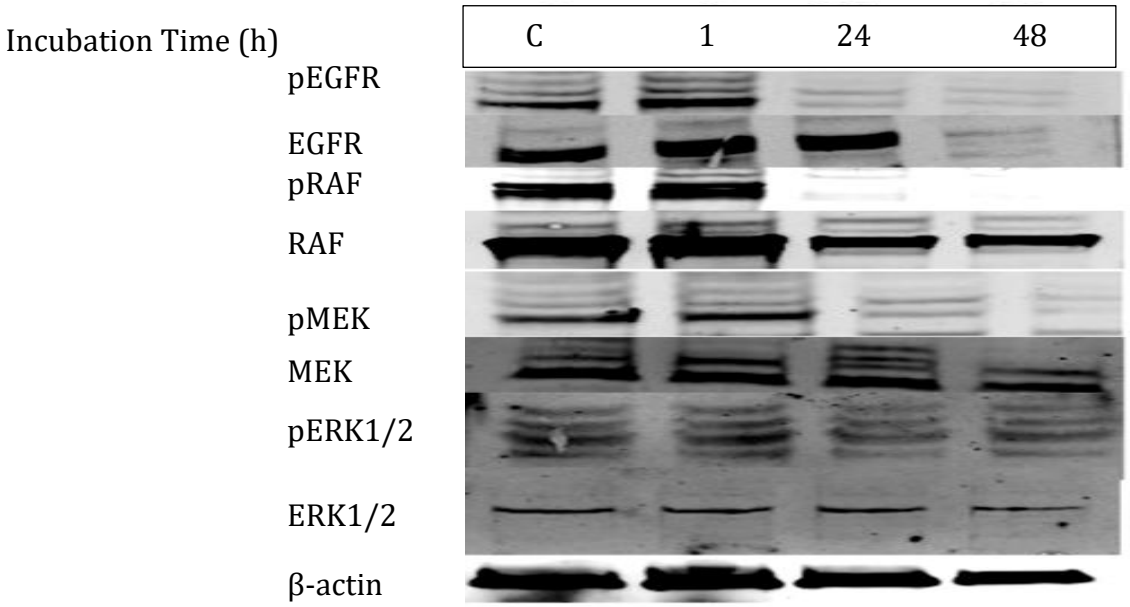

Figure 3.9 Changes in the expression level of EGFR signaling proteins by MMA132. Western blot analyses of EGFR and downstream signaling pathway activation. The HepG2-R cells line were treated with the correspondent IC_{50} of MMA132 (13 µm) for 1, 24, 48 hrs, β-Actin used as a loading control. Each experiment was performed in triplicate. The values above indicate relative expression level compared with control.

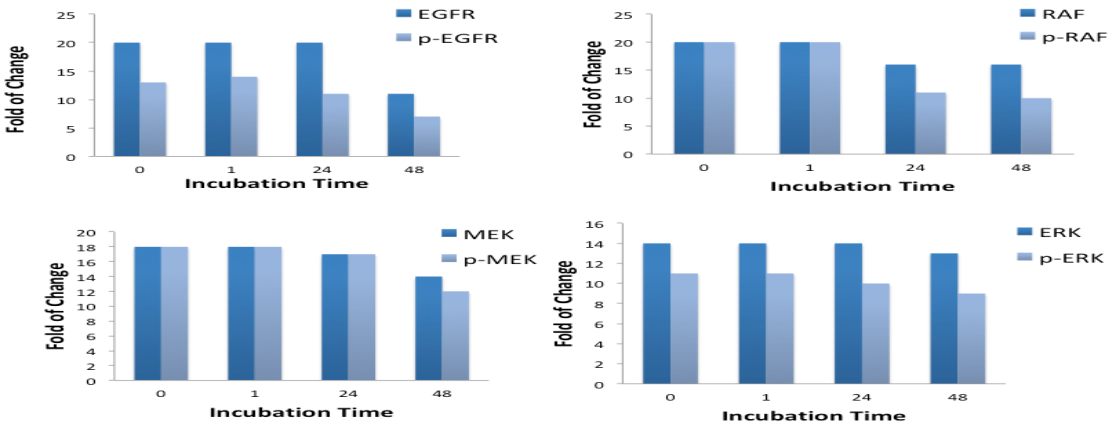

Figure 3.10 Histogram showing the inhibition of EGFR signaling pathway by MMA132

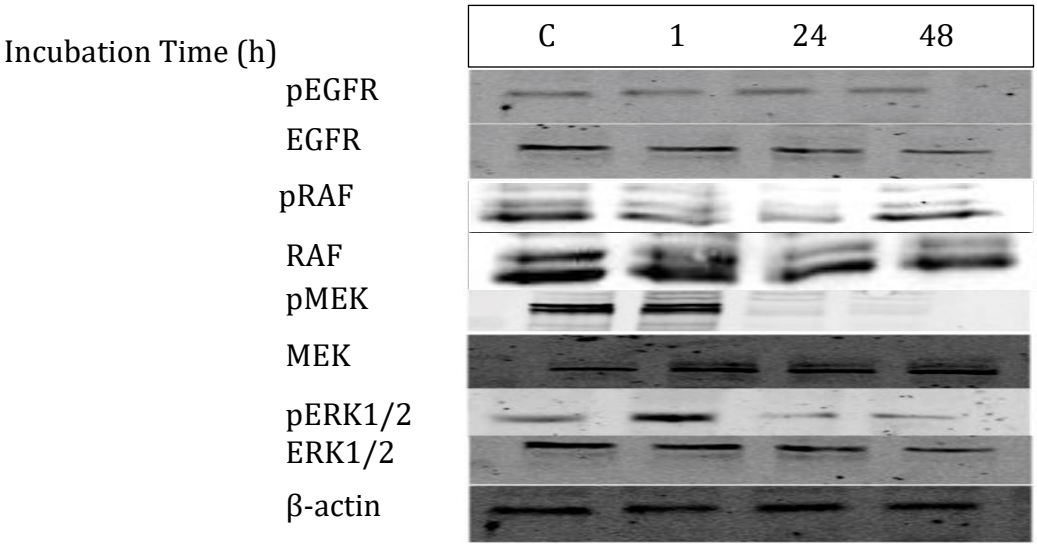

Figure 3.11 Changes in the expression level of EGFR signaling proteins by MMA102. The HepG2-R cells line were treated with the correspondent IC_{50} of MMA102 (20 µm) for 1, 24, 48 hrs, β-Actin used as a loading control. Each experiment was performed in triplicate. The values above indicate relative expression level compared with control

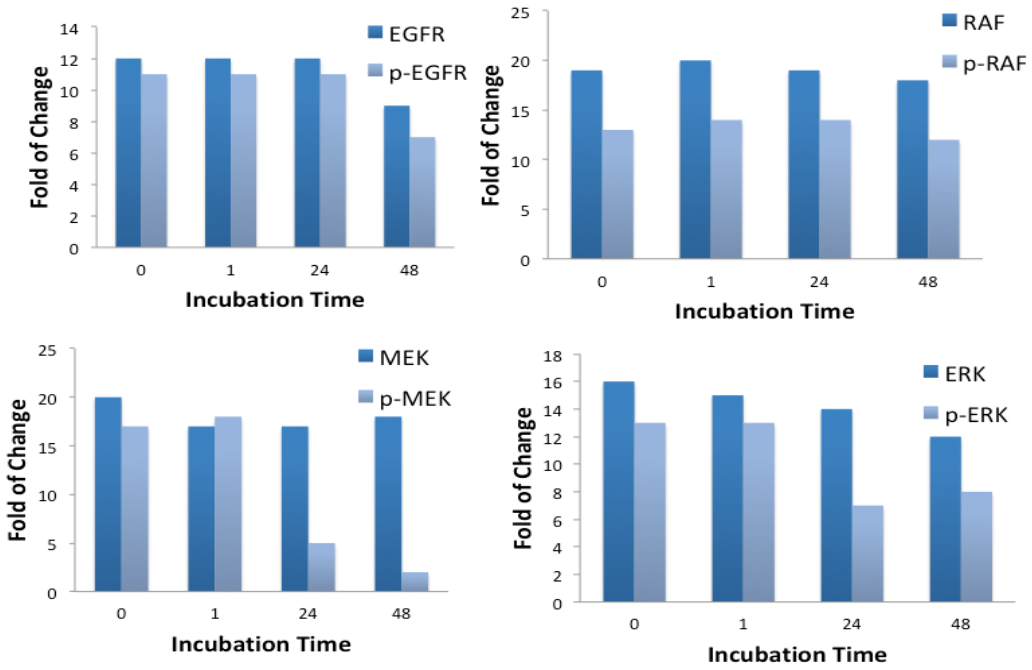

Figure 3.12 Histogram showing the inhibition of EGFR signaling pathway by MMA102

3.9 Discussion

Hepatocellular carcinoma (HCC) is the most common histological form of primary liver cancer, the tumor cells retain the features of hepatocytic differentiation. Erlotinib is the most effective chemotherapy drugs for HCC treatment. The low levels of survival in HCC patients is related to resistance of the HCC cell to this drug. This is a big challenge in HCC treatment in this time[68]. In the present study, Erlotinib alone showed an inhibitory effect on sensitive HepG2 cell. Moreover, in this study, development of resistant HepG2 cell line to Erlotinib (standard drug), cells were characterized by quantifying cell viability at different concentrations of Erlotinib. Erlotinib showed no cytotoxic activity against HEpG2-R after 48 hrs incubation with 25 μM, which confirms the resistance properties of HepG2 to Erlotinib and leads to investigate the cytotoxicity effect of CIEA compounds on resistant HepG2 cells line. The novel compounds MMA132 and MMA102 were effective on resistant cells more than Erlotinib itself in sensitive cells. Also, MMA132 and MMA102 showed antiproliferative activity against HepG2-R cells with 13 μM and 20 μM, respectively, after 48 hrs of incubation. This clearly demonstrated that MMA132 and MMA102 significantly has cytotoxic activity to inhibit resistance to Erlotinib.

MMA132 and MMA102 activity against HCC were through cell cycle arrest at G2/M phase, which is different from the effect of both MMA132 and MMMA102 on sensitive HepG2 cells line which was G1 arrest. For resistant cells, treatment of HepG2-R cells with 13 μM of MMA132 led to arrest 38.0% of the cells in control at G2 phase to increase 44.8% of cells that are treated with MMA132 in the G_2 phase, and to 41.7% with cells treated with MMA102 after 24 hrs of incubation with 13 μM and 20 μM of MMA132 and MMA102, respectively. In contrast, HepG2 sensitive cells were completely stopped at G1 phase at different concentration of MMA132 and MMA102. These results indicate that cell cycle progression was significantly blocked in the G2 phase when resistant cells were treated with MMA132 and MMA102, respectively, comparing to DMSO control. Zhai at al., in a different study have found that in HepG2 resistant to adriamycin (ADM), the number of cells in S-phase and G1 was significantly decreased (5. 6%) in HepG2/Adm while those G2-phase increased (24%)[69].Change the behavior of resistance cells in cycle

arrest to stop the cell cycle at G2 phase confirmed the resistance properties of cells to Erlotinib.

Inhibition the cell migration of resistant hepatocellular carcinoma cells (HepG2-R) by MMA132 and MMA102, the wounds were allowed to heal with DMSO treatment control and the migration was inhibited after 48 hrs with both MMA13 and MMA1022. Furthermore, we measured the cell migration in HepG2-R cells treated with 13 μM of MMA132 and 20 μM of MMA102. It was found that MMA132 inhibited the cell migration and *in vitro* invasion of HepG2-R cells compared with controls. In addition, MMA102 still has activity to inhibit cell migration against HepG2-R cell line but less than sensitive one. Generally, in Comparison with Erlotinib in sensitive cell, MMA132 treatment with resistance HepG2 cell line has activity to inhibit the cell migration after 48 hrs more than Erlotinib in sensitive cell.

The effect of MMA132 and MMA102 on the EGFR pathway against HepG2-R was different form sensitive cells. MMA 132 doesn't have a clear activity in inhibition of the phosphorylation level of ERK, which clearly demonstrated the behavior of cell cycle arrest at G2 phase because the role of ERK pathway to regulate G1 phase. In contrast MMA132 has a huge inhibition on total EGFR and p-EGFR. It also inhibited the phosphorylation level of MEK and RAF while MMA102 decreased the pERK1/2 and PMEK levels in the resistant HCC cell line. Hence, these results show the effect of CIEA on the EGFR signaling pathway in HepG2-R.

MEK/ERK induced proliferation, drug resistance. MEK also influences chemotherapeutic drug resistance[67]. Moreover, it has been observed that overexpression of MEK is associated with a worse prognosis of drug resistance. Both MMA132 and MMA102 decrease the phosphorylation level of MEK after 24 hrs, and MMA102 inhibited the p-MEK by 100% after 48, which make our CIEA a promising candidate drug for drug resistance. In addition, ERK in EGFR signaling pathway in caner plays role in inhibit the apoptosis and increases cell growth. The data presented that MMA132 and MMA102 inhibited the phosphorylation form of ERK which means decreasing the cancer cell growth and inhibiting the drug resistance.

In conclusion, this study confirmed the antiproliferative activity of MMA132 and MMA102 in drug resistance and demonstrated the mechanism of potential drug candidate for treatment and solve the drug resistant in hepatocellular carcinoma.

3.10 Summary and conclusions

Our study indicates that prolonged exposure of the Hepatocellular carcinoma cell line HepG2 to erlotinib selects for a subpopulation of erlotinib resistant cells which are enriched in stem cell markers and possess stem cell properties *in vitro*. Furthermore, these cells were found to be less sensitive to erlotinib treatment as determined by cell viability. Resistant HepG2 showed resistant properties to erlotinib after 3 months of maintenance in Erlotinib. HepG2 cells also showed decrease in the number of cells in G1 phase from 38% in control to 26.1% in MMA132 treatment and 30.1 in MMA102 treatment which is different from the sensitive HepG2 cell line. Our studies indicated that both MMA132 and MMA102 inhibit the HepG2-R cell migration after 48 hrs of incubation.

3.11 References

1. Rezaei, M.; Cao, J. H.; Friedrich, K.; Kemper, B.; Brendel, O.; Grosser, M.; Adrian, M.; Baretton, G.; Breier, G.; Schnittler, H. J., The expression of VE-cadherin in breast cancer cells modulates cell dynamics as a function of tumor differentiation and promotes tumor-endothelial cell interactions. *Histochemistry and Cell Biology* **2018,** *149* (1), 15-30.
2. Housman, G.; Byler, S.; Heerboth, S.; Lapinska, K.; Longacre, M.; Snyder, N.; Sarkar, S., Drug Resistance in Cancer: An Overview. *Cancers* **2014,** *6* (3), 1769-1792.
3. Assanhou, A. G.; Li, W. Y.; Zhang, L.; Xue, L. J.; Kong, L. Y.; Sun, H. B.; Mo, R.; Zhang, C., Reversal of multidrug resistance by co-delivery of paclitaxel and lonidamine using a TPGS and hyaluronic acid dual-functionalized liposome for cancer treatment. *Biomaterials* **2015,** *73*, 284-295.
4. Samuel, P.; Mulcahy, L. A.; Furlong, F.; McCarthy, H. O.; Brooks, S. A.; Fabbri, M.; Pink, R. C.; Carter, D. R. F., Cisplatin induces the release of extracellular vesicles from ovarian cancer cells that can induce invasiveness and drug resistance in bystander cells. *Philos Trans R Soc Lond B Biol Sci* **2018,** *373* (1737).
5. Elshaier, Y. A.; Shaaban, M. A.; El Hamid, M. K. A.; Abdelrahman, M. H.; Abou-Salim, M. A.; Elgazwi, S. M.; Halaweish, F., Design and synthesis of pyrazolo [3, 4-d] pyrimidines: Nitric oxide releasing compounds targeting hepatocellular carcinoma. *Bioorganic & medicinal chemistry* **2017,** *25* (12), 2956-2970.
6. Ai, X. H.; Sun, Y. J.; Wang, H. D.; Lu, S., A systematic profile of clinical inhibitors responsive to EGFR somatic amino acid mutations in lung cancer: implication for the molecular mechanism of drug resistance and sensitivity. *Amino Acids* **2014,** *46* (7), 1635-1648.
7. Pollutri, D.; Patrizi, C.; Marinelli, S.; Giovannini, C.; Trombetta, E.; Giannone, F. A.; Baldassarre, M.; Quarta, S.; Vandewynckel, Y. P.; Vandierendonck, A.; Van Vlierberghe, H.; Porretti, L.; Negrini, M.; Bolondi, L.; Gramantieri, L.; Fornari, F., The epigenetically regulated miR-494 associates with stem-cell phenotype and induces sorafenib resistance in hepatocellular carcinoma. *Cell Death Dis* **2018,** *9* (1), 4.
8. Niederst, M. J.; Engelman, J. A., Bypass mechanisms of resistance to receptor tyrosine kinase inhibition in lung cancer. *Sci Signal* **2013,** *6* (294), re6.

9. Liu, Q.; Yu, S.; Zhao, W.; Qin, S.; Chu, Q.; Wu, K., EGFR-TKIs resistance via EGFR-independent signaling pathways. *Molecular cancer* **2018,** *17* (1), 53.
10. Engelman, J. A.; Zejnullahu, K.; Mitsudomi, T.; Song, Y.; Hyland, C.; Park, J. O.; Lindeman, N.; Gale, C. M.; Zhao, X.; Christensen, J.; Kosaka, T.; Holmes, A. J.; Rogers, A. M.; Cappuzzo, F.; Mok, T.; Lee, C.; Johnson, B. E.; Cantley, L. C.; Janne, P. A., MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* **2007,** *316* (5827), 1039-43.
11. Sequist, L. V.; Martins, R. G.; Spigel, D.; Grunberg, S. M.; Spira, A.; Janne, P. A.; Joshi, V. A.; McCollum, D.; Evans, T. L.; Muzikansky, A.; Kuhlmann, G. L.; Han, M.; Goldberg, J. S.; Settleman, J.; Iafrate, A. J.; Engelman, J. A.; Haber, D. A.; Johnson, B. E.; Lynch, T. J., First-line gefitinib in patients with advanced non-small-cell lung cancer harboring somatic EGFR mutations. *J Clin Oncol* **2008,** *26* (15), 2442-9.
12. Bardelli, A.; Corso, S.; Bertotti, A.; Hobor, S.; Valtorta, E.; Siravegna, G.; Sartore-Bianchi, A.; Scala, E.; Cassingena, A.; Zecchin, D.; Apicella, M.; Migliardi, G.; Galimi, F.; Lauricella, C.; Zanon, C.; Perera, T.; Veronese, S.; Corti, G.; Amatu, A.; Gambacorta, M.; Diaz, L. A., Jr.; Sausen, M.; Velculescu, V. E.; Comoglio, P.; Trusolino, L.; Di Nicolantonio, F.; Giordano, S.; Siena, S., Amplification of the MET receptor drives resistance to anti-EGFR therapies in colorectal cancer. *Cancer Discov* **2013,** *3* (6), 658-73.
13. Tang, C.; Jardim, D. L.; Hong, D., MET in ovarian cancer: metastasis and resistance? *Cell Cycle* **2014,** *13* (8), 1220-1.
14. Ou, S.-H. I.; Kwak, E. L.; Siwak-Tapp, C.; Dy, J.; Bergethon, K.; Clark, J. W.; Camidge, D. R.; Solomon, B. J.; Maki, R. G.; Bang, Y.-J., Activity of crizotinib (PF02341066), a dual mesenchymal-epithelial transition (MET) and anaplastic lymphoma kinase (ALK) inhibitor, in a non-small cell lung cancer patient with de novo MET amplification. *Journal of thoracic oncology* **2011,** *6* (5), 942-946.
15. Harvey, P.; Warn, A.; Newman, P.; Perry, L. J.; Ball, R. Y.; Warn, R. M., Immunoreactivity for hepatocyte growth factor/scatter factor and its receptor, met, in human lung carcinomas and malignant mesotheliomas. *J Pathol* **1996,** *180* (4), 389-94.
16. Yamada, T.; Matsumoto, K.; Wang, W.; Li, Q.; Nishioka, Y.; Sekido, Y.; Sone, S.; Yano, S., Hepatocyte growth factor reduces susceptibility to an irreversible epidermal

growth factor receptor inhibitor in EGFR-T790M mutant lung cancer. *Clin Cancer Res* **2010,** *16* (1), 174-83.

17. Stabile, L. P.; Lyker, J. S.; Land, S. R.; Dacic, S.; Zamboni, B. A.; Siegfried, J. M., Transgenic mice overexpressing hepatocyte growth factor in the airways show increased susceptibility to lung cancer. *Carcinogenesis* **2006,** *27* (8), 1547-55.
18. Yano, S.; Takeuchi, S.; Nakagawa, T.; Yamada, T., Ligand-triggered resistance to molecular targeted drugs in lung cancer: roles of hepatocyte growth factor and epidermal growth factor receptor ligands. *Cancer Sci* **2012,** *103* (7), 1189-94.
19. Zhang, J.; Cao, J.; Li, J.; Zhang, Y.; Chen, Z.; Peng, W.; Sun, S.; Zhao, N.; Wang, J.; Zhong, D.; Zhang, X.; Zhang, J., A phase I study of AST1306, a novel irreversible EGFR and HER2 kinase inhibitor, in patients with advanced solid tumors. *J Hematol Oncol* **2014,** *7*, 22.
20. Cretella, D.; Saccani, F.; Quaini, F.; Frati, C.; Lagrasta, C.; Bonelli, M.; Caffarra, C.; Cavazzoni, A.; Fumarola, C.; Galetti, M.; La Monica, S.; Ampollini, L.; Tiseo, M.; Ardizzoni, A.; Petronini, P. G.; Alfieri, R. R., Trastuzumab emtansine is active on HER-2 overexpressing NSCLC cell lines and overcomes gefitinib resistance. *Mol Cancer* **2014,** *13*, 143.
21. Momcilovic, M.; Bailey, S. T.; Lee, J. T.; Fishbein, M. C.; Magyar, C.; Braas, D.; Graeber, T.; Jackson, N. J.; Czernin, J.; Emberley, E.; Gross, M.; Janes, J.; Mackinnon, A.; Pan, A.; Rodriguez, M.; Works, M.; Zhang, W.; Parlati, F.; Demo, S.; Garon, E.; Krysan, K.; Walser, T. C.; Dubinett, S. M.; Sadeghi, S.; Christofk, H. R.; Shackelford, D. B., Targeted Inhibition of EGFR and Glutaminase Induces Metabolic Crisis in EGFR Mutant Lung Cancer. *Cell Rep* **2017,** *18* (3), 601-610.
22. Takezawa, K.; Pirazzoli, V.; Arcila, M. E.; Nebhan, C. A.; Song, X.; de Stanchina, E.; Ohashi, K.; Janjigian, Y. Y.; Spitzler, P. J.; Melnick, M. A.; Riely, G. J.; Kris, M. G.; Miller, V. A.; Ladanyi, M.; Politi, K.; Pao, W., HER2 amplification: a potential mechanism of acquired resistance to EGFR inhibition in EGFR-mutant lung cancers that lack the second-site EGFRT790M mutation. *Cancer Discov* **2012,** *2* (10), 922-33.
23. Yonesaka, K.; Zejnullahu, K.; Okamoto, I.; Satoh, T.; Cappuzzo, F.; Souglakos, J.; Ercan, D.; Rogers, A.; Roncalli, M.; Takeda, M.; Fujisaka, Y.; Philips, J.; Shimizu, T.; Maenishi, O.; Cho, Y.; Sun, J.; Destro, A.; Taira, K.; Takeda, K.; Okabe, T.; Swanson,

J.; Itoh, H.; Takada, M.; Lifshits, E.; Okuno, K.; Engelman, J. A.; Shivdasani, R. A.; Nishio, K.; Fukuoka, M.; Varella-Garcia, M.; Nakagawa, K.; Janne, P. A., Activation of ERBB2 signaling causes resistance to the EGFR-directed therapeutic antibody cetuximab. *Sci Transl Med* **2011,** *3* (99), 99ra86.

24. Sergina, N. V.; Rausch, M.; Wang, D.; Blair, J.; Hann, B.; Shokat, K. M.; Moasser, M. M., Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. *Nature* **2007,** *445* (7126), 437-41.
25. Telesco, S. E.; Shih, A. J.; Jia, F.; Radhakrishnan, R., A multiscale modeling approach to investigate molecular mechanisms of pseudokinase activation and drug resistance in the HER3/ErbB3 receptor tyrosine kinase signaling network. *Mol Biosyst* **2011,** *7* (6), 2066-80.
26. Engelman, J. A.; Janne, P. A., Mechanisms of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer. *Clin Cancer Res* **2008,** *14* (10), 2895-9.
27. Prigent, S. A.; Gullick, W. J., Identification of c-erbB-3 binding sites for phosphatidylinositol 3'-kinase and SHC using an EGF receptor/c-erbB-3 chimera. *EMBO J* **1994,** *13* (12), 2831-41.
28. Holbro, T.; Beerli, R. R.; Maurer, F.; Koziczak, M.; Barbas, C. F., 3rd; Hynes, N. E., The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. *Proc Natl Acad Sci U S A* **2003,** *100* (15), 8933-8.
29. Yarar, D.; Lahdenranta, J.; Kubasek, W.; Nielsen, U. B.; MacBeath, G., Heregulin-ErbB3-Driven Tumor Growth Persists in PI3 Kinase Mutant Cancer Cells. *Mol Cancer Ther* **2015,** *14* (9), 2072-80.
30. Greenfield, E.; Griner, E.; Reproducibility Project: Cancer, B., Registered report: Widespread potential for growth factor-driven resistance to anticancer kinase inhibitors. *Elife* **2014,** *3*.
31. Li, H.; Batth, I. S.; Qu, X.; Xu, L.; Song, N.; Wang, R.; Liu, Y., IGF-IR signaling in epithelial to mesenchymal transition and targeting IGF-IR therapy: overview and new insights. *Mol Cancer* **2017,** *16* (1), 6.

32. Cortot, A. B.; Repellin, C. E.; Shimamura, T.; Capelletti, M.; Zejnullahu, K.; Ercan, D.; Christensen, J. G.; Wong, K. K.; Gray, N. S.; Janne, P. A., Resistance to irreversible EGF receptor tyrosine kinase inhibitors through a multistep mechanism involving the IGF1R pathway. *Cancer Res* **2013,** *73* (2), 834-43.
33. Sequist, L. V.; Waltman, B. A.; Dias-Santagata, D.; Digumarthy, S.; Turke, A. B.; Fidias, P.; Bergethon, K.; Shaw, A. T.; Gettinger, S.; Cosper, A. K.; Akhavanfard, S.; Heist, R. S.; Temel, J.; Christensen, J. G.; Wain, J. C.; Lynch, T. J.; Vernovsky, K.; Mark, E. J.; Lanuti, M.; Iafrate, A. J.; Mino-Kenudson, M.; Engelman, J. A., Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci Transl Med* **2011,** *3* (75), 75ra26.
34. Ohashi, K.; Sequist, L. V.; Arcila, M. E.; Moran, T.; Chmielecki, J.; Lin, Y. L.; Pan, Y.; Wang, L.; de Stanchina, E.; Shien, K.; Aoe, K.; Toyooka, S.; Kiura, K.; Fernandez-Cuesta, L.; Fidias, P.; Yang, J. C.; Miller, V. A.; Riely, G. J.; Kris, M. G.; Engelman, J. A.; Vnencak-Jones, C. L.; Dias-Santagata, D.; Ladanyi, M.; Pao, W., Lung cancers with acquired resistance to EGFR inhibitors occasionally harbor BRAF gene mutations but lack mutations in KRAS, NRAS, or MEK1. *Proc Natl Acad Sci U S A* **2012,** *109* (31), E2127-33.
35. Corcoran, R. B.; Ebi, H.; Turke, A. B.; Coffee, E. M.; Nishino, M.; Cogdill, A. P.; Brown, R. D.; Della Pelle, P.; Dias-Santagata, D.; Hung, K. E.; Flaherty, K. T.; Piris, A.; Wargo, J. A.; Settleman, J.; Mino-Kenudson, M.; Engelman, J. A., EGFR-mediated re-activation of MAPK signaling contributes to insensitivity of BRAF mutant colorectal cancers to RAF inhibition with vemurafenib. *Cancer Discov* **2012,** *2* (3), 227-35.
36. Cross, D. A.; Ashton, S. E.; Ghiorghiu, S.; Eberlein, C.; Nebhan, C. A.; Spitzler, P. J.; Orme, J. P.; Finlay, M. R.; Ward, R. A.; Mellor, M. J.; Hughes, G.; Rahi, A.; Jacobs, V. N.; Red Brewer, M.; Ichihara, E.; Sun, J.; Jin, H.; Ballard, P.; Al-Kadhimi, K.; Rowlinson, R.; Klinowska, T.; Richmond, G. H.; Cantarini, M.; Kim, D. W.; Ranson, M. R.; Pao, W., AZD9291, an irreversible EGFR TKI, overcomes T790M-mediated resistance to EGFR inhibitors in lung cancer. *Cancer Discov* **2014,** *4* (9), 1046-61.
37. Janne, P. A.; Yang, J. C.; Kim, D. W.; Planchard, D.; Ohe, Y.; Ramalingam, S. S.; Ahn, M. J.; Kim, S. W.; Su, W. C.; Horn, L.; Haggstrom, D.; Felip, E.; Kim, J. H.; Frewer,

P.; Cantarini, M.; Brown, K. H.; Dickinson, P. A.; Ghiorghiu, S.; Ranson, M., AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer. *N Engl J Med* **2015,** *372* (18), 1689-99.

38. Wang, X. Q.; Ongkeko, W. M.; Chen, L.; Yang, Z. F.; Lu, P.; Chen, K. K.; Lopez, J. P.; Poon, R. T.; Fan, S. T., Octamer 4 (Oct4) mediates chemotherapeutic drug resistance in liver cancer cells through a potential Oct4-AKT-ATP-binding cassette G2 pathway. *Hepatology* **2010,** *52* (2), 528-39.
39. Friese-Hamim, M.; Bladt, F.; Locatelli, G.; Stammberger, U.; Blaukat, A., The selective c-Met inhibitor tepotinib can overcome epidermal growth factor receptor inhibitor resistance mediated by aberrant c-Met activation in NSCLC models. *American Journal of Cancer Research* **2017,** *7* (4), 962-972.
40. Chen, K. F.; Chen, H. L.; Tai, W. T.; Feng, W. C.; Hsu, C. H.; Chen, P. J.; Cheng, A. L., Activation of phosphatidylinositol 3-kinase/Akt signaling pathway mediates acquired resistance to sorafenib in hepatocellular carcinoma cells. *J Pharmacol Exp Ther* **2011,** *337* (1), 155-61.
41. Chaft, J. E.; Oxnard, G. R.; Sima, C. S.; Kris, M. G.; Miller, V. A.; Riely, G. J., Disease flare after tyrosine kinase inhibitor discontinuation in patients with EGFR-mutant lung cancer and acquired resistance to erlotinib or gefitinib: implications for clinical trial design. *Clin Cancer Res* **2011,** *17* (19), 6298-303.
42. Zhai, B.; Sun, X. Y., Mechanisms of resistance to sorafenib and the corresponding strategies in hepatocellular carcinoma. *World J Hepatol* **2013,** *5* (7), 345-52.
43. Shen, D.; Pastan, I.; Gottesman, M. M., Cross-resistance to methotrexate and metals in human cisplatin-resistant cell lines results from a pleiotropic defect in accumulation of these compounds associated with reduced plasma membrane binding proteins. *Cancer Res* **1998,** *58* (2), 268-75.
44. Zahreddine, H.; Borden, K. L., Mechanisms and insights into drug resistance in cancer. *Frontiers in Pharmacology* **2013,** *4*, 28.
45. Michael, M.; Doherty, M. M., Tumoral drug metabolism: overview and its implications for cancer therapy. *J Clin Oncol* **2005,** *23* (1), 205-29.
46. Rodriguez-Antona, C.; Ingelman-Sundberg, M., Cytochrome P450 pharmacogenetics and cancer. *Oncogene* **2006,** *25* (11), 1679-91.

47. Rivlin, N.; Brosh, R.; Oren, M.; Rotter, V., Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis. *Genes Cancer* **2011,** *2* (4), 466-74.
48. Soengas, M. S.; Alarcon, R. M.; Yoshida, H.; Giaccia, A. J.; Hakem, R.; Mak, T. W.; Lowe, S. W., Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. *Science* **1999,** *284* (5411), 156-9.
49. Townsend, D. M.; Tew, K. D., The role of glutathione-S-transferase in anti-cancer drug resistance. *Oncogene* **2003,** *22* (47), 7369-75.
50. Cumming, R. C.; Lightfoot, J.; Beard, K.; Youssoufian, H.; O'Brien, P. J.; Buchwald, M., Fanconi anemia group C protein prevents apoptosis in hematopoietic cells through redox regulation of GSTP1. *Nat Med* **2001,** *7* (7), 814-20.
51. Stavrovskaya, A. A., Cellular mechanisms of multidrug resistance of tumor cells. *Biochemistry (Mosc)* **2000,** *65* (1), 95-106.
52. Doss, S.; Robertson, J.; Adam, J., Lapatinib or trastuzumab in combination with an aromatase inhibitor for first-line treatment of metastatic hormone-receptor-positive breast cancer that overexpresses HER2. *Lancet Oncology* **2012,** *13* (8), 766-767.
53. Xu, S.; Liu, X.; Liu, R. W.; Shi, T.; Li, X. F.; Zhong, D. S.; Wang, Y.; Chen, G.; Chen, J., Concurrent epidermal growth factor receptor T790M secondary mutation and epithelial-mesenchymal transition in a lung adenocarcinoma patient with EGFR-TKI drug resistance. *Thoracic Cancer* **2017,** *8* (6), 693-697.
54. Sauna, Z. E.; Ambudkar, S. V., Characterization of the catalytic cycle of ATP hydrolysis by human P-glycoprotein. The two ATP hydrolysis events in a single catalytic cycle are kinetically similar but affect different functional outcomes. *J Biol Chem* **2001,** *276* (15), 11653-61.
55. Borst, P.; Elferink, R. O., Mammalian ABC transporters in health and disease. *Annu Rev Biochem* **2002,** *71*, 537-92.
56. Bonanno, L.; Favaretto, A.; Rosell, R., Platinum Drugs and DNA Repair Mechanisms in Lung Cancer. *Anticancer Research* **2014,** *34* (1b), 493-501.
57. Sarkar, S.; Faller, D. V., Telomere-Homologous G-Rich Oligonucleotides Sensitize Human Ovarian Cancer Cells to TRAIL-Induced Growth Inhibition and Apoptosis. *Nucleic Acid Therapeutics* **2013,** *23* (3), 167-174.

58. Correction: Hydroxychloroquine Inhibits Autophagy to Potentiate Antiestrogen Responsiveness in ER+ Breast Cancer. *Clin Cancer Res* **2016,** *22* (11), 2825.
59. Dasgupta, S.; Srinidhi, S.; Vishwanatha, J. K., Oncogenic activation in prostate cancer progression and metastasis: Molecular insights and future challenges. *Journal of carcinogenesis* **2012,** *11*.
60. Shang, Y.; Cai, X.; Fan, D., Roles of epithelial-mesenchymal transition in cancer drug resistance. *Curr Cancer Drug Targets* **2013,** *13* (9), 915-29.
61. Sarkar, S.; Goldgar, S.; Byler, S.; Rosenthal, S.; Heerboth, S., Demethylation and re-expression of epigenetically silenced tumor suppressor genes: sensitization of cancer cells by combination therapy. *Epigenomics* **2013,** *5* (1), 87-94.
62. Ghosh, G.; Lian, X.; Kron, S. J.; Palecek, S. P., Properties of resistant cells generated from lung cancer cell lines treated with EGFR inhibitors. *BMC Cancer* **2012,** *12*, 95.
63. El-Senduny, F. F.; Badria, F. A.; EL-Waseef, A. M.; Chauhan, S. C.; Halaweish, F., Approach for chemosensitization of cisplatin-resistant ovarian cancer by cucurbitacin B. *Tumor Biology* **2016,** *37* (1), 685-698.
64. Belfrage, A. K.; Abdurakhmanov, E.; Åkerblom, E.; Brandt, P.; Oshalim, A.; Gising, J.; Skogh, A.; Neyts, J.; Danielson, U. H.; Sandström, A., Discovery of pyrazinone based compounds that potently inhibit the drug-resistant enzyme variant R155K of the hepatitis C virus NS3 protease. *Bioorganic & Medicinal Chemistry* **2016,** *24* (12), 2603-2620.
65. Trepat, X.; Chen, Z.; Jacobson, K., Cell Migration. *Compr Physiol* **2012,** *2* (4), 2369-92.
66. El-Senduny, F. F.; Badria, F. A.; El-Waseef, A. M.; Chauhan, S. C.; Halaweish, F., Approach for chemosensitization of cisplatin-resistant ovarian cancer by cucurbitacin B. *Tumour Biol* **2016,** *37* (1), 685-98.
67. McCubrey, J. A.; Steelman, L. S.; Chappell, W. H.; Abrams, S. L.; Wong, E. W. T.; Chang, F.; Lehmann, B.; Terrian, D. M.; Milella, M.; Tafuri, A.; Stivala, F.; Libra, M.; Basecke, J.; Evangelisti, C.; Martelli, A. M.; Franklin, R. A., ROLES OF THE RAF/MEK/ERK PATHWAY IN CELL GROWTH, MALIGNANT TRANSFORMATION AND DRUG RESISTANCE. *Biochim Biophys Acta* **2007,** *1773* (8), 1263-84.

68. Bréchot, C.; Gozuacik, D.; Murakami, Y.; Paterlini-Bréchot, P., Molecular bases for the development of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). *Seminars in Cancer Biology* **2000,** *10* (3), 211-231.
69. Zhai, B. J.; Wu, F.; Shao, Z. Y.; Hu, K.; Wang, Z. B., [Establishment of human multidrug-resistant hepatocellular carcinoma cell line (HepG2/Adm) and biological characteristics evaluation]. *Ai Zheng* **2004,** *23* (4), 391-5.

Chapter Four

Study the role of multidrug resistance associated proteins (MRPs) against sensitive HEPG2 and resistant HEPG2R cell lines

4.1 Introduction

Cancer cells have ability to develop resistance to structurally and mechanistically unrelated drugs over a period of time[1]. Chemotherapy is one of the major treatment modalities available for cancer patients. Unfortunately, during this course of treatment, cancer cells start develop resistance to functionally and structurally different anticancer drugs by either acquired (due to host factors) or intrinsic (due to genetic or epigenetic) mechanisms[2] . Later on, when the cells selected for resistance to a single cytotoxic drug, tumor cells may become resistant against an entire range of drugs with different chemical structures and cellular targets, a phenomenon called multidrug resistance (MDR)[3].

Many studies about MDR suggested that drug transport is a carefully controlled process, and this process was later found to be regulated by members of the ATP binding cassette (ABC) transporter family of proteins. P-glycoprotein (P-gp)/ABCB1 was the first identified ABC transporter[4] . In addition, this protein has been confirmed to play a role in many cellular functions. Recently, has been identified 49 different ABC transporters in humans, though there are more in bacteria and parasites[5] .

ABC transporters have been identified into seven subfamilies, ABCA to G, depends on sequence similarities. Of them the major ABC transporters involved in MDR development are ABC subfamily B member 1 [(ABCB1/P-glycoprotein (P-gp)], ABC subfamily G member 2 [ABCG2, also known as breast cancer resistance protein (BCRP)/mitoxantrone resistance protein (MXR)/placenta-specific ABC protein (ABCP)], and ABC subfamily C member 1 (ABCC1/MRP1)[6] .

The ABC transporter superfamily includes membrane proteins that include a wide variety of substrates across cellular membranes. An increasing number of chemotherapeutic drugs transported by ABC transporters have been recognized since the discovery of P-gp [1] **(Figure 4.1)**[7].

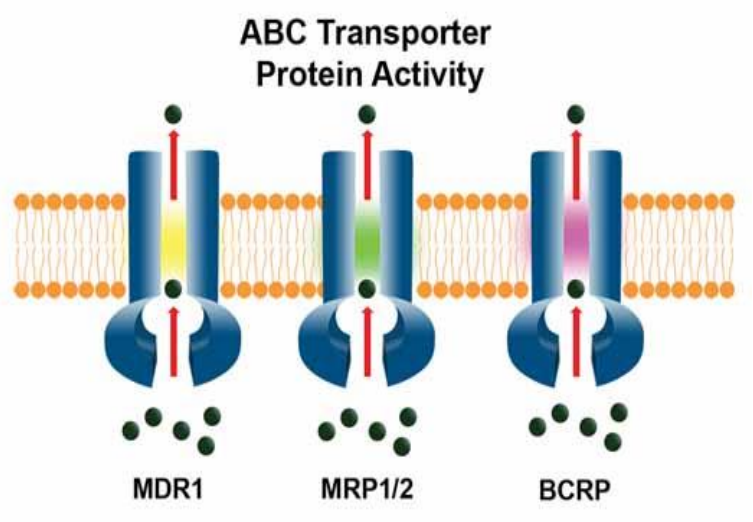

Figure 4.1 ABC transporters proteins in multidrug resistance that mediate chemotherapeutic drug resistance.

The MRP subfamily, the C subset of the ABC transporter superfamily, is classified to thirteen members, and nine of these are primarily involved in MDR **(Table4.1)**[8].These nine MRPs have been established as ATP dependent efflux transporters based on functional characterization, localization, and cloning studies for endogenous substances and xenobiotics. The other three members of the MRP subfamily, namely ABCC7/cystic fibrosis transmembrane conductance regulator (CFTR), ABCC8/sulfonylurea receptor SUR1), and ABCC9/SUR2, are not involved in conferring MDR[2]. The role of ABCC7 is to regulate chloride channel, whereas ABCC8 and ABCC9 are intracellular ATP sensors and regulate the specific K + channel permeability[9] .

Table 4.1 Summary of MRP members involved in MDR

MRP Member	Alternative name	Amino acid identity with MRP (%)	Tissue distribution
MRP1	ABCC1	100	Ubiquitous
MRP2	ABCC2, cMOAT, cMRP	50	Liver, kidney, gut
MRP3	ABCC3, MOAT-D, cMOAT-2	58	Liver, pancreas, kidney
MRP4	ABCC4, MOAT-B	41	Prostate, lung, muscles
MRP5	ABCC5, MOAT-C, Pabcc11	38	Ubiquitous

The nine main MRPs can be classified into two groups. One has a typical ABC transporter structure and is composed of two membranes spanning domains (MSD) with nucleotide binding domains (NBD1 and NBD2) in between **(Figure 1.7).** These can be referred to as a "short MRPs" and include MRP4, MRP5, MRP8, and MRP9 (ABCC4, 5, 11 and 13, respectively). The other group, which include MRP1, 2, 3, 6 and 7 (ABCC1, 2, 3, 6 and 7, respectively), have an additional MSD (MSD0) and are referred as "long MRP[10.]

Many studies have been confirmed that overexpression of MRP1 is associated with an increased transport rate of a range of substrates that are conjugated to glutathione (GSH), glucuronide, or sulfate[11,12]. This transporter mechanisms are known as glutathione conjugate (GS-X) pumps, or multi-specific organic anion transporters[3]. Besides organic anions MRP1 can also transport neutral and basic cytotoxic drugs not known to be conjugated to GSH or other negatively charged compounds[13]. Notwithstanding, MRP1 requires the presence of intracellular glutathione for the transport of these drugs. It is credible that MRP2 could play a role in drug resistance, just as MRP1 does. Studies with mutant rats (TR– /GY or EHBR) that lack the MRP2 protein in the hepatocanalicular membrane and transfection studies with MRP2 cDNA showed that the substrate specificity of MRP2 is very similar to that of MRP1[14], and suggested that MRP2 is able to transport several anticancer drugs[15].

4.2 ABC transporters as multidrug resistance mechanisms

The adenosine triphosphate (ATP)-binding cassette (ABC) transporters are found in all organisms. These transporters proteins have been reported to have a role in multidrug resistance in cancer. ABC transporters were so named because of the presence of a conserved ATP-binding domain which provides the energy required for a conformational change[16].

Figure 4.2 explains the general drug efflux mechanism of MDR transporters in cancer cells. First, the anticancer drug molecules penetrate the plasma membrane through passive diffusion (Figure 5.2a). Next the drug molecules bind to the TMDs, the NBDs are activated. Subsequently, ATP hydrolysis causes a major conformational change of the MDR transporter, which ultimately transports the drug molecules into the extracellular space (Figure 5.2b). This active transport of chemotherapeutic drugs out of the cell, severely reduces the amount of drug molecules accumulated in the cancer cells and the effectiveness of chemotherapy[17].

The protection of normal tissues such as kidney, liver, pancreas, and the endothelium of blood vessels of the brain is the vital role of ABC transporters. Among these transporters, the ones that are most likely to mediate chemotherapeutic drug resistance are permeability-glycoprotein (P-gp), multidrug resistance-associated protein-1 (MRP1) and breast cancer resistance protein (BCRP) **(Figure 1.5)**[18].

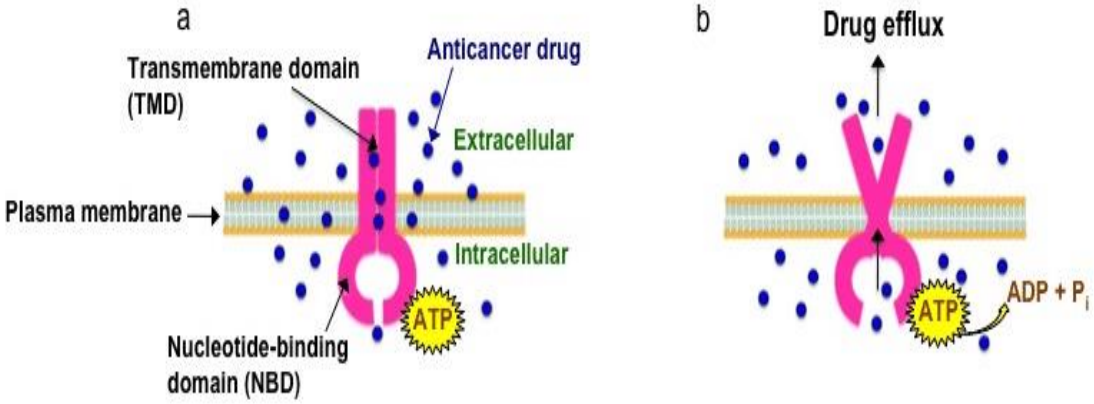

Figure 4.2 S diagram of the general drug efflux mechanism of an MDR transporter in the plasma membrane of a cancer cell. (a) Drug molecule enters the plasma membrane through passive diffusion. (b) The efflux pump, energized by the hydrolysis of a bound ATP molecule, ejects the drug molecules out of the cell. Redrawn based on the schematic diagram published in Cancer Control.

4.3 MRP2/ABCC2

MRP2 is the second member of the MRP subfamily of ABC transporter. The first time MRP2 was cloned from rat hepatocyte and was named as a hepatocellular canalicular multiple organic anion transporter (cMOAT)[19]. In addition, MRP2 shares 49% amino acid identity with MRP1 but it has a different expression pattern. While MRP1 is widely expressed in many tissues, MRP2 is mainly expressed in the apical (canalicular) hepatocyte plasma membrane, small intestine, and renal proximal tubules (Table 1)[2]. mRNA is present in the peripheral nerves, gallbladder, placental trophoblasts, and CD4 + lymphocyte[2, 20,21].

Because of the similarity between MRP2 and MARP1, it was believed to confer resistance to similar anticancer drugs as well. This hypothesis was created based on an experiment in which an antisense RNA construct was introduced into human hepatocellular carcinoma HepG2 cells, causing in increased sensitivity to several anticancer drugs such as cisplatin, vinblastine, sorafenib, doxorubicin, and Erlotinib[19].

MRP2 have been demonstrated TO transport vinblastine in polarized Madin Darby canine kidney epithelial (MDCK) cells, proposing a potential role for MRP2 in vresistance.

In addition, transfected cells also conferred resistance to cisplatin, etoposide, doxorubicin, and epirubicin .This phenomenon is convincible to suggestion a potential role for MRP2 in drug resistance such as Erlotinib against HepG2 resistance cell line[15].

The expression of MRP2 has been reported in several human tumor cell lines of lung, gastric, renal, and colorectal cancers[22] . Moreover, few cisplatin and doxorubicin resistant cell lines have shown overexpression of MRP2[23]. Recently, Korita[24], suggest that efficacy of cisplatin-based chemotherapy in patients with hepatocellular carcinoma depends upon MRP2 expression level[24].

4.4 Physiological and pharmacological function of MRP2

Many studies have been confirmed that MRP2 is important from a pharmacological function. Firstly, MRP2 has essential role in the intracellularly formed glucuronide and GSH-conjugates of clinically important drugs[25]. Secondly, MRP2 is inhibition of the ATP-dependent excretion of bile also involved in the biliary excretion of non-conjugated anionic drug[26]. For example, the efficient biliary excretion of pravastatin, an HMG-CoA reductase inhibitor, this process is mediated by MRP2[27]. After exhibiting its pharmacological action in the liver, pravastatin is then excreted into the bile via MRP2 without metabolic conversion[28]. Thus, efficient biliary excretion by MRP2 plays an important role in the entero-hepatic circulation, which is responsible for maintaining significant plasma concentrations of drugs. The mechanism for the substrate recognition by MRP2 still remains to be clarified, although Han et al.[29] suggested the importance of the non-polar surface area in determining the affinity using a series of methotrexate analogues[29].

Many tumor tissues have been reported that MRP2 is also expressed in some of them such as ovarian carcinoma[30], colorectal carcinoma[31], leukemia[32], and hepatocarcinoma[33]. Since the transfection of MRP2 cDNA to mammalian cells results in the conquest of drug resistance against antitumor drugs such as etoposide, vincristine, cisplatin, doxorubicin, Sorafenib and Erlotinib[34], it is possible that the tumor cells overexpressing MRP2 gain the multidrug resistance. For example , in colorectal carcinoma, a significant correlation has been observed between MRP2 mRNA levels and cisplatin

resistance , Although it has been assumed that GSH is required for the excretion of P-gp substrate antitumor drugs , the precise mechanism for the transport still remains un clear[35].

Depends on all these previous studies, we hypothesized that MRP2 expression level in HepG2-R to Erlotinib should be increase.

Aim of this study: Therefore, is convincible to suggestion a potential role for MRP2 in drug resistance such as Erlotinib against HepG2 resistance cell line and Studying the role of multidrug resistance associated protein (MRPs) against sensitive HEPG2 cell line and resistant HEPG2R cell line**.**

4.5 Material and Methods

4.5.1 Detection of expression levels of MRP2 on sensitive and resistant HepG2 cell line

Western blot analysis of HepG2 protein lysate for resistant and sensitive cells line were prepared and done as previously described in western blot method page 63. Cells were lysed without any treatment with 1X RIPA buffer(Thermoscientific™Pierce™) containing protease and phosphatase inhibitors (Thermo Scientific™). The BCA protein assay was used to quantify total protein concentration. An amount of 20 µg was loaded onto 8% SDSPAGE per well, blotted onto 0.45micron nitrocellulose membrane for 120 minutes at a current of 400mA. Because MRP2 is a large protein ,10% methanol was used in the transfer buffer instead of 20%. Subsequently, blocking was done with 5% BSA (Sigma Aldrich), and incubated with MRP2 primary antibodies overnight at 4°C. The membrane was subjected to the corresponding IR-conjugated secondary antibodies. The membrane was developed by using LiCOR odyssy imager. β-actin was used as a positive control.

4.5.2 Study the proliferation effect of MK571 on HepG2-R alone and in combination with MMA 132 and MMA 102

The resistance HepG2-R cancer cells (A2780) were seeded in 96-well plate as $5*10^4$ cells/mL (100 µL/well). A serial dilution of MK571(Cayman Chemical (Ann Arbor, MI) was added after overnight incubation of the cells at 37°C and 5% CO2 at a final

concentration of 25 and 15µM, and DMSO (Acros Organics) was used as a control (0.1 %). The cells were incubated with the MK571 for 48 hrs. After that 15 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide (MTT) (5 mg/mL PBS) (Hyclone™ L aboratories, Inc) were added to each well and the plate was incubated for another 4 hrs. The formazan crystals were solubilized by 100 µL acidified SDS solution (10% SDS/0.01 N HCl) (Fisher BioReagents). Absorbance was measured after 14 hrs of incubation at 37°C and 5% CO2 at 570 nm by Hidex Sense Microplate readers

The resistance HepG2-R cells were seeded as in cytotoxicity assay and incubated with MMMA132 and MMA102 for 24 hrs followed by addition of serial dilutions of MK571 (15 µL) then incubated for a total of 48 hrs. The viability of the cells was measured by MTT. The control was cells treated with DMSO[36].

4.6 Results

4.6.1 Detection of expression levels of MRP2 on sensitive and resistant HepG2 cell line

The level of MRP2 was detected in HepG2 protein lysate for both resistant and sensitive cells line. Cells were lysed without any treatment. Western blot of resistance HepG2-R protein lysate demonstrated that MRP2 was highly expressed in HepG2-R about 93% than sensitive cells. In contrast to the sensitive cells, the western blot of protein lysate for sensitive HepG2 showed lees level expression of MRP2 to be about 44% **(Figure 4.3**).

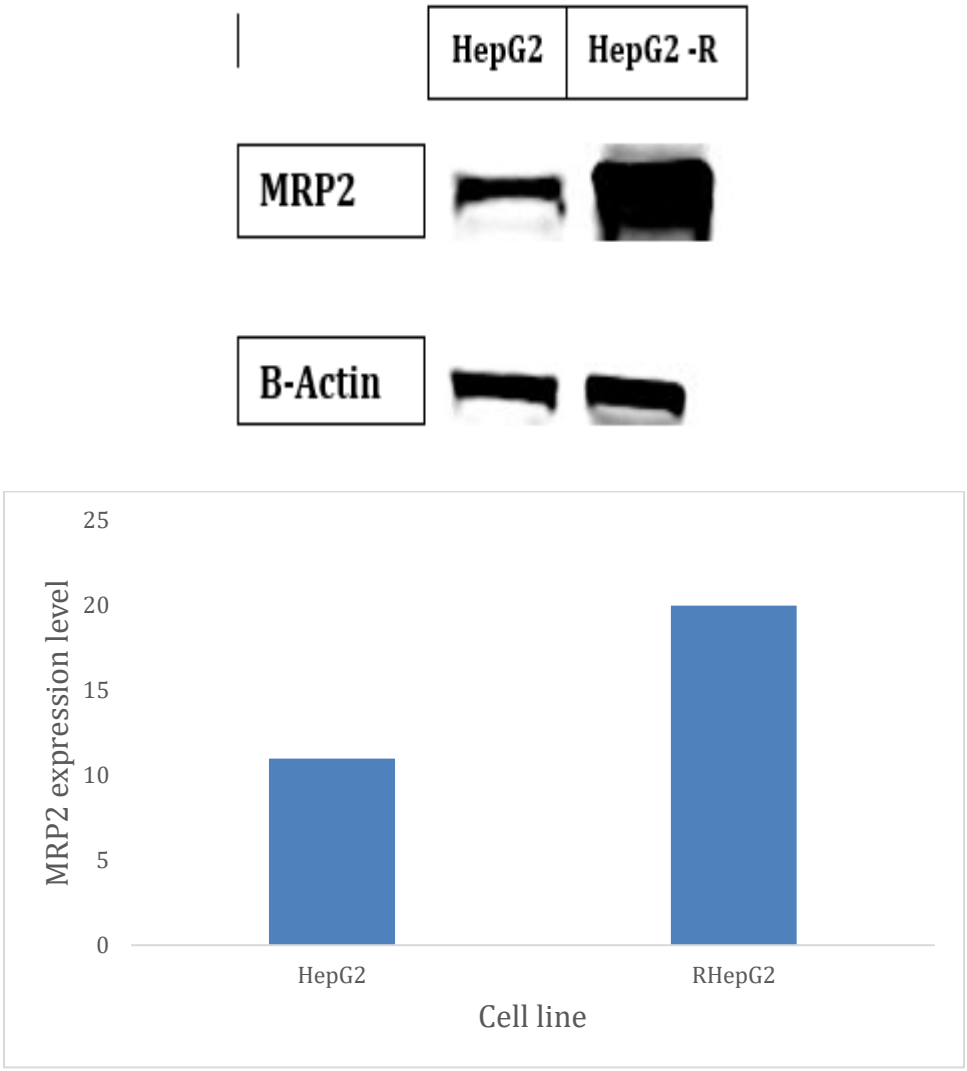

Figure 4.3 MRP2 expression level on resistant HepG2 and sensitive HepG2.

4.6.2 Study the proliferation effect of MK571(MRP1, MRP2 inhibitor) on HepG2-R alone and in combination with MMA 132 and MMA102

To determine the proliferation effect of MK571 (MRP1, MRP2 inhibitor) alone and its effect on combination treatment with CIEA analogs (MMA132 and MMA102) on the resistant hepatocellular carcinoma cancer cells, the cells were treated for 48 hrs with MK571 at a final concentration of 25 and 15 µM, and viability was determined by MTT assay. **Figure 4.4** and **table 4.2** show the cytotoxic effects of the MK571 on resistant HepG2-R cell lines. The IC_{50} values of each MK571 were 18.5 µM on both concentration

of MK571 (25 µM and 15 µM). After that, the proliferation activity of each MMA132 and MMA102 combined with MK571 was tested by pre-incubating the cells with MMA132 and MMA102, according to their respective IC_{50} values against HepG2-R (13 µM MMA132 and 20 µM MMA102), followed by incubation with serial dilutions of MK571. It was found that the most notably enhanced cell death was evident in cells treated with MMA132 (13 µM) combined with 15 µM of MK571 inhibitor, IC_{50} value was about 10 µM, comparing to MMA132 alone and MK571 alone on HepG2-R. In this case the presence of MK571 led to enhance the effect of MMA132 on HepG2-R and reducing cell viability after 48 hrs. With MMA102, MK571 did not have any effect in these cells line HepG2-R.

From our data, MMA132 has antiproliferative activity on multidrug resistance against HepG2-R cell line alone more than the stander MRP2 inhibitor MK571. It's 13 µM compared to MK571 (18.5 µM). Additionally, when it has been combined with MRP2 inhibitor, the cytotoxicity increased to 10 µM. In general, all these results make our CIEA as a promising potential novel drug candidate that play a role for MRP2 in drug resistance such as Erlotinib in this study against HepG2 resistance cell line.

Table 4.2 antiproliferation result against HepG2-R

Compound	**IC50(µM)**
MK571 (25µM)	18.5
MK571 (15µM)	19
MMA132+MK571	10
MMA102+MK571	18
MMA132	13
MMA102	20

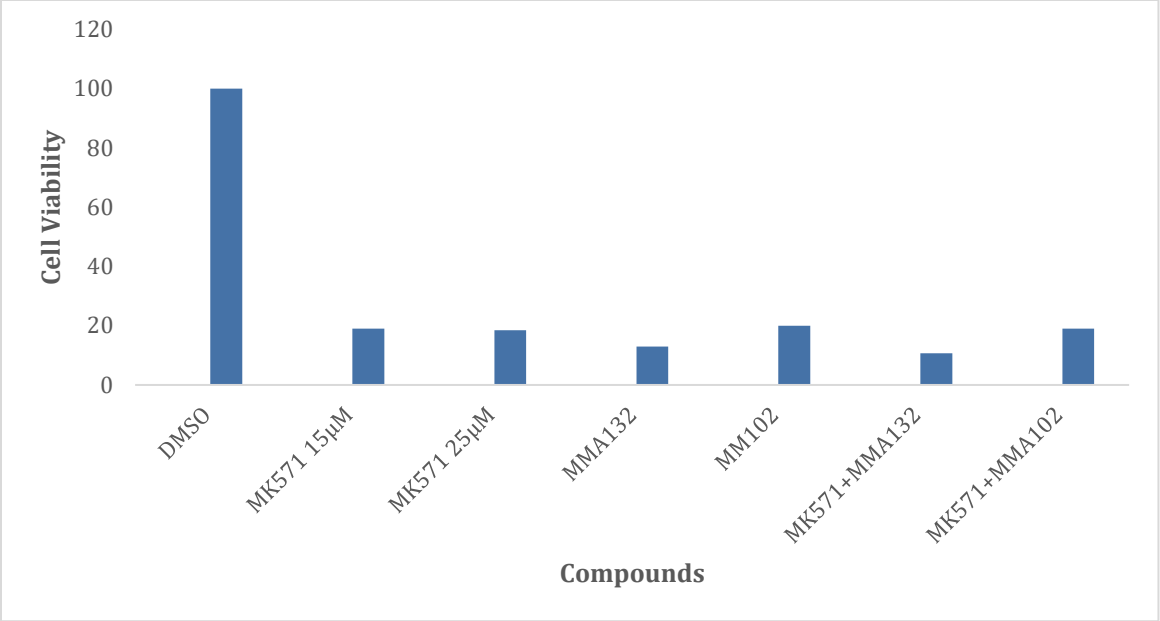

Figure 4.4 Cells pretreated with 13 µM and 20 µM of MMA132 and MMA102, respectively, then treated with serial dilutions of MK571. The total incubation time was 48 hrs. The cells treated with DMSO as a control.

4.7 Discussion

Previous studies showed that MRP2 plays a role in drug resistance, just as MRP1 does. Because MRP2 handles a wide range of conjugates similar to that of MRP1, it was believed to confer resistance to similar anticancer drugs as well[20]. Moreover, it has been suggested that MRP2 interfere in transporting several anticancer drugs[15]. In the present study, MRP2 expression level was different in comparison of sensitive cells to resistance cells.

MRP2 expression level was extremely low in sensitive HepG2 cell line and expressed two times less than resistance cell 44%. In contrast, the expression level of MRP2 was increased in resistant HepG2 cell line to Erlotinib 93% comparing to β-Actin as loading control. It has been reported before by Charls et al,2000, that MRP2 has been expressed in sensitive HepG2 cell line by western blot[37].

In addition, it was found an antisense RNA construct was introduced into human hepatocellular carcinoma HepG2 cells, resulting in enhanced sensitivity to several anticancer such as cisplatin[2].

These findings indicate that MK571 (MRP2 inhibitor) alone showed an inhibitory effect on resistant HepG2-R cancer cell lines 18.5 µM. Moreover, the combination of 13 µM of MMA132 with 15 µM of MK571 leads to a significant increase in the inhibitory effect of MK571 from 18.5 µM to 10 µM. These combinations of drugs were less effectively with MMA102 (20 µM) and the data showed almost no change of MK571 activity after this combination.

MMA132 showed a potential inhibitory activity when it combined with MK571 against HepG2-R as MRP2 inhibitor to enhance the activity of the stander inhibitor from 18.5 µM to 10 µM after 48 hrs incubation.

The difference in structure and functional between MMA102 and MMA132 which makes MM132 more active biologically, that MMA132 and MMA102 are diastereomers with binding mode with the receptor. MMA132, which possess the stereochemistry of cucurbitacin D side chain, showed an outstanding binding mode with EGFR by forming H-bond with MET:769: A, which is same amino acids residues that erlotinib binds to in EGFR to induce anti-cancer activity by H bonding with the same amino acid MET:769-A. Also, MMA132 has hydrophobic interactions with the amino acids residues inside the binding pocket while MMA102 which has the opposite stereochemistry of cucurbitacin D demonstrated less binding affinity towards the EGFR binding site only with a hydrophobic interaction mode with the EGFR binding pocket. In general, this result proved the significant activity of MMA132 more than MMA102.

4.8 Summary and Conclusion

In summary, MRP2 has been highly expressed in resistance HepG2 cell line to Erlotinib. In contrast the sensitive HepG2 cell line, the level of MRP2 was extremally low comparing to resistance cell line to Erlotinib. In addition, MK571 (MRP2 inhibitor) showed moderate activity against resistant HepG2 cell (18.5 µM). Also, combination 13 µM of MMA 132

with 15 µM of MK571 enhanced the activity of MK571 from 18.5 µM to 10 µM. On the other hand, combination of 20 µM of MMA102 with 15 µM does not change the activity of MK571.

In the meantime, cytotoxicity of MK571 enhanced significantly in combination with 13 µM of MMA132. A significant increase of MK571 cytotoxicity from 18.5 µM to 10 µM. This clearly demonstrate the potential anticancer activity of MMA132 in controlling HepG2-R to Erlotinib.

4.9 References

1. Sun, Y. L.; Patel, A.; Kumar, P.; Chen, Z. S., Role of ABC transporters in cancer chemotherapy. *Chinese Journal of Cancer* **2012,** *31* (2), 51-7.
2. Sodani, K.; Patel, A.; Kathawala, R. J.; Chen, Z. S., Multidrug resistance associated proteins in multidrug resistance. *Chinese Journal of Cancer* **2012,** *31* (2), 58-72.
3. Evers, R.; de Haas, M.; Sparidans, R.; Beijnen, J.; Wielinga, P. R.; Lankelma, J.; Borst, P., Vinblastine and sulfinpyrazone export by the multidrug resistance protein MRP2 is associated with glutathione export. *British Journal of Cancer* **2000,** *83* (3), 375-383.
4. Juliano, R. L.; Ling, V., A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1976,** *455* (1), 152-162.
5. Vasiliou, V.; Vasiliou, K.; Nebert, D. W., Human ATP-binding cassette (ABC) transporter family. *Human Genomics* **2009,** *3* (3), 281.
6. El-Awady, R.; Saleh, E.; Hashim, A.; Soliman, N.; Dallah, A.; Elrasheed, A.; Elakraa, G., The Role of Eukaryotic and Prokaryotic ABC Transporter Family in Failure of Chemotherapy. *Frontiers in Pharmacology* **2016,** *7*.
7. Zou, X.-Q.; Peng, S.-M.; Hu, C.-P.; Tan, L.-F.; Deng, H.-W.; Li, Y.-J., Furoxan nitric oxide donor coupled chrysin derivatives: Synthesis and vasculoprotection. *Bioorganic & Medicinal Chemistry Letters* **2011,** *21* (4), 1222-1226.
8. Dean, M.; Allikmets, R., Complete characterization of the human ABC gene family. *J Bioenerg Biomembr* **2001,** *33* (6), 475-9.
9. Keppler, D., Multidrug resistance proteins (MRPs, ABCCs): importance for pathophysiology and drug therapy. *Handb Exp Pharmacol* **2011,** (201), 299-323.
10. Cole, S. P.; Bhardwaj, G.; Gerlach, J. H.; Mackie, J. E.; Grant, C. E.; Almquist, K. C.; Stewart, A. J.; Kurz, E. U.; Duncan, A. M.; Deeley, R. G., Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* **1992,** *258* (5088), 1650-4.
11. Leier, I.; Jedlitschky, G.; Buchholz, U.; Cole, S. P.; Deeley, R. G.; Keppler, D., The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. *J Biol Chem* **1994,** *269* (45), 27807-10.

12. Jedlitschky, G.; Leier, I.; Buchholz, U.; Hummel-Eisenbeiss, J.; Burchell, B.; Keppler, D., ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2. *Biochem J* **1997,** *327 (Pt 1)*, 305-10.
13. Cole, S. P.; Sparks, K. E.; Fraser, K.; Loe, D. W.; Grant, C. E.; Wilson, G. M.; Deeley, R. G., Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Res* **1994,** *54* (22), 5902-10.
14. Jedlitschky, G.; Leier, I.; Buchholz, U.; Barnouin, K.; Kurz, G.; Keppler, D., Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. *Cancer Research* **1996,** *56* (5), 988-994.
15. Cui, Y.; Konig, J.; Buchholz, J. K.; Spring, H.; Leier, I.; Keppler, D., Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. *Mol Pharmacol* **1999,** *55* (5), 929-37.
16. Choi, C.-H., ABC transporters as multidrug resistance mechanisms and the development of chemosensitizers for their reversal. *Cancer cell international* **2005,** *5* (1), 30.
17. Thomas, H.; Coley, H. M., Overcoming multidrug resistance in cancer: an update on the clinical strategy of inhibiting p-glycoprotein. *Cancer control* **2003,** *10* (2), 159-165.
18. Gottesman, M. M.; Fojo, T.; Bates, S. E., Multidrug resistance in cancer: role of ATP–dependent transporters. *Nature Reviews Cancer* **2002,** *2* (1), 48.
19. Buchler, M.; Konig, J.; Brom, M.; Kartenbeck, J.; Spring, H.; Horie, T.; Keppler, D., cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. *J Biol Chem* **1996,** *271* (25), 15091-8.
20. Kool, M.; de Haas, M.; Scheffer, G. L.; Scheper, R. J.; van Eijk, M. J.; Juijn, J. A.; Baas, F.; Borst, P., Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Res* **1997,** *57* (16), 3537-47.

21. Rost, D.; Konig, J.; Weiss, G.; Klar, E.; Stremmel, W.; Keppler, D., Expression and localization of the multidrug resistance proteins MRP2 and MRP3 in human gallbladder epithelia. *Gastroenterology* **2001,** *121* (5), 1203-8.
22. Narasaki, F.; Oka, M.; Nakano, R.; Ikeda, K.; Fukuda, M.; Nakamura, T.; Soda, H.; Nakagawa, M.; Kuwano, M.; Kohno, S., Human Canalicular Multispecific Organic Anion Transporter (cMOAT) Is Expressed in Human Lung, Gastric, and Colorectal Cancer Cells. *Biochemical and Biophysical Research Communications* **1997,** *240* (3), 606-611.
23. Taniguchi, K.; Wada, M.; Kohno, K.; Nakamura, T.; Kawabe, T.; Kawakami, M.; Kagotani, K.; Okumura, K.; Akiyama, S.-i.; Kuwano, M., A Human Canalicular Multispecific Organic Anion Transporter (cMOAT) Gene Is Overexpressed in Cisplatin-resistant Human Cancer Cell Lines with Decreased Drug Accumulation. *Cancer Research* **1996,** *56* (18), 4124-4129.
24. Korita, P. V.; Wakai, T.; Shirai, Y.; Matsuda, Y.; Sakata, J.; Takamura, M.; Yano, M.; Sanpei, A.; Aoyagi, Y.; Hatakeyama, K.; Ajioka, Y., Multidrug resistance-associated protein 2 determines the efficacy of cisplatin in patients with hepatocellular carcinoma. *Oncol Rep* **2010,** *23* (4), 965-72.
25. Suzuki, H.; Sugiyama, Y., Single nucleotide polymorphisms in multidrug resistance associated protein 2 (MRP2/ABCC2): its impact on drug disposition. *Adv Drug Deliver Rev* **2002,** *54* (10), 1311-1331.
26. Suzuki, H.; Sugiyama, Y., Excretion of GSSG and glutathione conjugates mediated by MRP1 and cMOAT/MRP2. *Semin Liver Dis* **1998,** *18* (4), 359-76.
27. Suzuki, H.; Sugiyama, Y., Transporters for bile acids and organic anions. *Pharm Biotechnol* **1999,** *12*, 387-439.
28. Sasaki, M.; Suzuki, H.; Ito, K.; Abe, T.; Sugiyama, Y., Transcellular transport of organic anions across a double-transfected Madin-Darby canine kidney II cell monolayer expressing both human organic anion-transporting polypeptide (OATP2/SLC21A6) and multidrug resistance-associated protein 2 (MRP2/ABCC2). *Journal of Biological Chemistry* **2002,** *277* (8), 6497-6503.

29. Niinuma, K.; Kato, Y.; Suzuki, H.; Tyson, C. A.; Weizer, V.; Dabbs, J. E.; Froehlich, R.; Green, C. E.; Sugiyama, Y., Primary active transport of organic anions on bile canalicular membrane in humans. *Am J Physiol* **1999,** *276* (5 Pt 1), G1153-64.
30. Arts, H. J.; Katsaros, D.; de Vries, E. G.; Massobrio, M.; Genta, F.; Danese, S.; Arisio, R.; Scheper, R. J.; Kool, M.; Scheffer, G. L.; Willemse, P. H.; van der Zee, A. G.; Suurmeijer, A. J., Drug resistance-associated markers P-glycoprotein, multidrug resistance-associated protein 1, multidrug resistance-associated protein 2, and lung resistance protein as prognostic factors in ovarian carcinoma. *Clin Cancer Res* **1999,** *5* (10), 2798-805.
31. Hinoshita, E.; Uchiumi, T.; Taguchi, K.; Kinukawa, N.; Tsuneyoshi, M.; Maehara, Y.; Sugimachi, K.; Kuwano, M., Increased expression of an ATP-binding cassette superfamily transporter, multidrug resistance protein 2, in human colorectal carcinomas. *Clin Cancer Res* **2000,** *6* (6), 2401-7.
32. van Der Kolk, D. M.; Vellenga, E.; van Der Veen, A. Y.; Noordhoek, L.; Timmer-Bosscha, H.; Ossenkoppele, G. J.; Raymakers, R. A.; Muller, M.; van Den Berg, E.; de Vries, E. G., Deletion of the multidrug resistance protein MRP1 gene in acute myeloid leukemia: the impact on MRP activity. *Blood* **2000,** *95* (11), 3514-9.
33. Nies, A. T.; Konig, J.; Pfannschmidt, M.; Klar, E.; Hofmann, W. J.; Keppler, D., Expression of the multidrug resistance proteins MRP2 and MRP3 in human hepatocellular carcinoma. *Int J Cancer* **2001,** *94* (4), 492-9.
34. Guo, A.; Marinaro, W.; Hu, P.; Sinko, P. J., Delineating the contribution of secretory transporters in the efflux of etoposide using Madin-Darby canine kidney (MDCK) cells overexpressing P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP1), and canalicular multispecific organic anion transporter (cMOAT). *Drug Metab Dispos* **2002,** *30* (4), 457-63.
35. Ishikawa, T.; Kuo, M. T.; Furuta, K.; Suzuki, M., The human multidrug resistance-associated protein (MRP) gene family: from biological function to drug molecular design. *Clin Chem Lab Med* **2000,** *38* (9), 893-7.
36. El-Senduny, F. F.; Badria, F. A.; El-Waseef, A. M.; Chauhan, S. C.; Halaweish, F., Approach for chemosensitization of cisplatin-resistant ovarian cancer by cucurbitacin B. *Tumour Biol* **2016,** *37* (1), 685-98.

37. Morrow, C. S.; Smitherman, P. K.; Townsend, A. J., Role of multidrug-resistance protein 2 in glutathione S-transferase P1-1-mediated resistance to 4-nitroquinoline 1-oxide toxicities in HepG2 cells. *Mol Carcinog* **2000,** *29* (3), 170-8.