Republic of Iraq
Ministry of Higher Education and Scientific Research
University of Babylon
College of Medicine
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Effect of hydroxychloroquine and metformin on the cytotoxicity of cisplatin on colorectal and lung cancer cell lines .

A Thesis Submitted to the Council of College of Medicine-University of Babylon in partial of the Requirements for the Degree of master's in pharmacology/ pharmacology and toxicology

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1443 A.H

2022 A.D

بسم الله الرحمن الرحيم

يرفع الله الذين آمنوا منكم والذين

أوتوا العلم درجات ۚ والله بما

تعملون خبير ﴿١١﴾

صدق الله العليَ العظيم

سورة المجادلة اية١١

Certification

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Dedication

I dedicate this project to God Almighty, my creator, my strong pillar, my source of inspiration, wisdom, knowledge and understanding. He has been the source of my strength throughout this program. Also dedicate my thesis work to my family. A special feeling of gratitude to my lovely husband and parents, who encouraged me all the way. Sisters and brothers never left my side were very special. I also dedicate this thesis to many friends who have supported me throughout the process, and will always appreciate all they have done, along with the hardworking and respected supervisors and all who's inspired me .

Istabraq saeed abbas

Acknowledgements

First, I want to thank God for helping me and giving me strength, willingness, and patience to finish what I have started. I would like to express my great appreciation to Prof. Dr. Nisreen J. Muhammed & Prof Dr. Rana Ayad Ghaleb for their supervision, help, continuous advice, and efforts for helping me.

I would like to express my deepest gratitude and thanks to Dr. Qaisar Nema Madhlum and Dr. hamed najy for help and contribution to the laboratory.

I would like to express my deepest gratitude to the dean of Medicine College and head of Pharmacology department, for their kindness and help. Thanks go to all the staff in the Department of Pharmacology, College of Medicine, University of Babylon.

I owe a special thanks to my family, who supported me and helped me throughout my life, and during this study. Their love, emotion, and moral has been and always be the source of my inspiration, I dedicate this work to them all.

Summary

The study done in the postgraduate research laboratory, cell culture laboratory in the College of Medicine\ University of Babylon during the period from January 2021- December 2021.

- The study aimed to determine the cytotoxic effect of cisplatin, metformin and hydroxychloroquine as single or in combination with each other after determination of cytotoxic concentration on lung cancer A549 cell line and colorectal cancer SW480 cell line.

- study the effect of autophagy induction or inhibition on CDKN2A.

The experiment

Part 1 : the cytotoxicity assay

First experiment:-

* The 96-well plates were seeded with human lung cancer A549 cell line and colorectal cancer SW480 cell line in a seeding density of $5*10^5$ and the wells of the plate (except one column from each plate which let without treatment as a control), exposed to 200 µL of six serial dilutions of cisplatin as follow (750 µg/ml, 375 µg/ml, 187.5µg/ml 93.75 ,µg/ml , 46.875 µg/ml and 23.437 µg/ml). The plates were covered with the plastic lids and incubated for 24 hours. Afterward, the plates were washed with 200 µl of a sterile PBS, and the effect of the cisplatin on cell lines growth was assessed by crystal violet cytotoxicity assay .

* Same procedure done by using metformin at serial dilutions of (500µg/ml , 250µg/ml , 125 µg/ml , 62.5 µg/ml , 31.25µg/ml and 15.8µg/ml) and incubate for 24 hours, then viability test was done using crystal violate assay.

* The same procedure was used but with hydroxychloroquine serial dilutions (500µg/ml , 250µg/ml , 125 µg/ml , 62.5

µg/ml , 31.25µg/ml and 15.8 µg/ml) and incubate for 24 hours, then crystal violate assay was done to measure viability.

Second experiment: the same procedure used for all combination and the effect of combination on cell viability don by crystal violate assay .

* Combination of cisplatin serial dilution and hydroxychloroquine 25 µg/ml.

* combination of cisplatin serial dilution and hydroxychloroquine 125 µg/ml.

* combination of cisplatin serial dilution and metformin 10 µg/ml.

* combination of cisplatin serial dilution and metformin 50 µg/ml , then by crystal violet assay measure the viability of cell.

The results showed that there were significant ($p \leq 0.05$) decrease in viability percentage of A549 cell line in all concentration used of cisplatin except (23.347 , 46. 875) µg/ml.

While significant ($p \leq 0.001$) decrease in the viability percentage of SW480 cell line for all concentration in comparison with control group

The result showed that there were significant ($p \leq 0.05$) increase in viability percentage of A549 cell line with high concentration (500µg/ml) metformin only.

while metformin decrease viability percentage significantly ($p \leq 0.001$) of SW480 cell line when used at high concentrations (250 and 500µg/ml) only in comparison with the control group

The result showed that there is significant ($p \leq 0.001$, $p \leq 0.05$) decrease in cell viability percentage of A549 and SW480 cell lines in

all concentrations used of hydroxychloroquine in comparison with control group except (31.25µg/ml and 15.8 µg/ml)

The result of combination of hydroxychloroquine and cisplatin at different concentration showed that there were significant decrease ($p \leq 0.001$) in viability percentage of A549 cell line with all concentrations of cisplatin and constant concentration of hydroxychloroquine (25 µg/ml) in comparison with the control group.

when the combination used on SW480 the results showed that there were significant decrease ($p \leq 0.001$) in viability percentage in all combinations except for (750cisplatin +25 hydroxychloroquine and 375cisplatin +25 hydroxychloroquine) .

The result of combination of hydroxychloroquine and cisplatin at different concentration showed that there were significant ($p \leq 0.001$) decrease in viability percentage of both A549 and SW480 cell line with all concentrations of cisplatin and constant concentration of hydroxychloroquine (125 µg/ml) in comparison with the control group

There were significant ($p \leq 0.001$) decrease in viability percentage of A549 cell line at higher concentrations of cisplatin (750 , 375 , 187.5 and 93.75 µg/ml) and constant concentration of metformin in comparison with the control .

The results also showed that there were significant ($p \leq 0.001$) decrease in viability percentage of SW480 cell line at all concentrations of cisplatin with constant concentration of metformin (10 µg/ml) .

There were significant ($p \leq 0.05$) decrease in viability percentage of A549 cell line only at high concentrations of cisplatin (750 and 375µg/ml) and constant concentration of metformin 50 µg /ml.

There were significant ($p \leq 0.001$) decrease in viability percentage of SW480 cell line at all concentrations of cisplatin with constant concentration of metformin (50 µg/ml) .

Part II: determine the level of Cyclin Dependant Kinase inhibitor 2A (CDKN2A)

By using the supernatant of the first part of experiment for each sample (A549) , by using (CDKN2A) kit procedure and by ELISA technique to determine the effects of autophagy induction and inhibition on CDKN2A concentration .

Then by statistical analysis the obtained results were:-

There were significant decrease in CDKN2A concentration when use cisplatin alone or in combination with hydroxychloroquine 25µg/ml or 125µg/ml.

Metformin when use in combination with cisplatine, it decrease the activity of cisplatin on CDKN2A concentration , and the concentration of CDKN2A increase significantly when use low concentration of cisplatin with metformin 10 or 50µg/ml.

When use metformin alone at high concentration, there were significant increase in CDKN2A concentration and decrease with low concentrations.

When hydroxychloroquine use alone, there were significant decrease in CDKN2A concentration as the concentration of hydroxychloroquine increase.

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List of abbreviations

Abbreviation	Meaning
ABC	ATP biending casette
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATGs	Autophagy related gene
ATP	Adenosine triphosphate
BCA	Bacillus caldovelox arginase mutant
BCRP	Breast cancer resistance protein
℃	Centigrade
CDKN2A	CYCLIN DEPENDENT KINASE INHIBITOR 2A
CDKs	Cyclin kinase dependant
Cm	Centimeters
CNS	Central nervous system
CO_2	Carbon dioxide

COX-2	cyclooxygenase-2
CR	Calorie restriction
CRC	Colorectal cancer
CQ	chloroquine
CT or CAT	Computed tomography
DDW	deionized distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMH	1,2-Dimethylhydrazine
DMSO	dimethyl-sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	ethylenediamine tetra-acetic acid
EGFR	Epidermal growth factor receptor
FBS	Fetal Bovine Serum
g	Gram
GISTS	Gastrointestinal stromal tumors
HCQ	Hydroxychloroquine
IF	Imunoflorescence
LCC	Large cell carcinoma
LC3-I	Light chain 3-I
mg	Milligram

ml	Milliliter
MMP9	metalloproteinase-9
MMS	Matrix mitaloprotinases
MPFs	Medium progression – free survival
MTOR	Mechanistic target of rapamycin
NSCC	Nonsmall cell lung cancer
P53	Tumor suppressor gene
PBS	Phosphate buffer saline
PI	Propidium iodide
PCOS	Polycystic ovarian syndrome
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SCLS	Small cell lung cancer
SCC	Squamous cell carcinoma
T2D	Type 2 diabetes
VEGFR2	Vascular endothelial growth factor receptor
µg	Microgram

Chapter One

Introduction
and
Literature Reviews

1.1 introduction

Autophagy is a physiological cellular process for the degradation and elimination of misfolded proteins and damaged organelles that functions in adaptation to starvation, development, cell death, and tumor suppression, one of the important mechanisms of autophagy is an intracellular degradation pathway mediated by double membrane vesicles called autophagosomes. These autophagosomes deliver degraded cytoplasmic components to the lysosome to be recycled during stressful conditions. This mechanism of autophagy is essential for protecting cells from damaged proteins, to shield cell organelles from toxins, to maintain cell metabolism and energy homeostasis, and to promote cell survival (Kraft et al., 2009).

Recently, it has been reported that defects of autophagy are associated with genomic damage, metabolic stress, and tumorigenesis, dysfunction of autophagy leads to cell death, cancer, neurodegenerative, and other diseases (Mizushima et al., 2008).

In cancer biology, autophagy plays dual roles in tumor promotion and suppression and contributes to cancer-cell development and proliferation. Some anticancer drugs can regulate autophagy. Therefore, autophagy-regulated chemotherapy can be involved in cancer-cell survival or death. Additionally, the regulation of autophagy contributes to the expression of tumor suppressor proteins or oncogenes (Yun & Lee, 2018).

Aim of Study

1- Study the effect of chemotherapy (cisplatin) ,autophagy Inhibitor (hydroxychloroquine) and inducer (metformin) drugs , on two cancer cell line A549 (lung cancer) and SW480 (colon cancer) .

2- Study the effect of combination of (cisplatin) with autophagy Inhibitor (hydroxychloroquine) and with autophagy inducer (metformin) on both cancer cell lines.

3- Measurement of CDKN2A level in the presence of chemotherapy (cisplatin) with autophagy Inhibitor (hydroxychloroquine) and autophagy inducer (metformin).

1.2 Cancer

Cancer is an illness in which some of the body's cells grow uncontrollably and spread to other parts of the body (Mueller et al., 2018).

Cancer is a multifactorial disease, with genetics being an important contributing etiologic factor. In particular, mutations influencing DNA repair genes, cell cycle regulators and cell-death pathways are the major genetic causes of malignancies. recent research emphasizes the role of environmental factors in the emergence of oncological diseases. risk factors or risk habits for oncological diseases exist (eg, bad nutrition, no physical activity, smoking, and alcohol or junk food intake) (Saletta et al., 2015).

1.3 Lung cancer

Lung cancer, which is also known as carcinoma of the lung or pulmonary carcinoma, and is derived from epithelial cells, is a

malignant lung tumor characterized by uncontrolled cell growth in the tissues of the lung (Kanwal et al., 2017).

In some regions, particularly Asia, indoor air pollution and occupational exposures play a greater role in female lung cancer. Similar to the US, there is significant geographical and ethnic variation in lung cancer incidence and mortality within regions. Higher income countries have comparatively improved survival rates than low income countries. Of particular concern for the future is the recent rise of cigarette consumption in countries like China, where 65% of men initiate smoking by their mid-20s, presaging an epidemic of lung cancer in the next few decades (de Groot et al., 2018).

1.3.1 Lung cancer classification

Lung cancers are traditionally divided into

- non–small cell carcinoma (NSCC) with the former accounting for 80% of the cases
- small cell carcinoma (small cell lung carcinoma, SCLC), for the remaining 20%. (Kanwal, Ding and Cao, 2017).

SCLCs behave aggressively and are treated nonsurgically in most cases, whereas NSCCs are managed by a combination of surgery and adjuvant therapy. recognition of the diversity of NSCC has led to its subclassification, culminating in the 2015 World Health Organization (WHO) classifications Major types of NSCC include adenocarcinoma, squamous cell carcinoma (SSC), and large cell carcinoma (LCC) (Zheng, 2016).

1.4 Colorectal cancer

Colorectal cancer (CRC) is already the third leading cause of cancer death in the world, and its incidence is steadily rising in developing nations CRC is a type of gastrointestinal malignancy originating from either the colon or the rectum. Although both forms can be simply defined as colon or rectal cancers, depending on their origin, they are often merged because of the many biologically and clinically common features (Rawla et al., 2019).

Adenocarcinoma is the most frequent colorectal malignancy (up to 95% of cases), followed by carcinoid tumors, gastrointestinal stromal tumors (GISTs), lymphomas and sarcomas (Mattiuzzi et al., 2019)

Colorectal adenocarcinoma, CRC usually emerges from the glandular, epithelial cells of the large intestine. The cancer arises when certain cells of the epithelium acquire a series of genetic or epigenetic mutations that confer on them a selective advantage. With abnormally heightened replication and survival, these hyperproliferative cells give rise to a benign adenoma, which may then evolve into carcinoma and metastasize over decades.the CRC is more incident among men than women and 3–4 times more common in developed than in developing nations. Age-standardized (world) incidence rates per 100,000 of CRC in both sexes is 19.7, in males is 23.6, and in females is 16.3 (Rawla et al., 2019)

1.5 Autophagy

Autophagy is a physiological and dynamic process dependent on the formation of double-membrane vesicles to maintain metabolic homeostasis by capturing intracellular constituents, in-

cluding redundant or unnecessary proteins, injured or aged organelles, and later degrading them in lysosomes. Basal autophagy is widely accepted as a mechanism of cell survival under conditions of nutrient deprivation because the lysosome-released breakdown products are recycled into metabolic and biosynthetic pathways (Liu et al., 2020).

Autophagy is a process that allows cells to sequester cytoplasmic contents, through the formation of double-membrane vesicles (autophagosomes), and targets them for degradation, through their fusion with lysosomes, creating single-membrane autolysosomes. Emerging evidence indicates that autophagy is a self-protective cellular mechanism, which provides energy through the degradation and recycling of cytoplasmic contents and promotes cell survival in response to a variety of stimuli. In addition to inducing apoptosis, antineoplastic agents can also induce autophagy in cancer cells, and under certain conditions, autophagy and apoptosis seem to interact, either concurrently or sequentially, to positively or negatively determine cell fate (Zou et al., 2013) .

Autophagy can be general (non-selective) or selective. General autophagy packages portions of the cytoplasm into autophagosomes and delivers them to lysosomes for degradation. In contrast, selective autophagy works by recognizing specific targets, such as damaged cell organelles, protein aggregates, and intracellular pathogens(Yun & Lee, 2018).

Autophagy is also a cytoprotective mechanism against various environmental stresses, such as oxidant stress, endoplasmic reticulum stress, and viral or bacterial infection, by eliminating damaged and toxic cellular components and products. However, autophagy plays a dual role in both tumor-suppressing and -

promoting activity in cancer initiation, development, progression, and treatment by preventing the toxic accumulation of oncogenic signaling substances from carcinogenic factors, such as genomic injury to suppress cancer initiation. By contrast, cancer cells tend to utilize autophagy-mediated recyclable biomolecules to meet the increased metabolic energy demand of survival and proliferation and take advantage of the engulfment capability to overcome micro-environmental stress, which facilitates tumorigenesis and aggressiveness. It has been shown that cancer cells are more autophagy-dependent than normal tissues. Thus, targeting autophagy directly is a therapeutic strategy for cancer therapy (Liu et al., 2020).

Autophagy in cancer treatment is also context-dependent and complicated. There are generally two effects of autophagy in response to anticancer drugs or ionizing radiation treatment in cancer cells. One effect is the cytotoxic function known as autophagic cell death, also named type II programmed cell death. It is a non-apoptotic form of programmed cell death caused by over activated autophagy. Anticancer treatment induces robust autophagy of cancer cells to self-digestion until death. Many natural compounds and synthetic agents exhibit their anticancer effects through triggering autophagic cell death. Moreover, the activation of autophagy-related signaling may implicate the suppression of certain other cancer therapeutic target to help against cancer, such as tumor invasion and migration and tumor angiogenesis (Liu et al., 2020).

The other effect is the cytoprotective function, which is a drug resistance mechanism resulting in a clinical obstacle to successful cancer treatment and leads to a poor prognosis of the pa-

tients. The cancer cells initiate autophagy to escape from the damage of drugs or radiation. Efforts to inhibit treatment-induced autophagy has therefore attracted great interest to improve cancer therapy efficiency. By combining the antineoplastic agents, the application of autophagy inhibitors is considered beneficial to increase the susceptibility of cancer cells to therapeutic agents that induce autophagy (Liu et al., 2020).

1.5.1 Mechanism of autophagy

The process of autophagy begins with the formation of a membrane known as a phagophore (A double membrane) that encloses and isolate the cytoplasmic components during macroautophagy. Closure of the phagophore around the cellular components forms a double membrane called an autophagosome, the autophagosome then fuses with a lysosome, form single membrane autolysosome which utilizes acid hydrolase enzymes for degradation of its contents. The breakdown products are then recycled and utilized to promote cell survival, primarily through production of ATP (Settembre et al., 2013) as shown in (figure 1-1).

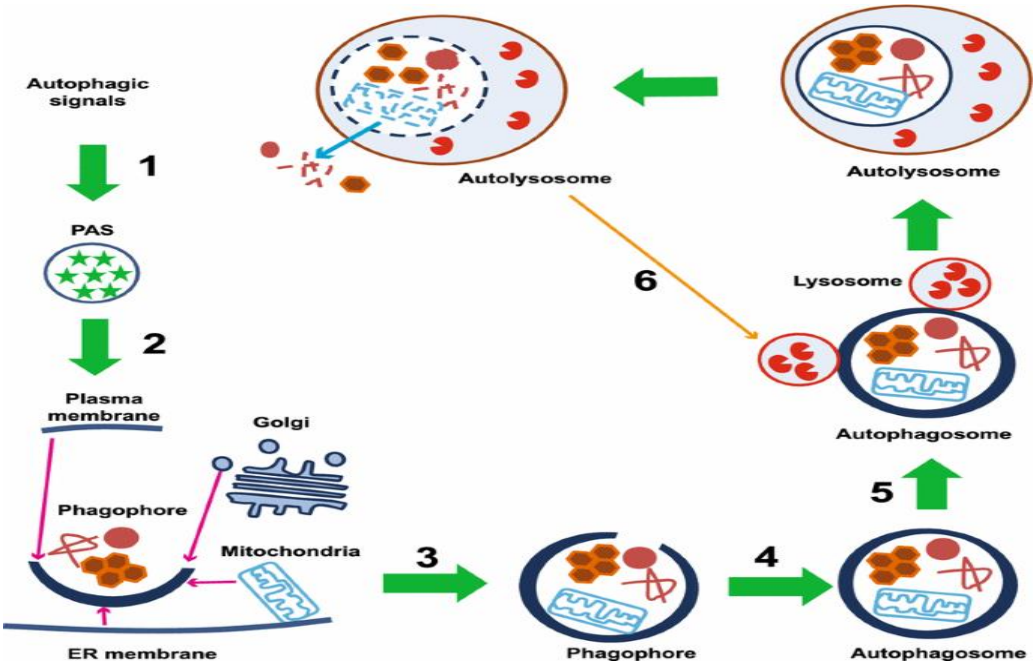


Figure (1-1) mechanism of autophagy(Hou et al., 2022)

Autophagy occurs under many physiological conditions. Notably it has been demonstrated that autophagy is critical during mammalian development and also during the period of nutrient deprivation immediately after birth. At the cellular level, autophagy is crucial in removing unfolded proteins and damaged or superfluous organelles such as mitochondria, peroxisomes, ribosomes, endoplasmic reticulum, or endosomes, and therefore acts as a ‘‘quality control’’ process. Under starvation and stress, autophagy is dramatically induced to recycle cellular components and to supply the building blocks so that cellular homeostasis is maintained. Autophagy is thus viewed as a cell survival process in response to stress (Pasquier, 2016).

1.5.2 Role of autophagy in cancer

Autophagy is a complex cellular process that appears to serve multiple roles depending on the cancer type and physiologic context. In normal healthy cells, autophagy may act as a tumor suppressor by clearing DNA damage and allowing the cell to correct problems before malignant transformation. In contrast, in established cancers, autophagy is believed to promote growth by allowing cancer cells to survive metabolic stress as the tumor microenvironment becomes hypoxic, acidotic and nutrient deprived. These findings support the “double edge sword” of targeting autophagy in cancer, whereby the autophagy may be beneficial in preventing development of malignancy in healthy or premalignant tissues, but it promotes cancer growth in established tumors (H.-Y. Chen & White, 2011).

Autophagy is considered a cytoprotective function in cancer therapy under certain conditions and is a drug resistance mechanism that represents a clinical obstacle to successful cancer treatment and leads to poor prognosis in cancer patients. Because certain clinical drugs and agents in development have cytoprotective autophagy effects, targeting autophagic pathways has emerged as a potential smarter strategy for cancer therapy. Efforts to inhibit treatment-induced autophagy has therefore attracted great interest to improve cancer therapy efficiency. By combining the antineoplastic agents, the application of autophagy inhibitors is considered beneficial to increase the susceptibility of cancer cells to therapeutic agents that induce autophagy. by inhibiting autophagy; cancer cells can then be driven to apoptotic cell death. Therefore, the addition of autophagy inhibition to chemotherapy may improve outcomes by promoting apoptotic cell death and decreasing resistance to treatment (Liu et al., 2020).

Autophagy-competent tumors may activate autophagy as an adaptive response to anticancer agents, in which case autophagy may act as a treatment resistance mechanism prolonging tumor cell survival. In this case, concurrent inhibition of autophagy is expected to deprive cancer cells of an essential coping mechanism, and, thus, enhance the efficacy of anticancer drugs. Given that apoptosis-defective cancer cells rely on autophagy for survival under metabolic stress, in contrast to tumors with intact apoptosis which may undergo rapid apoptotic cell death when stressed, it is also expected that autophagy inhibition will likely be therapeutically more beneficial in the treatment of tumors with apoptosis defects, but functional autophagy. Furthermore, the higher metabolic demands of rapidly proliferating cancer cells may render

these cells “addicted” to autophagy for survival, and, consequently, more vulnerable to autophagy inhibition than normal cells, a concept that can be exploited for preferential tumor cell killing and reduction of undesired treatment side-effects(N. Chen & Karantza-Wadsworth, 2009).

1.5.3 Autophagy inhibitor

Throughout the past decade, distinct approaches based on the inhibition of autophagy have been conceived and evaluated (in vitro and in vivo) for their ability to (1) mediate therapeutic effects as standalone interventions, or (2) boost the antineoplastic activity of conventional or targeted chemotherapeutics. autophagy was disabled pharmacologically, by the administration of (1) lysosomotropic agents including chlroquine, hydroxychloroquine and monensin, all of which inhibit the fusion of autophagosomes with lysosomes and their degradation (2) class III PI3K inhibitors, such as 3-methyladenine, wortmannin and pyrvinium (3) the V-type ATPase inhibitor bafilomycin A1, which inhibits lysosomal acidification and hence the degradation of autophagosomes . All these interventions have been shown to exert anticancer effects or to boost the activity of conventional antineoplastic regimens(Manic et al., 2014).

1.5.4 Hydroxychloroquine

The anti-malarial drug chloroquine a 4-alkylamino-substituted quinoline family member and a derivative, hydroxychloroquine are medications that have been used for a long time. Their most common use is for the treatment and prophylaxis of malaria. However, these antimalarial drugs are known to also have anti-inflammatory and antiviral effects and are used for sev-

eral chronic diseases such as systemic lupus erythematosus with low adverse effects. The antiviral action of hydroxychloroquine and chloroquine has been a point of interest to different researchers due to its mechanism of action. Several in vitro studies have proven their effectiveness on severe acute respiratory syndrome virus and currently both in vitro and in vivo studies have been conducted on 2019 novel coronavirus (covid-19) (Sinha & Balayla, 2020).

Hydroxychloroquine also has been shown to have antineoplastic effects in numerous preclinical experiments when combined with other agents. hydroxychloroquine inhibits autophagy by preventing the lysosome from degrading and recycling the materials engulfed in the autophagosome (Boone et al., 2018).

Hydroxychloroquine is the most commonly studied autophagy inhibitor in cancer and the sole autophagy inhibitor to be studied in clinical trials. Hydroxychloroquine is inexpensive, has high oral bioavailability and has an established safety profile due to use for treatment of malaria, lupus, and rheumatoid arthritis, facilitating its use in patients with lung and pancreatic cancer. hydroxychloroquine functions as a lysosomal inhibitor by blocking acidification of the lysosome, thereby preventing fusion of the autophagosome, inhibiting breakdown of its contents and blocking autophagy as shown in (figure 1.2).

Because it is a late inhibitor of autophagy, chloroquine treatment results in accumulation of autophagosomes and results in an increase in the autophagy marker LC3-II (Boone et al., 2018) .

Autophagy inhibition with HCQ can augment the cytotoxicity of a number of chemotherapies and targeted therapies. Many

preclinical studies have reported that the concept of combining HCQ with chemotherapies or radiotherapies may enhance the effect of anticancer, providing rationale for launching cancer clinical trials involving HCQ. Thus, the deliberate attempt to inhibit autophagy therapeutically to overcome resistance to chemotherapy has been accomplished through the publication of the first seven clinical trials (Shi et al., 2017) **.**

For clinical trials, hydroxychloroquine was chosen over chloroquine as an autophagy inhibitor because it is less toxic than chloroquine at peak concentrations. Thus high doses of hydroxychloroquine could be combined safely with other cytotoxic chemotherapies. 1200 mg hydroxychloroquine daily (600 mg twice a day), which was the highest dosage allowed by the food and drug administration, were well tolerated without the production of excess toxicity. That is no recurrent dose-limiting toxicities were observed when 200–1200 mg hydroxychloroquine were combined with antineoplastic agent in patients with advanced solid malignancies(Chude & Amaravadi, 2017)**.**

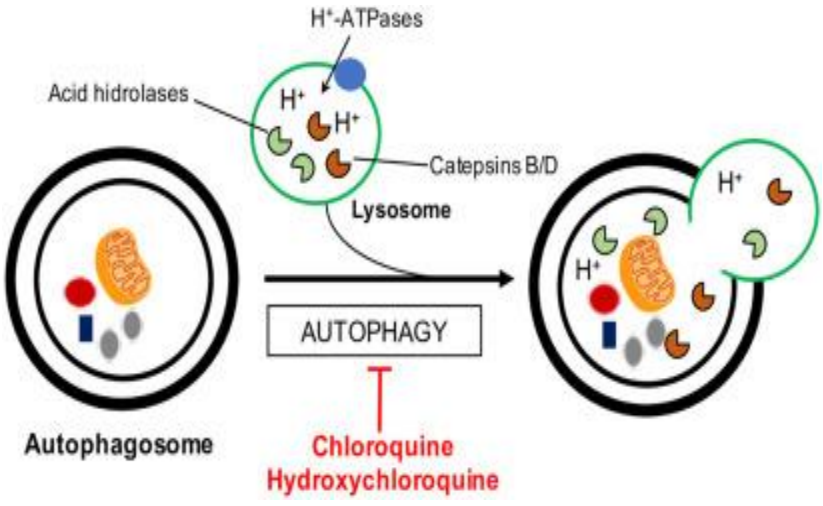


Figure (1-2) Chloroquine and hydroxychloroquine as autophagy inhibitor (Ferreira et al., 2021)**.**

1.5.5 Autophagy inducer

Considerable enthusiasm has emerged for the development of autophagy-inducing agents for the prevention or treatment of diseases in which the upregulation of autophagy is thought to be clinically beneficial. A common underlying pathophysiologic event in these diseases is the accumulation of harmful contents inside the cell — damaged organelles, protein aggregates, lipid droplets, or pathogens. In these circumstances, the pharmacologic (or non-pharmacologic) enhancement of autophagy-mediated delivery of deleterious structures for lysosomal destruction may be beneficial. Non-pharmacologic interventions such as caloric restriction and regular exercise induce autophagy and may improve overall health. In addition to caloric restriction, other nutritional factors such as the consumption of coffee and vitamin D, may also influence health through autophagy induction. Caffeine-induced autophagy reduces hepatic steatosis in mice with nonalcoholic fatty liver disease and protects against human prion protein–mediated neurotoxicity in cultured cells. Vitamin D is a potent inducer of autophagy, and through an autophagy-dependent mechanism it inhibits HIV and *M. tuberculosis* replication in human macrophages and kills human breast tumor cells. Defects in vitamin D–induced autophagy might therefore underlie the epidemiologic associations between vitamin D deficiency and adverse health outcomes, including susceptibility to certain cancers and infectious diseases. (De Santi et al., 2019).

Several drugs currently approved by the FDA induce autophagy (such as lithiuim, rapamycin, metformin, carbamazepine, clonidine, verapamil, vitamin D and statins) but generally have

pleiotropic actions, making it difficult to parse out the role of autophagy induction in their therapeutic actions in patients. FDA-approved compounds have been “repurposed” for use in preclinical models of diseases that are believed to respond favorably to autophagy enhancement, e.g., mTOR inhibitors in neurodegenerative diseases, EGFR and other tyrosine kinase inhibitors in diabetic nephropathy and neurodegenerative diseases, carbamazepine in α1-antitrypsin deficiency, trifluoperazine in *Salmonella* infection, and statins in *M. tuberculosis* infection.several approved and/or experimental drugs, together with natural compounds, have been reported to induce autophagy in different cancer types (De Santi et al., 2019).

1.5.6 Metformin

Metformin has been the first-line anti-hyperglycemic agent for patients newly diagnosed with type 2 diabetes, especially for obese diabetic patients. Its glucose-lowering effect of metformin mainly results from decreasing hepatic gluconeogenesis, reducing intestinal glucose absorption, improving glucose uptake of cells, and helping peripheral tissues utilize glucose (e.g. skeletal muscle and adipose tissue) via decreasing insulin resistance . Multiple pathways in these processes had been affirmed, such as inhibiting the mitochondrial respiratory chain (complex I) and mitochondrial glycerophosphate dehydrogenase, activating adenosine monophosphate-activated protein kinase (AMPK), inhibiting glucagon action of elevating cyclic adenosine monophosphate by reducing the activation of protein kinase A, and stimulating glucagon-like peptide-1 secretion which targets its receptors on the surface of

pancreatic β-cells and promoting insulin secretion by affecting gut microbiota , etc (De Santi et al., 2019).

Along with the antidiabetic effect on diabetes, metformin also owns other beneficial effects, such as anti-aging effects, aging-related pathologies (e.g. metabolic effects, cardiovascular disease, inflammation, cancer, modulation of the microbiome and neurological diseases), remedial actions for polycystic ovarian syndrome (PCOS), a potential therapeutic tool for thyroid diseases etc. In view of aging promoting the development of aging-related diseases, the reason of metformin preventing aging-related disease was attributed to several aspects of improving aging: improving nutrient-sensing (activating AMPK); suppressing pro-inflammatory reaction via preventing the differentiation of monocytes to macrophages; improving metabolism via modulating the gut microbiota; protecting genome (reducing cancer occurrence); rescuing protein misfolding; lowering oxidative stress via improving mitochondrial biogenesis (muscle); inducing stem cell rejuvenation capacity (in neuron); reducing telomere shortening (aging) etc. Therefore, these effects resulted from integrative contributions of one to multiple organs or tissues targeted by metformin. Other effects of metformin (in PCOS and thyroid) were not full clarified(Lu et al., 2021) **.**

Metformin, a biguanide anti-diabetic drug, is able to trigger autophagy by AMPK activation and subsequent inhibition of mTOR, which is one of major inhibitor of the autophagic flux. epidemiological studies showed that the use of metformin in diabetic patients is associated with a decrease in various types of cancer incidence, most significantly in pancreatic cancer, hepatocellular carcinoma, and colon cancer. However, whether metformin has

activity against cancer in non-diabetics still has to be demonstrated. It has been hypothesized that metformin could act with both direct and indirect mechanisms, primarily decreasing glucose, IGF-1 and insulin signaling, thereby creating an unfavorable environment for tumor growth that is similar to that created by caloric restriction. Through AMPK activation and mTORC signaling inhibition, metformin suppresses protein synthesis and cell proliferation (De Santi et al., 2019).

Later, analyses of retrospective data from patients affected by T2D indicated that metformin was associated with a 30% reduction in cancer incidence. As extensively recently reviewed, the cancer prevention effect of metformin was associated with autophagy induction in premalignant cell. This suggest that metformin may prevent cancer cell promotion or angiogenesis by autophagy induction effect so metformin could induce autophagic cell death during tumor promotion. But the classical experimental approach demonstrate the activation of autophagy lead to drug resistance in cancer treatment (De Santi et al., 2019).

Despite autophagy induction by metformin is considered a survival mechanism for cancerous cells in the hostile tumor microenvironment, it could prevent chronic tissue stress that can induce cellular damage to proteins, organelles and DNA, inhibiting cancer initiation and progression (De Santi et al., 2019).

1.7 Cisplatin

Cisplatin, cisplatinum, or *cis*-diamminedichloroplatinum (II), is a well-known chemotherapeutic drug. It has been used for treatment of numerous human cancers including bladder, head and neck, lung, ovarian, and testicular cancers. It is effective against

various types of cancers, including carcinomas, germ cell tumors, lymphomas, and sarcomas. Its mode of action has been linked to its ability to crosslink with the purine bases on the DNA; interfering with DNA repair mechanisms, causing DNA damage, and subsequently inducing apoptosis in cancer cells. Cisplatin is a metallic (platinum) coordination compound with a square planar geometry. It is a white or deep yellow to yellow-orange crystalline powder at room temperature. Cisplatin is stable under normal temperatures and pressures, but may transform slowly over time to the *trans*-isomer. Cisplatin has a molecular weight of 301.1 gm/mol, a density of 3.74 g/cm^3, a melting point of 270° C, a log K_{ow} of -2.19 and a water solubility of 2.53 g/L at 25° C. Cisplatin was first synthesized by M. Peyrone in 1844 and its chemical structure was first elucidated by Alfred Werner in 1893. However, Since the identification of cis-dichlorodiammineplatinum (II) (cisplatin, *r)* as the agent responsible for this activity, much interest has been generated in the use of coordination complexes of platinum, palladium, and other noble metals in the treatment of cancer (Fennell et al., 2016)**.**

Cisplatin is clinically proven to combat different types of cancers including sarcomas, cancers of soft tissue, bones, muscles, and blood vessels. Although such cancers have recently received better prognosis and therefore have become less life threatening, significant challenges remain with regard to their cure. Also, because of drug resistance and considerable side effects, combination therapy of cisplatin with other cancer drugs have been applied as novel therapeutic strategies for many human cancers (Huang et al., 2017)**.**

Cisplatin has been especially interesting since it has shown anticancer activity in a variety of tumors including cancers of the ovaries,' testes,' lung and solid tumors of the head and neck. It was discovered to have cytotoxic properties in the 1960s, and by the end of the 1970s it had earned a place as the key ingredient in the systemic treatment of germ cell cancers. Among many chemo-therapy drugs that are widely used for cancer, Cisplatin is one of the most compelling ones. It was the first FDA-approved platinum compound for cancer treatment in 1978. This has led to interest in platinum (II) - and other metal-containing compounds as potential anticancer drugs (Huang et al., 2017).

Long-term chemotherapeutic exposure is required for the effective cancer therapy. Cisplatin exposure has been long known to result in cisplatin resistance in many cancers. drug resistance greatly reduces its efficacy. recent studies have shown that au-tophagy plays a crucial role in chemotherapy resistance . Autoph-agy is a self-protection mechanism that occurs as an emergency response but can cause cell death. It is precisely this self-protection ability that increases the resistance of cancer cells to chemotherapeutic drug with cisplatin (Sirichanchuen et al., 2012)as shown in figure 1-3.

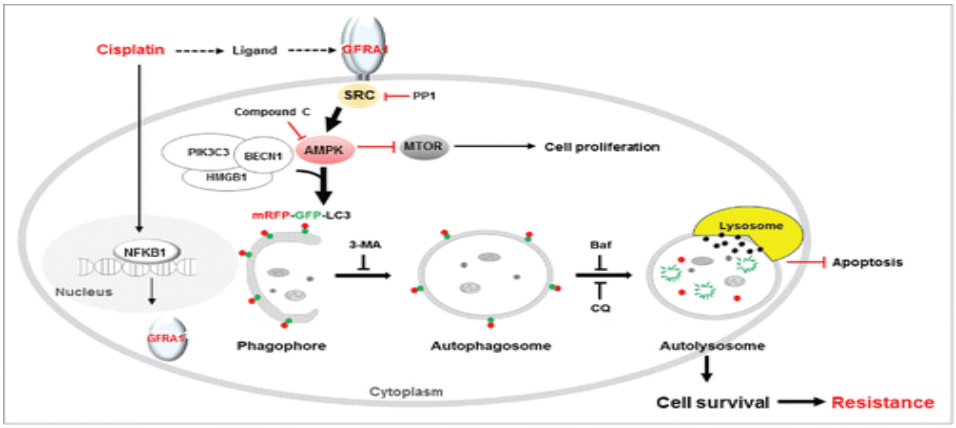


Figure 1-3 cisplatin resistance by autophagy induction (Kim et al., 2017)

For current regimens, cisplatin is usually administered in combination with third-generation cytotoxic agents at a cumulative dosage of 50–100 mg/m2 every 3 weeks. Cisplatin-based chemotherapy reduced the risk of death by 27%, improved 1-year survival by 10% and increased median survival by 1.5 months compared with supportive care (Fennell et al., 2016).

Cisplatin is one of the most emetogenic drugs used, with considerable variability between individuals. Systematic use of a three-drug combination of a neurokinin 1 receptor antagonist, a 5-hydroxytryptamine-3 (5-HT3) receptor antagonist, and dexamethasone improves control of acute emesis, but control of delayed emesis is often suboptimal (Fennell et al., 2016).

Anemia can also occur during treatment with cisplatin. Several mechanisms can led to anemia, including depletion of intrinsic erythropoietin production (caused by peritubular renal cell depletion), reduced bone marrow stem cell activity and the absence of the stem cell reaction to administered erythropoietin (Fennell et al., 2016).

1.7 Cyclin-Dependent Kinase Inhibitor 2A (CDKN2A)

The CDKN2A gene is located within the frequently deleted chromosomal region 9 of p21. The CDKN2A gene encodes two different proteins, read from alternate reading frames of a common second exon. The portion of the gene that encodes p16 is composed of three exons, and p16 is a recognized tumor suppressor because of its role in preventing progression through the G1 cell cycle checkpoint. the other protein encoded by the CDKN2A locus, p14, has negative regulatory effects on growth as it serves to stabilize p53. As p53 is activated, it interacts with various downstream targets that can arrest cyclin-dependent kinases at G1

and G2 checkpoints as well as initiate apoptosis. Both p16- and p14-mediated growth arrests appear to be involved in preventing neoplastic transformation (Zhao et al., 2016).

The CDKN2A tumor suppressor can induce potent growth arrest or cell death in response to hyper proliferative oncogenic stimuli. Stimulated CDKN2A expression in normal cells induces G1 and G2 cell cycle arrest or apoptosis. Animals lacking CDKN2A are highly prone to tumor formation, and their embryonic fibroblasts do not senesce, continuing to cycle after DNA damage (Zhao et al., 2016).

Several genetic and epigenetic aberrations of CDKN2A lead to enhanced tumorigenesis and metastasis with recurrence of cancer and poor prognosis. In fact, loss of tumor suppressor genes and their encoded proteins through deletion, inactivating mutations, epigenetic silencing or post-translational modification results in tumorigenesis. For these reasons, the restoration of genetic and epigenetic reactivation of CDKN2A is a practical approach for the prevention and therapy of cancer (Zhao et al., 2016).

1.8 Cell culture

Cells, removed from animal tissue or whole animals, will continue to grow if supplied with nutrients and growth factors. This process is called cell culture. It occurs in vitro (‘in glass’) and allows single cells to act as independent units much like any microorganism such as bacteria or fungi. The cells are capable of division by mitosis and the cell population can continue growth until limited by some parameter such as nutrient depletion. Cultures normally contain cells of one type (e.g. fibroblasts). The cells in the culture may be genetically identical (homogeneous population)

or non-identical (heterogeneous population). A homogeneous population of cells derived from a single parental cell is called a clone. Therefore all cells within a clonal population are genetically identical (J. Butler, 2004) .

The cell culture environment are ideal for the growth of numerous microorganisms recognized by a significant change in the pH of the culture medium (as identified by a color shift in the medium) and the sudden appearance of turbidity or colonies of fungal organisms and the cells themselves are prone to genetic changes. three critical characteristics of cell cultures that are fundamental to the assurance of good-quality cell culture work. These are

• Purity—The cells are free from microbiological contamination.

• Identity—The cells are what they are claimed to be.

• Stability—The genotype and phenotype remain stable during growth and passage in vitro (Morris et al., 2007).

1.8.1 Primary cultures

Are derived directly from excised, normal animal tissue and cultures either as an explant culture or following dissociation into a single cell suspension by enzyme digestion. Such cultures are initially heterogeneous but later become dominated by fibroblasts. Primary cultures have not been passaged, as soon as they are passaged they become a cell line and are no longer primary (LLC, 2016) .

1.8.2 Cell line

After the first subculture, the primary culture becomes known as a cell line. Cell lines derived from primary cultures have a lim-

ited life span (they are finite), and as they are passaged, cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population (Education, 2016) .

1.8.3 Cell strain

If a subpopulation of a cell line is positively selected from the culture by cloning or some other method, this cell line becomes a cell strain. A cell strain often acquires additional genetic changes subsequent to the initiation of the parent line (Philippeos et al., 2012).

1.8.4 Continuous Cultures

Continuous cultures are comprised of a single cell type that can be serially propagated in culture either for a limited number of cell divisions (approximately thirty) or otherwise indefinitely. Cell lines of a finite life are usually diploid and maintain some degree of differentiation. Continuous cell lines that can be propagated indefinitely generally have this ability because they have been transformed into tumour cells. Tumour cell lines are often derived from actual clinical tumours, but transformation may also be induced using viral oncogenes or by chemical treatments (Dilnessa & Zeleke, 2017)

1.8.5 Advantages Of Cell Culture

Cell culture allows for the control of the physiochemical enviroment (pH, temperature, osmotic pressure, and O2 and CO 2 tension).This provides many advantages as cytology and immunostaining can be easily performed, quantitation is straight forward, and experiments can be performed with reduced volumes which lowers the costs. Cultures may be exposed to a reagent at a

low and defined concentration with direct access to the cell . Tissue samples are regularly heterogeneous; however, after one or two passages, cultured cell lines transfer and the selective pressure of the culture conditions tends to produce a homogeneous culture of the most vigorous cell type. This, therefore, allows for experimental replicates to be very similar, reducing statistical variance (D. Park et al., 2015).

1.8.6 Limitations

Culture techniques need to be carried out under strict aseptic conditions, as common contaminants (e.g. bacteria, moulds, and yeast) grow more rapidly than mammalian cells. Other limitations include the high cost of consumables and media, cross-contamination, and dedifferentiation and selection. Dedifferentiation is the overgrowth of undifferentiated cells that produces a loss of the phenotypic characteristics typical of the tissue from which the cells have been isolated. This can be overcome under the right conditions with selective media (Philippeos et al., 2012).

1.8.7 Cryopreservation of Cell Lines

The aim of cryopreservation is to enable stocks of cells to be stored to prevent the need to have all cell lines in culture at all times. It is invaluable when dealing with cells of limited life span. The basic principle of successful cryopreservation and resuscitation is a slow freeze at a rate of –1°C to –3°C per minute and thaw quickly by incubation in a 37°C water bath for 3-5 minutes (LLC, 2016) .

The maintenance of cells in a frozen state—usually in liquid nitrogen. Cryopreservative Compounds which prevent cell damage during the freezing and thawing include glycerol or dimethylsulfoxide (DMSO) (M. S. Butler, 2004) .

1.9 human non-small cell lung cancer A549 Cell line

The A549 is the most commonly used human non-small cell lung cancer cell line for both basic research and drug discovery. The A549 cell line consists of hypotriploid alveolar basal epithelial cells. This cell line was first developed by D. J. Giard et al. in 1972 by removing and culturing pulmonary carcinoma tissue from the explanted tumor of a 58-year-old caucasian male, the A549 cells are squamous in nature and are responsible for the diffusion of water and electrolytes across the alveoli. When grown in vitro, these cells grow as a monolayer, adhering to the culture flask. These cells have the ability to synthesize lecithin and contain a high percentage of unsaturated fatty acids that are responsible for the maintenance of membrane phospholipids (B. Zhang et al., 2017).

1.10 human colorectal cancer SW480 cell line

SW480 [SW-480] cells isolated from the large intestine epithelial cell of a male patient with colorectal adenocarcinoma. SW480 Derived from a grade 3-4 colon adenocarcinoma. The initial cultures contained a mixture of epithelial and bipolar cells, but subsequently epithelial cells predominated. SW480 cell line was established from the primary tumor before metastasis

Chapter Two

Materials and Methods

2. Materials and Methods

The study was done during the period from January -Septemper, 2021 in the tissue culture of post graduate lab at the College of Medicine\ University of Babylon.

2.1. Materials

2.1.1. Chemicals

The chemicals used in this study are listed in (Table 2.1) with their suppliers.

Table (2.1) List of Chemicals Used in The Study

Chemical	Company	Country
Alcohol spray (ethanol 70%)	Aljoud	Iraq
Crystal violet	Sigma	USA
Dimethyl sulfoxide (DMSO)	Roth	Germany
Fetal bovine serum (FBS)	Capricorn	Germany
Liquid nitrogen	Clever	USA
Phosphate buffer saline(PBS) packets	BioPLUS chemicals	USA
RPMI 1640 medium w/L-glutamine, 25mM HEPES (powder)	Gibco	UK

Sodium bicarbonate powder	Ludeco	Belgium
Trypsin- Ethylenediaminetet-raacetic acid (EDTA) powder	US Biological life science	USA
Pencillin- streptomycin solu-tion	Capricorn	Germany

2.1.2. Equipment

The equipment used in the study is listed in (Table 2.2) with their suppliers.

Table (2.2) List of Equipment Used in The Study

Equipment	Company	Country
Sterile freezing vial (1.5 ml)	Biofil	Australia
Autoclave	Jeiotech	Korea
micropipettes (different sizes)	Dragon-Med	India
Cell culture flask (25ml)	SPL	Korea
Cell culture plate (96- wells)	SPL	Korea
Deep freezer -80°C	Labtech	Korea
Digital camera	Sony	Japan
Disposable face masks	KY	China

Disposable gloves	Fe	Malaysia
Distiller	ROWA	Germany
Double distillation water stills	GFL	Germany
Electric oven	Memmert	Germany
ELISA Reader	Human	Germany
Eppendorf tube	Eppendorf	Germany
Freezer -20 °C	Mettler	Switzerland
Incubator	Memmert	Germany
Inverted microscope	T.C Meiji techno	Japan
Laminar air flow cabinet	Labtech	Korea
Liquid nitrogen container GT38	Air Liquide	France
Magnetic stirrer	Scotech	Germany
Millipore filter (0.45, 0.22μm)	Biofil	Australia
Paraffin film	Analab	Ireland
Refrigerator	Arcelik	Turkey
Sensitive Balance	Labtech	Korea

Ultrasonic	Binder	Germany
Water bath	Minilyotrap	England
Whatman Filter Paper	Sigma	USA
Disposable Surgical Gown	Kangbao	China
Face shield	Zangchin	China
Conical flask of different size	Kangbao	China
Marker	Stedidlar	Germany
Printer	HP	China
Cover head	Kangbao	China
Cover shoes	Kangbao	China
Syringe 5 ml	MED	China

2.1.3 Assay kit and drugs

the assay kits used in this study include:

Table 2.3 ELISA assay kit and drugs used in study.

CDKN2A Kitt	Elabscience	USA
metformin	Sigma-aldrich	Germany
hydroxychloroquin	Sigma-aldrich	Germany
cisplatin	Accord	UK

2.1.4. Cell lines

Frozen vials of A549 cell lines and SW480 were obtained from Tissue Culture laboratory in the College of Medicine / University of Babylon.

2.2. Methods

2.2.1. Preparation of reagents

2.2.1.1 Phosphate Buffer Saline (PBS)

According to the BioWorld manufacturer manual, the PBS was prepared through dissolving only one packet in 500 ml of deionized distilled water (DDW) with continuous stirring by a magnetic stirrer at room temperature resulting in PH value of 7.45 without need for adjustment. Autoclaving is required for complete sterilization and stored in a closed bottle until use to keep sterile. (Trusted and In, 2008).

2.2.1.2 Trypsin-(EDTA) Solution

As indicated by US Biological headings, a weight of a 10.1 gm of trypsin-EDTA powder was dissolved in 0.9 Liter of double distilled water (DDW) with continuous mixing at room temperature. 7.2 of PH value should be reached and complete the volume to 1 Liter by DDW, the solution was sterilized through using Millipore filters of 0.45 and 0.22 µm respectively, after that, the solution was kept at (- 20C˚) of temperature.

2.2.1.3 Preparation of crystal violet solution

Crystal Violet powder 0.5gm dye dissolved in 20 ml of 99% methanol and mix with 80 ml of DDW.

Crystal Violet powder dye should be kept in a tightly closed original package at temperature between 15°C and 25°C. Keep in dry places, do not freeze, and avoid exposure to direct sunlight. Expiry date is stated on the product's label (Mobini and Poursafar, 2019)

2.2.4. Preparation of Tissue Culture Medium

Liquid RPMI-1640 medium was prepared according to US Biologics from RPMI-1640 medium powder as the following: Weighing RPMI-1640 powder to obtain 16.353 grams and dissolved in 900ml of ddH2O without heating, 2 g of sodium bicarbonate can be added if required with gentle stirring to adjust pH additional water was added to obtain 1 liter then filtered using 0.22-micron membrane. Penicillin-Streptomycin at 1% was added and 10% fetal bovine serum also added, then filter-sterilized using 0.22-micron membrane filter. The mixture contains heat labile compounds that can be damaged with autoclaving. The prepared media should be kept at 4°C and used within a short period of time.

2.2.4.1. Preparation of Serum-Free Medium

Roswell Park Memorial Institute developed this medium, thus the acronym of this media became RPMI. RPMI-1640 medium has been used for the culture of SW480 and A549 cell lines and it promoted the growth of many types of cell cultures.

Liquid RPMI-1640 medium was prepared from powdered RPMI-1640 medium according to the Gibco product manual as the following:

From the RPMI-1640 powdered medium, 10.43 g was dissolved in nearly 900 ml of DDW in a volumetric flask. The other components include: 2 g of sodium bicarbonate powder or as needed and 1.25 ml of gentamicin stock solution had been added with continuous stirring, then the volume was completed by adding DDW to complete the volume to one liter and the medium pH adjusted to 7.4. The sterilization was performed by 0.45 and 0.22 μm filters. Distribute the medium aseptically into sterile containers, then stored at 2-8ºC until use

2.2.4.2. Preparation of Serum-Medium

Serum-medium was prepared as described in 2.2.4.1 with the addition of 10% FBS.

2.2.4.3. Preparation of Freezing Medium

Ten ml of freezing medium were prepared from the following constituents: six ml serum-free medium, three ml of FBS, and one ml of DMSO were added drop by drop with the mixture. The solution was stored in between uses around (- 20˚C)

2.2.5. Preparation of A549 and SW480 Cell LineS for Cytotoxicity Assay

A549 and SW480 cell lines in the frozen vials growth were maintained in a 25 ml culture flask, with a complete growth medium containing 10% FBS and antibiotics and incubated at 37˚C.

2.2.6. Thawing of A549 cell Line and SW480 cell line

Remove the cryo vial from the liquid nitrogen and thaw the cells quickly by placing into a 37°C water bath while agitating gently, remove after 60 seconds .few ice crystals should leave after thawing. Decontaminate the vial by spraying with 70% ethanol. Gently re-suspend the cells in the vial and transfer the cell suspension into a 15 mL sterile conical tube containing 5 mL of pre-warmed media and centrifuge the cells at 200x g for 3 minutes to pellet. Aspirate out the supernatant without disturbing the cell pellet. Re-suspend the cell pellet in fresh, pre-warmed culture media and transfer the cells into a culture vessel. Incubate the culture at 37°C, at least 24 hours before processing the cells for downstream experiments (Phelan and May, 2017).

2.2.7. Harvesting and Sub-Culturing of A549 Cell Line and sw480 cell line

Harvesting is a technique that uses proteolytic enzymes to detach adherent cells from the surface of a cell culture vessel. First, the growth medium in the vessel was aspirated and discarded. Phosphate-buffered saline (PBS) was used to wash the cells twice. Afterwards, the enzymatic harvesting solution was added to the vessel. After 15 minutes, the proteolytic reaction was neutralized by adding the serum containing culture medium. The cells in the tissue culture flasks were harvested by using

different enzymatic solutions composed of different concentrations of trypsin and EDTA (Viazzi *et al.*, 2015).

Sub culturing of Cells (Meleady and O'Connor, 2006).

1.Examine the condition of the cells using an inverted microscope with phase-contrast capabilities. Ensure that the cells are healthy and subconfluent and free of contamination.

2. Sanities the laminar flow cabinet by wiping the surface of the working area with 70% ethanol.

3. Remove the spent growth medium from the flask using a pipette and wash the monolayer with a sufficient volume of PBF to ensure the removal of all media from the flask.

4. Add an appropriate volume of the trypsin/EDTA solution to the flask and incubate at 37 c° to allow the cells to detach from the inside surface of the flask (within 2-10 min).

5. The cells were examined using an inverted microscope to ensure that all the cells are detached and in suspension. Gently tap the flask with the palm of the hand a couple of times to release any remaining detached cells.

6. Inactivate the trypsin by adding an equal volume of serum-containing media to the flask.

7. Divide the cell suspension into two flask and label each flask with cell line name, passage number, and date.

9. The cell line was incubated at 37C°for 24 hr.

2.2.8. stock solutions preparation

2.2.8.1. Preparation of cisplatin stock solution

Stock solution of cisplatin was obtained from cisplatin vial of 1mg/1ml and from this stock a serial dilution was made. This solution can kept and still stable at temperature 2-8°C.

2.2.8.2. Preparation of metformin stock solution

Stock solution of metformin was prepared from weighed (1mg) pure powder dissolved in (1ml) double distilled water and from this stock a serial dilution was made. This solution can kept and still stable at temperature -20°C.

2.2.8.3. Preparation of hydroxychloroquine stock solution

Stock solution of hydroxychloroquine was prepared from weighed (1mg) pure powder dissolved in (1ml) double distilled water with and from this stock a serial dilution was made.

2.3 Study plan

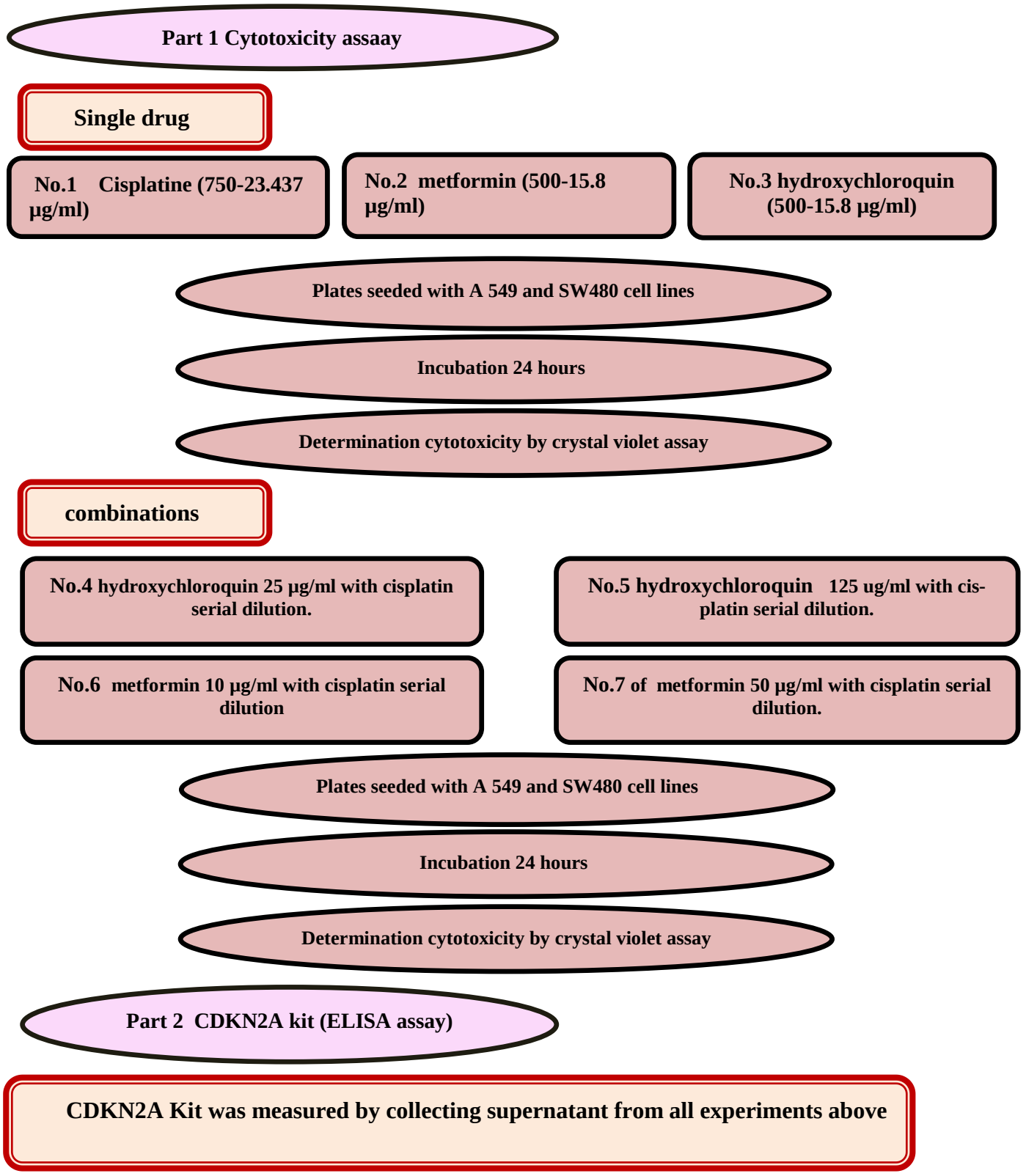


Figure (2.1) study plan

2.3.1 Cytotoxicity Assay

The cytotoxicity assay was applied to determine the cytotoxic concintrations of cisplatin, metformin and hydroxychloroquine and their combination on A549 cell line and SW480 cell line .

1. Effect of cisplatin on A549and SW480 cell line

The 96 well plates were seeded with human colorectal sw480 cells and another 96 well plates were seeded with non-small cell lung cancer A549 cells in seeding density of 5*10 and the wells of the plate (except one column from each plate which left without treatment as a control), exposed to 200 µL of six serial dilutions of cisplatin as follow (750 , 375 , 187.5 , 93.75 , 46.875 and 23.437 µg/ml), Then the plates were covered with the plastic lid and incubated for 24 hours. Afterward, the plates were washed with 200 µl of a sterile PBS, and the effect of the cisplatin on A549 cell line and sw480cell line growth was assessed by crystal violet assay.

2. Effect of metformin 500µg/ml on SW480 and A549 cell line

The 96 well plates were seeded with human colorectal sw480 cells and another 96 well plates were seeded with non-small cell lung cancer A549 cells in seeding density of 5*10 and the wells of the plate (except one column from each plate which left without treatment as a control), exposed to 200µL of six serial dilutions of metformin as follow (500 , 250 , 125 , 62.5 , 31.25 and 15.8 µg/ml). The plates were covered with the plastic lids and incubated for 24 hours. Afterward, the plates were washed with 200 µl of a sterile PBS, and the effect of the metformin on sw480 cell line and A549 cell line growth was assessed by crystal violet assay.

3. Effect of hydroxychloroquine 500µg/ml on SW480 and A549 cell line

The 96 well plates were seeded with human colorectal sw480 cells and another 96 well plates were seeded with non-small cell lung cancer A549 cells in seeding density of 5*10 and the wells of the plate (except one column from each plate which left without treatment as a control), exposed to 200 µL of six serial dilutions of hydroxychloroquine as follow (500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml and 15.8 µg/ml). The plates were covered with the plastic lids and incubated for 24 hours. Afterward, the plates were washed with 200 µl of a sterile PBS, and the effect of the hydroxychloroquine on sw480 cell line and A549 cell line growth was assessed by crystal violet assay.

4.Effect of hydroxychloroquine 25µg/ml in combination with cisplatin on SW480 and A549 cell line

The 96 well plates were seeded with human colorectal sw480 cells and another 96 well plates were seeded with non-small cell lung cancer A549 cells in seeding density of 5*10 and the wells of the plate (except one column from each plate which left without treatment as a control), exposed to 200 µL of six combination of hydroxychloroquine 25 µg/ml and six serial dilution of cisplatin as follow (750 , 375 , 187.5 , 93.75 , 46.875 and 23.437). Then the plates were covered with the plastic lids and incubated for 24 hours. Afterward, the plates were washed with 200 µl of a sterile PBS, and the effect of cisplatin in combination with low concentration hydroxychloroquine on sw480 cell line and A549 cell line growth was assessed by crystal violet assay.

5.Effect of hydroxychloroquine 125µg/ml in combination with cisplatin on SW480 cell line and A549 cell line

The 96 well plates were seeded with human colorectal sw480 cells and another 96 well plates were seeded with non-small cell lung cancer A549 cells in seeding density of 5*10 and the wells of the plate (except one column from each plate which left without treatment as a control), exposed to 200 µL of six combination of hydroxychloroquine 125 µg/ml and six serial dilution of cisplatin as done in last experiment and the effect of cisplatin in combination with high concentration hydroxychloroquine on sw480 cell line and A549 cell line growth was assessed by crystal violet cytotoxicity assay.

6. Effect of metformin 10µg/ml in combination with cisplatin on SW480 cell line and A549 cell line

The 96 well plates were seeded with human colorectal sw480 cells and another 96 well plates were seeded with non-small cell lung cancer A549 cells in seeding density of 5*10 and the wells of the plate (except one column from each plate which left without treatment as a control), exposed to 200 µL of six combination of metformin 10 µg/ml and six serial dilution of cisplatin as done in experiment 4 and 5 and the effect of cisplatin in combination with low concentration of metformin on sw480 cell line and A549 cell line growth was assessed by crystal violet assay.

7. Effect of metformin 50µg/ml in combination with cisplatin on SW480 cell line and A549 cell line

Same procedure with same six serial concentration of cisplatin with metformin 50µg/ml and the effect of cisplatin in combination with high concentration of metformin on sw480 cell line and A549 cell line growth was assessed by crystal violet assay.

2.3.2 Crystal Violet Assay for Determining the Viability of Cultured Cells

During cell death, adherent cells detach themselves from cell culture plates. This property can be used for cell death determinations. One simple method for detecting cell adherence is the staining of attached cells with crystal violet coloring, which links to proteins and DNA. Cells that undergo cell death lose adherence and are subsequently lost from the cell population, reducing the amount of crystal violet staining in a culture. This protocol describes a quick and reliable method for testing the impact of chemotherapeutics or other compounds on cell survival and inhibition of growth.

Procedure:

After finishing each experiment, the maintenance medium was discarded and the cells were washed by automatic pipette three times with 100 µl cold PBS, then we added 100 µL of 0.5% crystal violet staining solution to each well and incubated at room temperature for 20 min. Wash the plate in a gentle stream of tap water three times. We tilted the plate to avoid direct hitting of the cell monolayer by the stream of water. Aspire the water from the wells immediately after the wells have filled with water. We inverted the plate on filter paper after washing and gently flipped the plate to remove any remaining liquid. Air-dry the plate without its lid at room temperature for at least 2 h. Afterward, we measured the optical density of each well at 570 nm (OD 570) with a plate reader .

The percentage of inhibition was calculated according to the following equation:

Inhibition Rate (I.R)% = (optical density of control wells-optical density of test wells) / (optical density of control wells) X 100.

Viable cell number = (optical density of treatment wells X 105) (Chiang et. al., 2003).

2.4 CDKN2A kit

Table (2.4)List of contents of the ELISA assay kit

Micro ELISA Plate (Dismountable)	96T: 8 wells ×12 strips 48T: 8 wells ×6 strips
Reference Standard	96T: 2 vials 48T: 1 vial

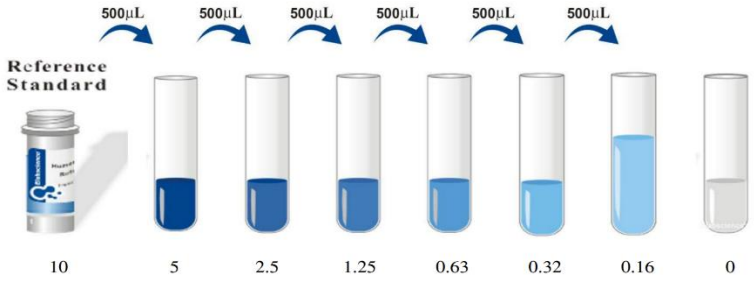
Concentrated Biotinylated Detection Ab (100×)	96T: 1 vial, 120 µL 48T: 1 vial, 60 µL
Concentrated HRP Conjugate (100×)	96T: 1 vial, 120 µL 48T: 1 vial, 60 µL
Reference Standard & Sample Diluent	1 vial, 20 mL
Biotinylated Detection Ab Diluent	1 vial, 14 mL
HRP Conjugate Diluent	1 vial, 14 mL
Concentrated Wash Buffer (25×)	1 vial, 30 mL
Substrate Reagent	1 vial, 10 mL
Stop Solution	1 vial, 10 mL
Plate Sealer	5 pieces
Manual	1 copy
Certificate of Analysis	1 copy

2.4.1 Reagent preparation

1. all reagents were brought to room temperature (18-25℃) before use. If the kit will not be used up in one assay, please only take out the necessary strips and reagents for present experiment and store the re-maining strips and reagents at required condition.

2. Wash Buffer: 30 mL of Concentrated Wash Buffer was diluted with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer. Note: if crystals have formed in the concentrate, warm it in a 40℃-water bath and mix it gently until the crystals have completely dissolved.

3. Standard working solution: Centrifugation of the standard at 10,000×g for 1 min was done then 1.0 mL of Reference Standard &Sample Diluent were added, let it stand for 10 min and invert it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 10 ng/mL(or add 1 mL of Reference Standard &Sample Diluent, let it stand for 1-2 min and then mix it thoroughly with a vortex meter of low speed. Bubbles generated during vortex could be removed by centrifuging at a relatively low speed). Then make serial dilutions as needed. The recommended dilution gradient is as follows: 10, 5, 2.5, 1.25, 0.63, 0.32, 0.16, 0 ng/mL. Dilution method: Take 7 EP tubes, add 500uL of Reference Standard & Sample Diluent to each tube. Pipette 500uL of the 10 ng/mL working solution to the first tube and mix up to produce a 5 ng/mL working solution. Pipette 500uL of the solution from the former tube into the latter one according to this step. The illustration below is for reference



Figure(2.2) serial dilution technique

4. Biotinylated Detection Ab working solution: the required amount was calculated before the experiment (100 μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the Concen-

trated Biotinylated Detection Ab at 800×g for 1 min, then dilute the 100× Concentrated Biotinylated Detection Ab to 1× working solution with Biotinylated Detection Ab Diluent(Concentrated Biotinylated Detection Ab: Biotinylated Detection Ab Diluent= 1: 99).

5. Concentrated Avidin-Horseradish Peroxidase (HRP) Conjugate working solution: Calculate the required amount before the experiment (100 µL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the Concentrated HRP Conjugate at 800×g for 1 min, then dilute the 100× Concentrated HRP Conjugate to 1× working solution with HRP Conjugate Diluent(Concentrated HRP Conjugate: HRP Conjugate Diluent= 1: 99).

2.4.2 Assay procedure

1. Determine wells for diluted standard, blank and sample. Add 100 µL each dilution of standard, blank and sample into the appropriate wells (It is recommended that all samples and standards be assayed in duplicate). Cover the plate with the sealer provided in the kit. Incubate for 90 min at 37℃. Note: solutions should be added to the bottom of the micro-ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

2. the liquid was decanted from each well, do not wash. Immediately add 100 µL of Biotinylated Detection Ab working solution to each well. Cover the plate with a new sealer. Incubate for 1 hour at 37°C.

3. the solution from each well was decanted, and 350 µL of wash buffer added to each well. Soak for 1 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step

and other wash steps. Make the tested strips in use immediately after the wash step. Do not allow wells to be dry.

4. 100 µL of HRP Conjugate working solution was added to each well. Cover the plate with a new sealer. Incubate for 30 min at 37°C.

5. the solution from each well was added , repeat the wash process for 5 times as conducted in step 3.

6. 90 µL of Substrate Reagent was added to each well. Cover the plate with a new sealer. Incubate for about 15 min at 37°C. Protect the plate from light.

Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30 min. Preheat the Microplate Reader for about 15 min before OD measurement.

7. 50 µL of Stop Solution was added to each well. Note: adding the stop solution should be done in the same order as the substrate solution.

8. Determinination the optical density (OD value) of each well was done at once with a micro-plate reader set to 450 nm.

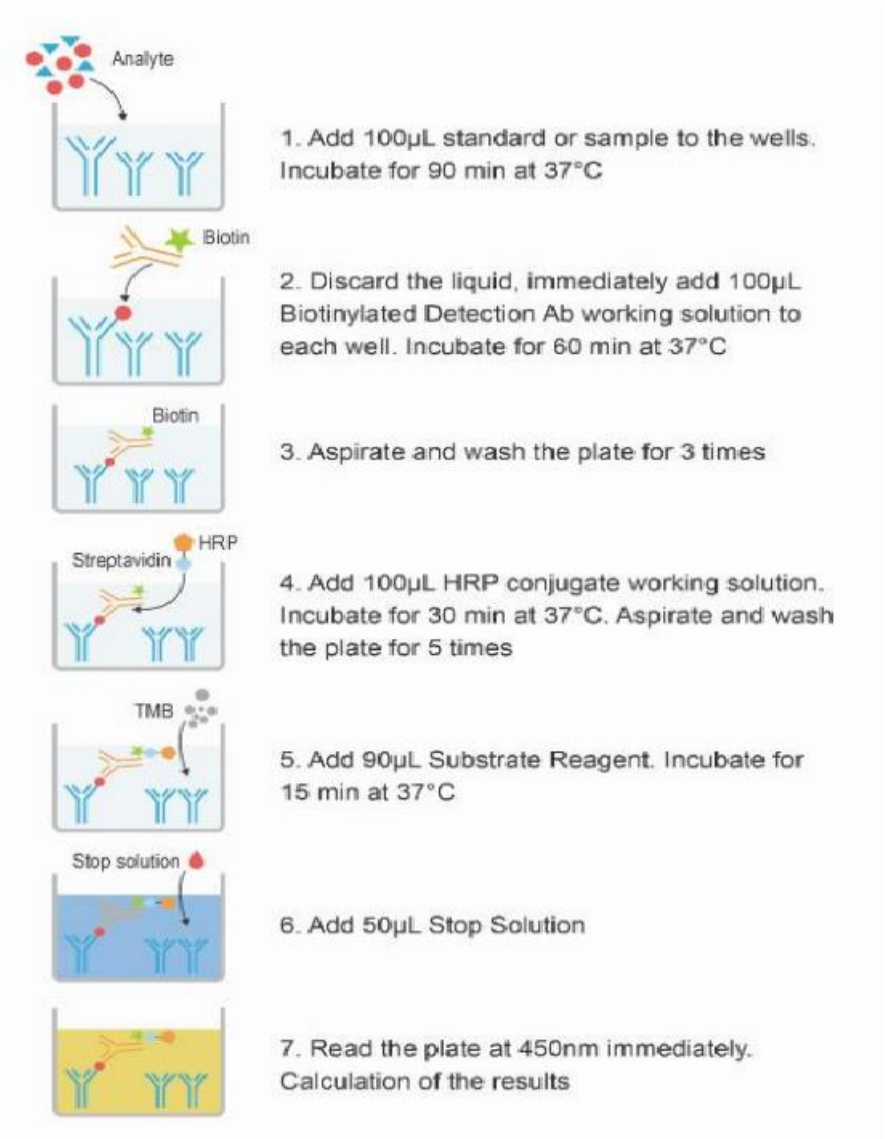


Figure (2.3) assay procedure

2.5 Statistical Analysis

All data were collected and analyzed by Microsoft Office Excel 2010 and Sigma plot version 13 software. ANOVA test was used to assess significant differences among the means of data.

Chapter Three

Results

3. Results

3.1 Cytotoxicity Assay

3.1.1 The effect of cisplatin on A549 and SW480 cell line:

The results showed that there were significant ($p \leq 0.05$) decrease in viability percentage of A549 cell line in all concentration used of cisplatin except (23.347 , 46. 875) µg/ml.

While significant ($p \leq 0.001$) decrease in the viability percentage of SW480 cell line for all concentration in comparison with control group as shown in (figure 3.1) .

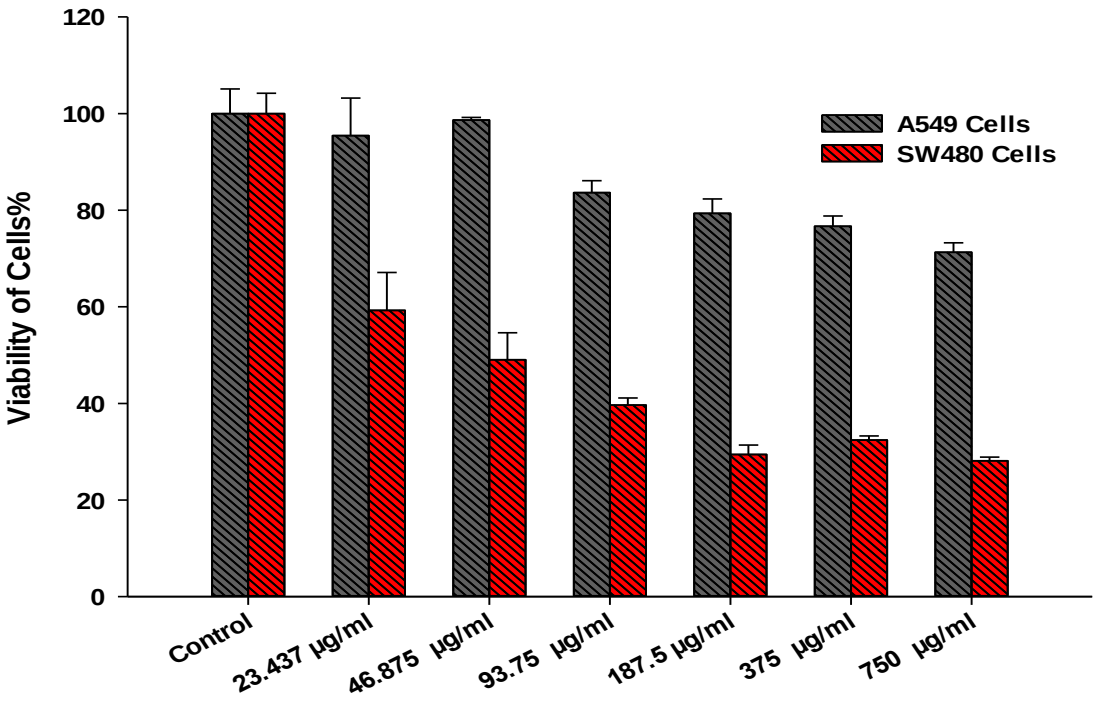


Figure (3.1) The effect of cisplatin on A549 and SW480 cell line

3.1.2 The effect of metformin on A549 and SW480 cell line

The result showed that there were significant ($p \leq 0.05$) increase in viability percentage of A549 cell line with high concentration (500µg/ml) metformin only.

while metformin decrease viability percentage significantly ($p \leq 0.001$) of SW480 cell line when used at high concentrations (250 and 500µg/ml) only in comparison with the control group as shown in (figure 3.2).

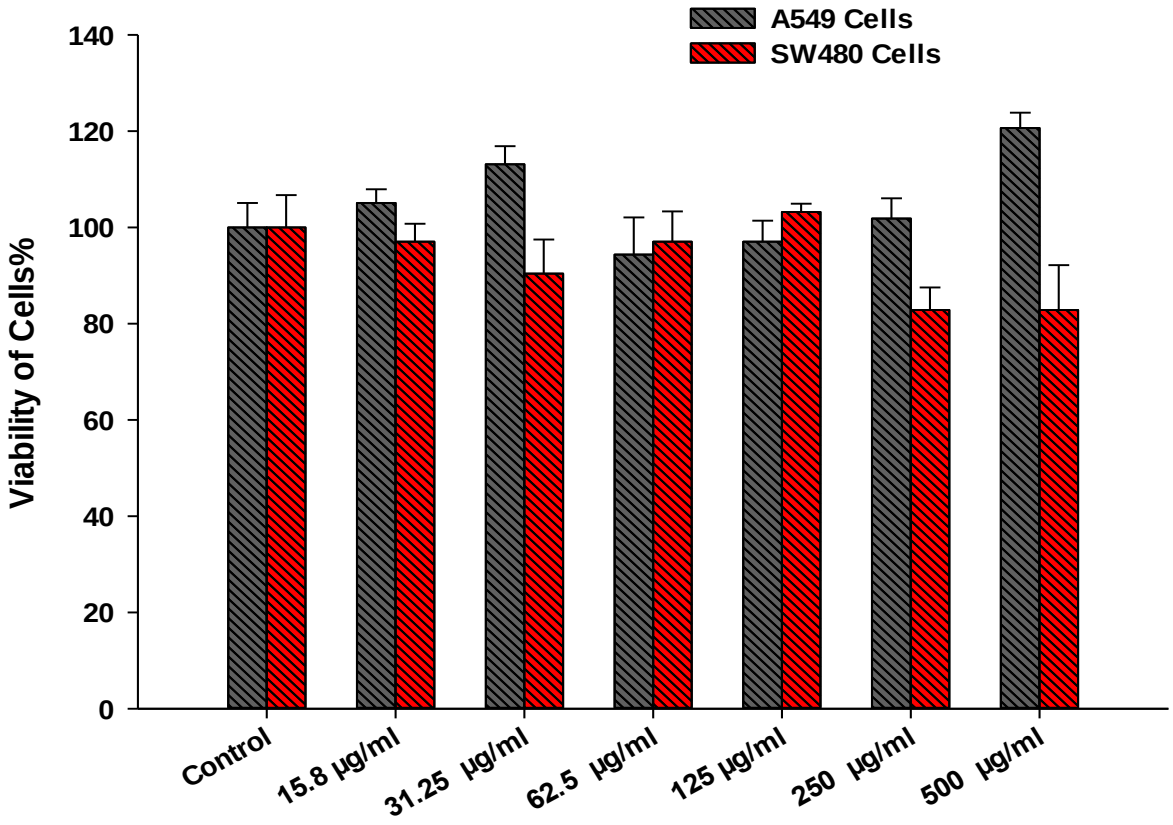


Figure (3.2) The effect of metformin on A549 and SW480 cell line

3.1.3 The effect of hydroxychloroquine on A549 and SW480 cell lines.

The result showed that there is significant ($p \leq 0.001$, $p \leq 0.05$) decrease in cell viability percentage of A549 and SW480 cell lines in all concentrations used of hydroxychloroquine in comparison with control group except (31.25µg/ml and 15.8 µg/ml) as shown in (figure 3.3).

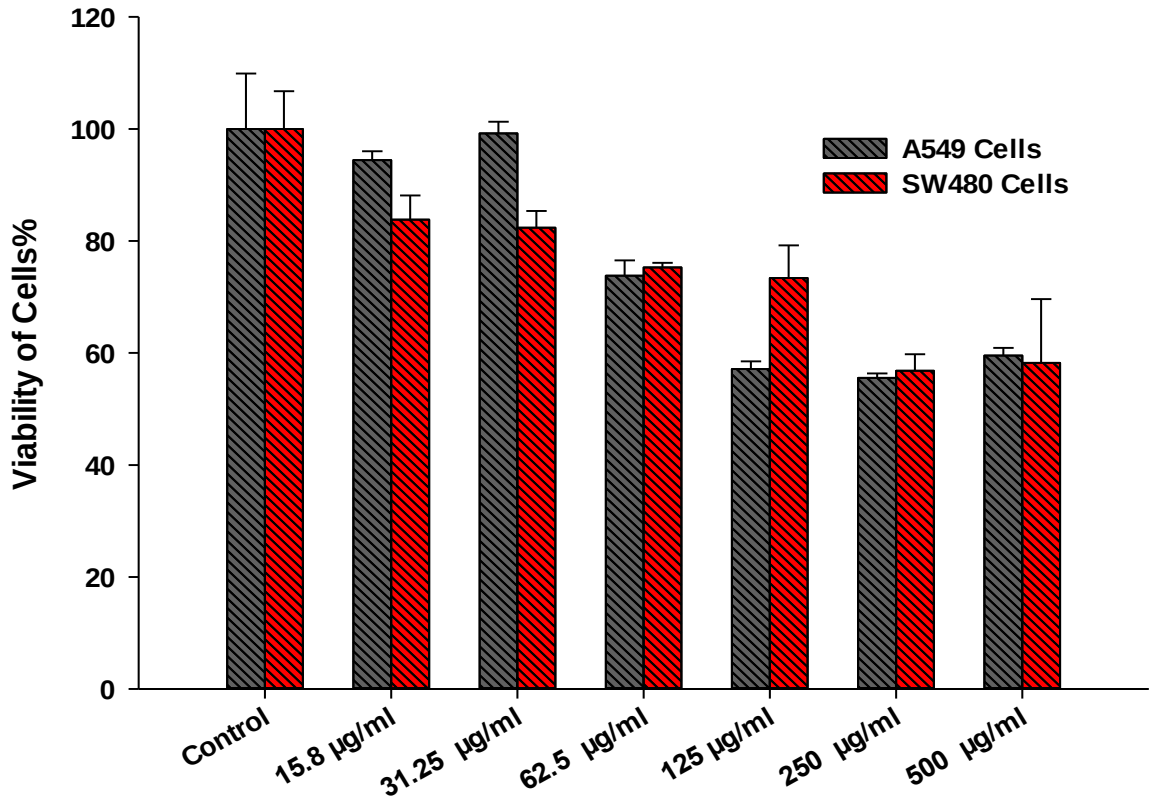


Figure (3.3) The effect of hydroxychloroquine on A549 cell line and SW480 cell line

3.1.4 The effect of hydroxychloroquine 25 µg/ml in combination with cisplatin on A549 and SW480 cell line

The result of combination of hydroxychloroquin and cisplatin at different concentration showed that there were significant decrease ($p \leq 0.001$) in viability percentage of A549 cell line with all concentrations of cisplatin and constant concentration of hydroxychloroquin (25 µg/ml) in comparison with the control group.

when the combination used on SW480 the results showed that there were significant decrease ($p \leq 0.001$) in viability percentage in all combinations except for (750cisplatin +25 hydroxychloroquin and 375cisplatin +25 hydroxychloroquin) as shown in (figure 3.4).

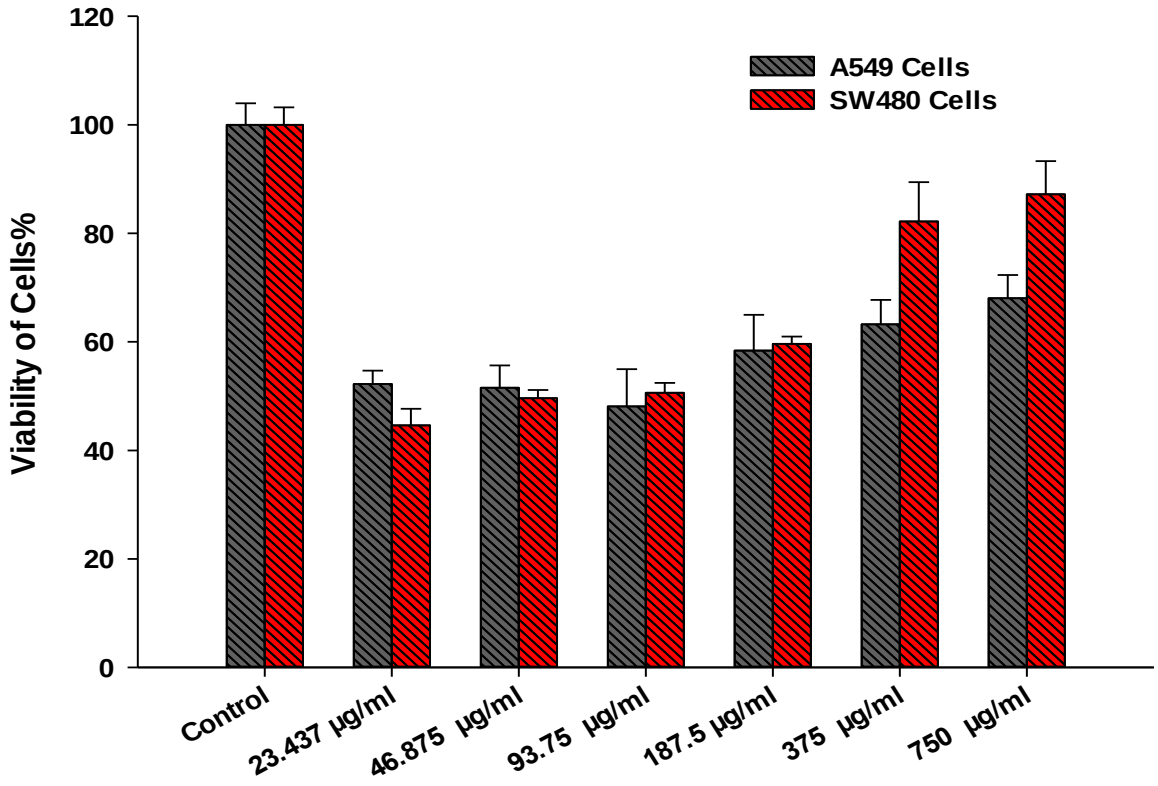


Figure (3.4)The effect of hydroxychloroquine 25 microgram/ml in combination with cisplatin on A549 and SW480 cell line

3.1.5 The effect of hydroxychloroquine 125 µg/ml in combination with cisplatin on A549 and SW480 cell line

The result of combination of hydroxychloroquine and cisplatin at different concentration showed that there were significant ($p \leq 0.001$) decrease in viability percentage of both A549 and SW480 cell line with all concentrations of cisplatin and constant concentration of hydroxychloroquin (125 µg/ml) in comparison with the control group as shown figure (3.5) .

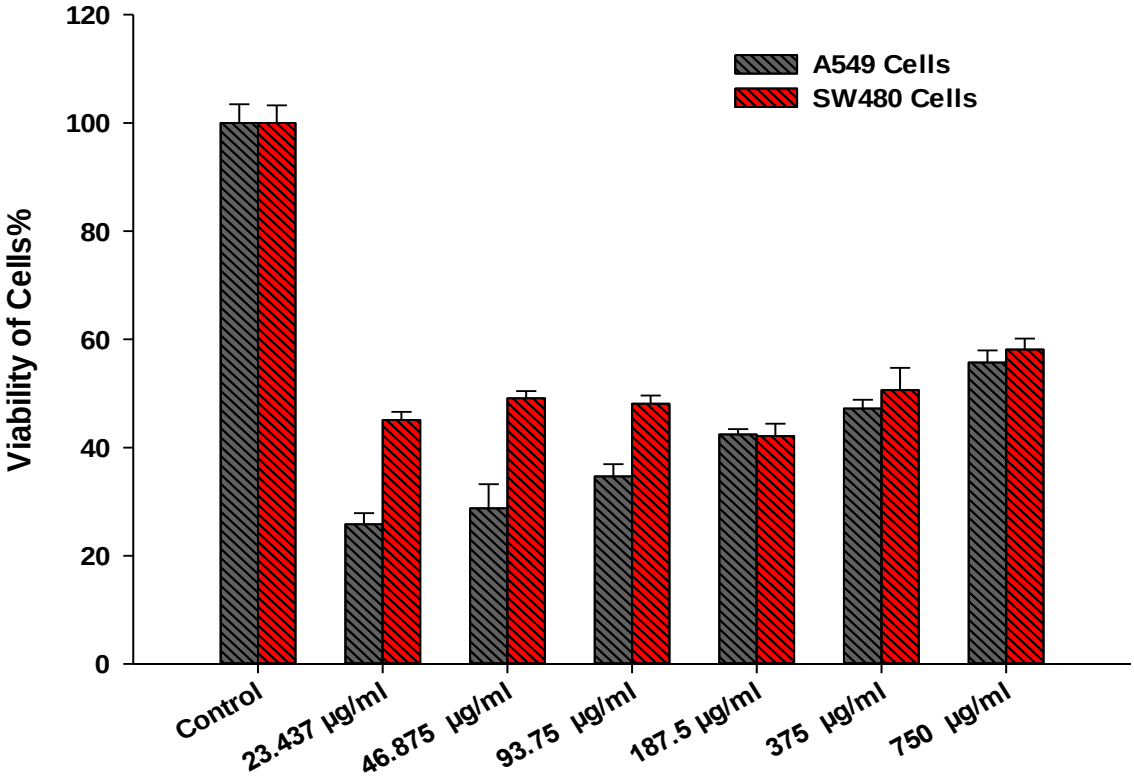


Figure (3.5) The effect of hydroxychloroquin 125 µg/ml in combination with cisplatin on A549 and SW480 cell line

3.1.6 The effect of metformin 10 µg /ml in combination with cisplatin on A549 and SW480 cell line

There were significant ($p \leq 0.001$) decrease in viability percentage of A549 cell line at higher concentrations of cisplatin (750 , 375 , 187.5 and 93.75 µg/ml) and constant concentration of metformin in comparison with the control .

The results also showed that there were significant ($p \leq 0.001$) decrease in viability percentage of SW480 cell line at all concentrations of cisplatin with constant concentration of metformin (10 µg/ml) as shown in (figure 3.6).

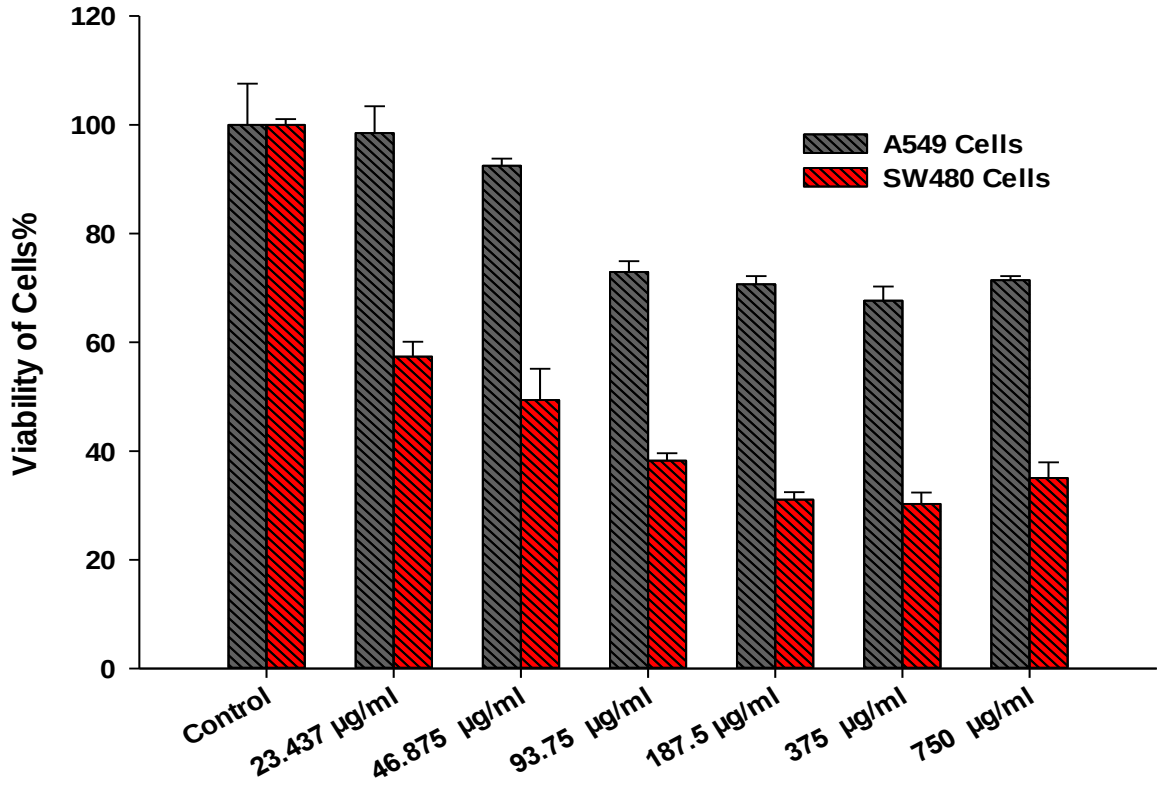


figure (3.6)The effect of metformin 10 µg /ml in combination with cisplatin on A549 and SW480 cell line

3.1.7 The effect of metformin 50 µg /ml in combination with cisplatin on A549 and SW480 cell line

There were significant ($p \leq 0.05$) decrease in viability percentage of A549 cell line only at high concentrations of cisplatin (750 and 375µg/ml) and constant concentration of metformin 50 µg /ml.

There were significant ($p \leq 0.001$) decrease in viability percentage of SW480 cell line at all concentrations of cisplatin with constant concentration of metformin (50 µg/ml) as shown in (figure 3.7).

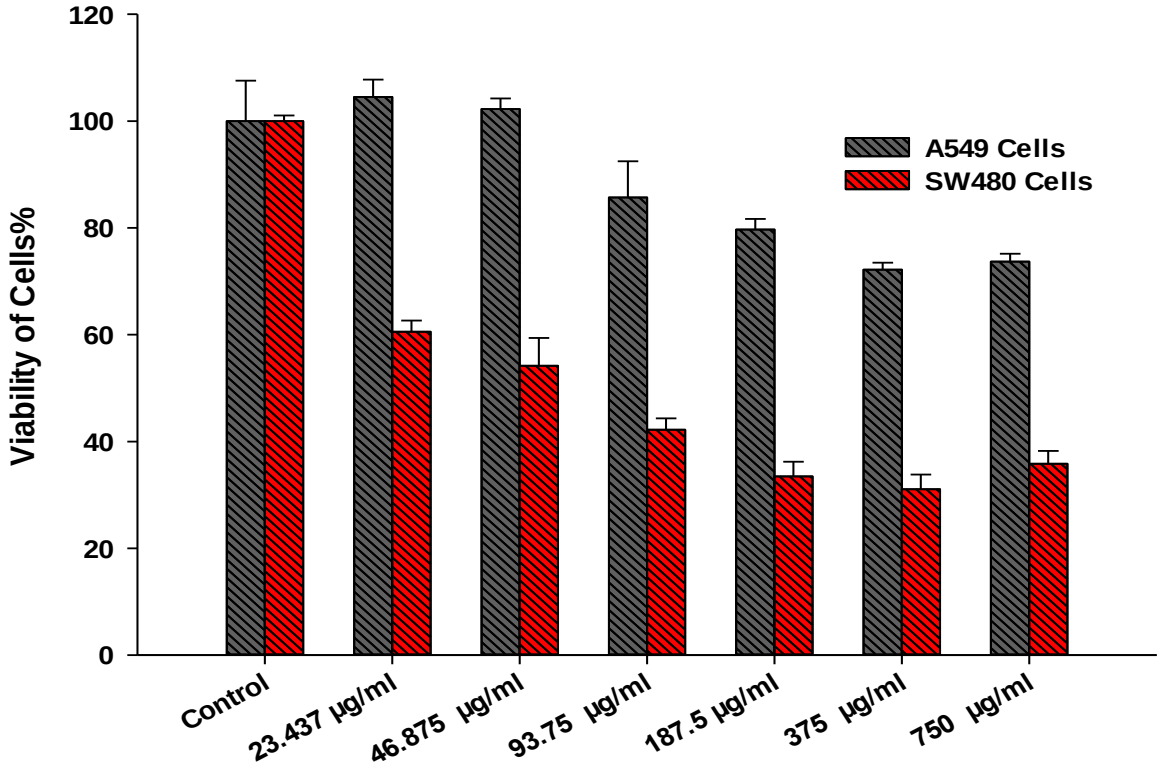
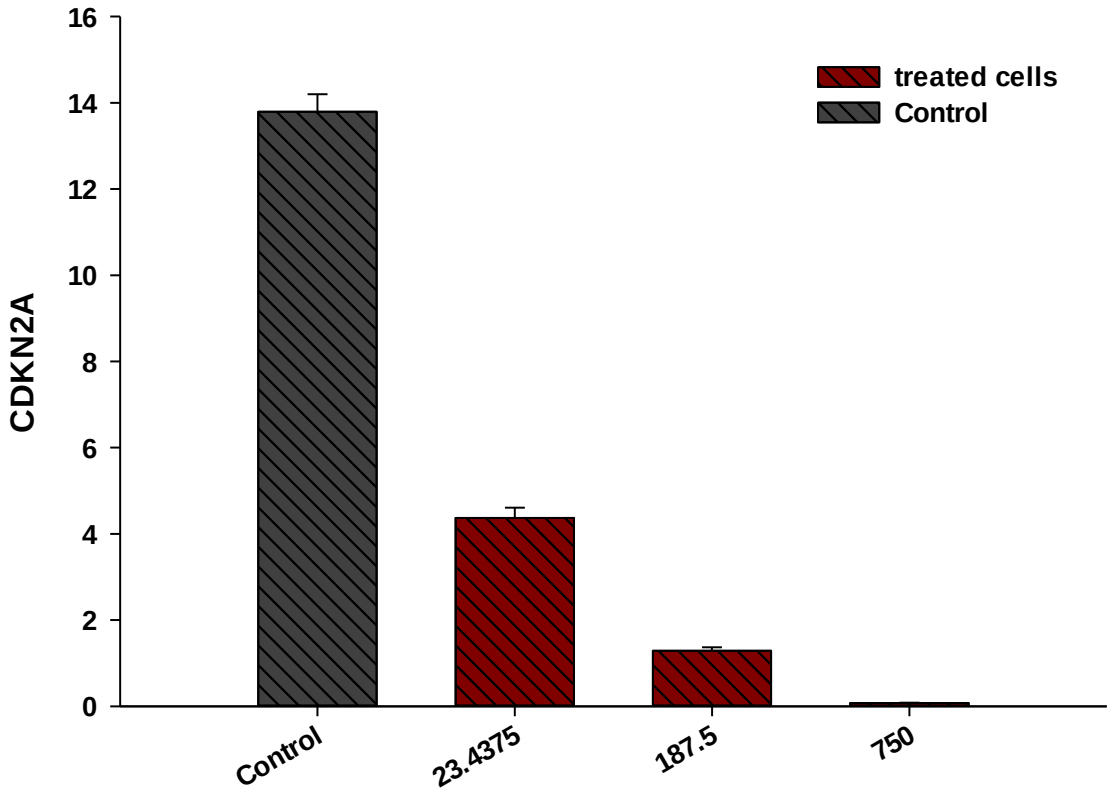


figure (3.7)The effect of metformin 50 µg /ml in combination with cisplatin on A549 and SW480 cell line

3.2 CDKN2A Kit result

3.2.1 Effect of cisplatin on CDKN2A

There were significant ($p \leq 0.001$) decrease in CDKN2A level as a result to cisplatin effect and the activity increase as the concentration of cisplatin increase as shown in figure (3.8).



Figure(3.8) effect of cisplatin on CDKN2A in A549 cell line.

3.2.2 Effect of metformin on CDKN2A

There were significant ($p \leq 0.001$) decrease in CDKN2A level in concentration 15.6 µg/ml and 62.5µg/ml as shown in (figure 3.9).

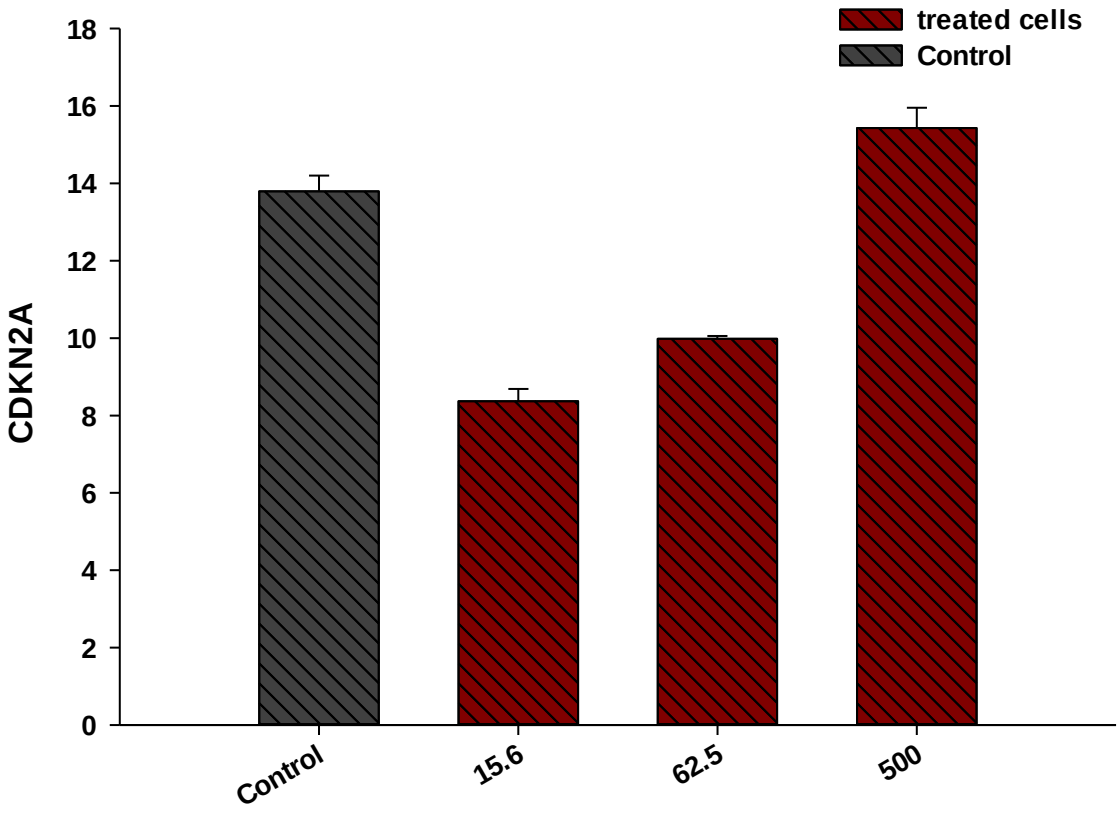


Figure (3.9) Effect of metformin on CDKN2A in A549cell line

3.2.3 Effect of hydroxychloroquine on CDKN2A.

There were significant ($p \leq 0.001$) decrease in CDKN2A level as the concentration of hydroxychloroquine increase (500 µg/ml) except for 62.5µg/ml as shown in figure (3.10).

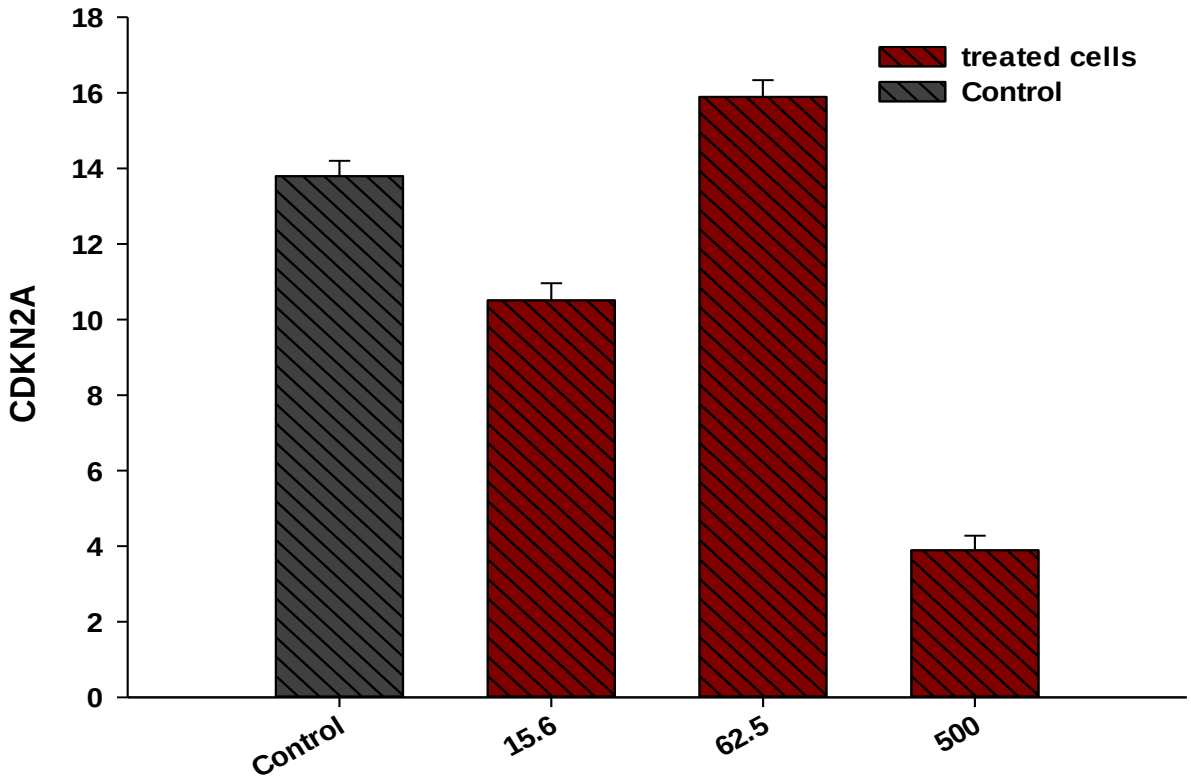


Figure (3.10) Effect of hydroxychloroquine on CDKN2A in A549 cell line.

3.2.4 effect of combination of hydroxychloroquin 25 µg/ml with cisplatin on CDKN2A.

There were significant ($p \leq 0.001$) decrease in CDKN2A level as the concentration of cisplatin increase in combination with constant concentration of hydroxychloroquine as shown in figure (3.11).

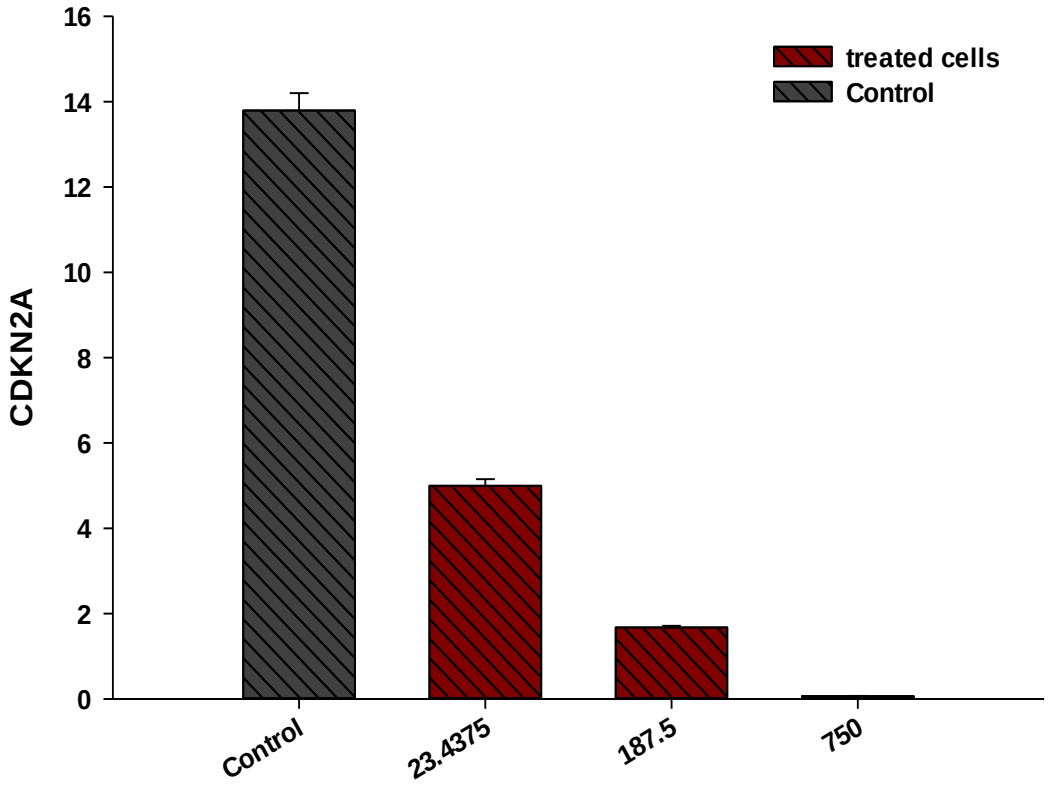


Figure (3.11)effect of combination of hydroxychloroquin 25 µg/ml with cisplatin on CDKN2A in A549 cell line.

3.2.5 effect of combination of hydroxychloquin 125µg/ml with cisplatin on CDKN2A

There were significant ($p \leq 0.001$) decrease in CDKN2A level as the concentration of cisplatin increase in combination with constant concentration of hydroxychloroquine as shown in (figure 3.12).

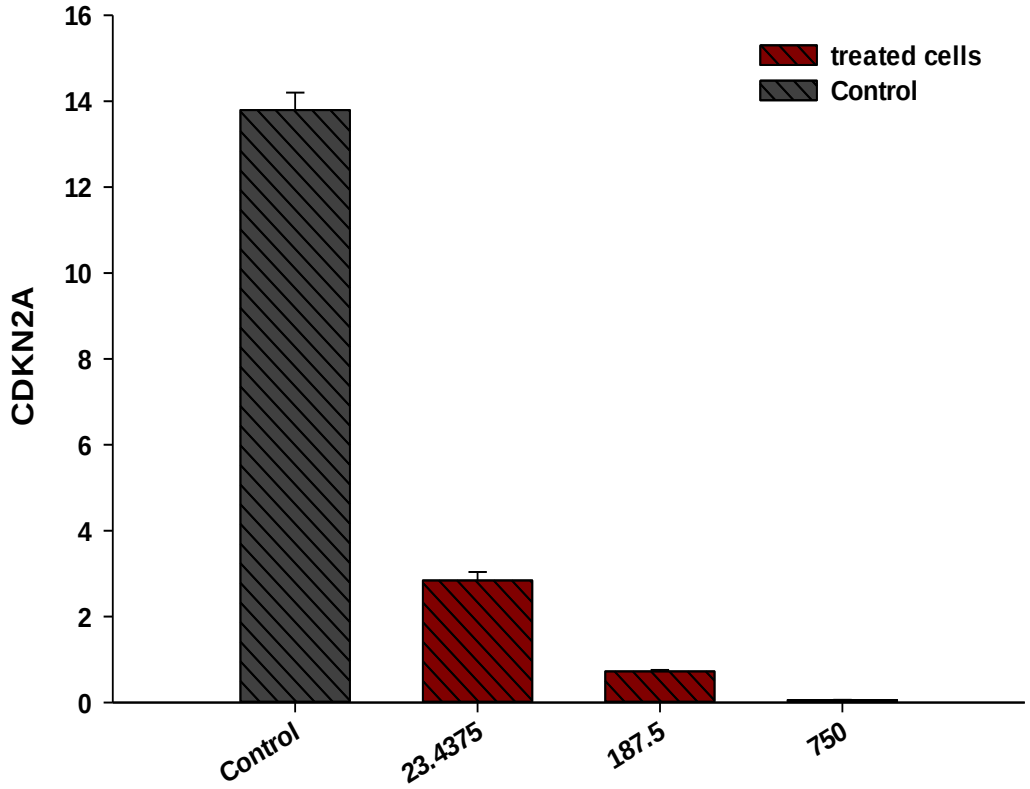


Figure (3.12) effect of combination of hydroxychloquin 125µg/ml with cisplatin on CDKN2A in A549 cell line

3.2.6 effect of combination of metformin 10µg/ml with cisplatin on CDKN2A

There were significant ($p \leq 0.05$) decrease in CDKN2A level as the concentration of cisplatin increase (187.5 and 750 µg/ml) in combination with constant concentration of metformin .

There was significant ($p \leq 0.05$) increase in CDKN2A level when the concentration of cisplatin equal to 23.437 µg/ml. due to effect of metformin as shown in figure (3.13).

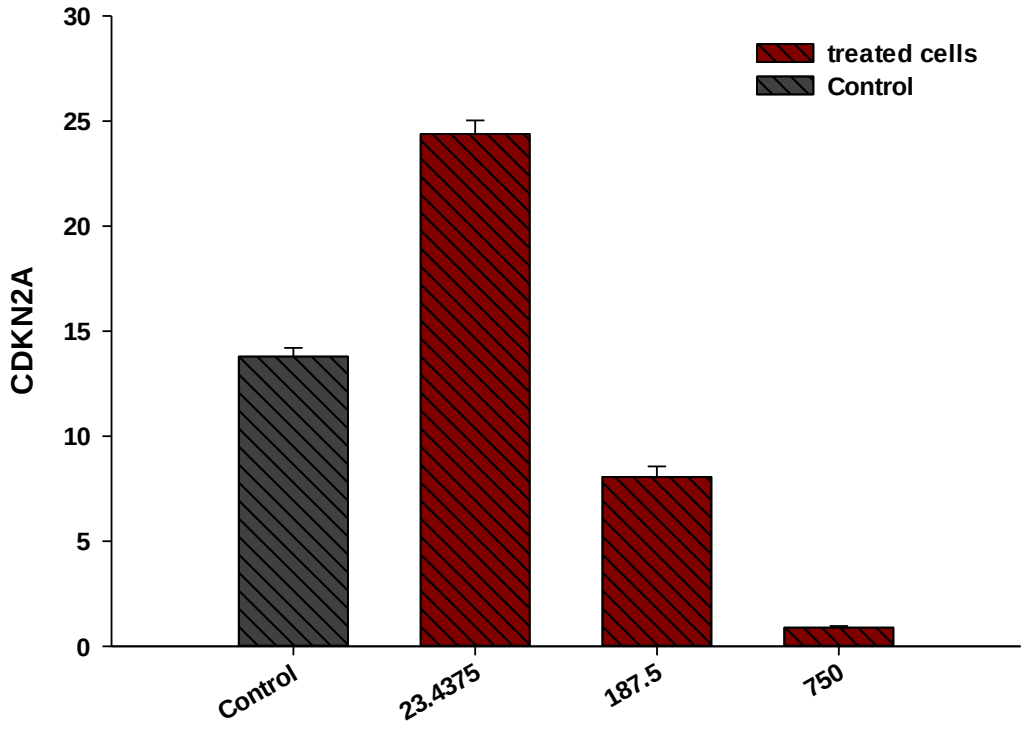


Figure (3.13)effect of combination of metformin 10µg/ml with cisplatin on CDKN2A in A549 cell line.

3.2.7 effect of combination of metformin 50 µg/ml with cisplatin on CDKN2A

There was significant ($p \leq 0.001$) increase in CDKN2A level when the concentration of cisplatin equal to 23.437 µg/ml. due to effect of metformin .

The level of CDKN2A decrease significantly ($p \leq 0.001$) when the concentration of cisplatin increased as shown in figure (3.14).

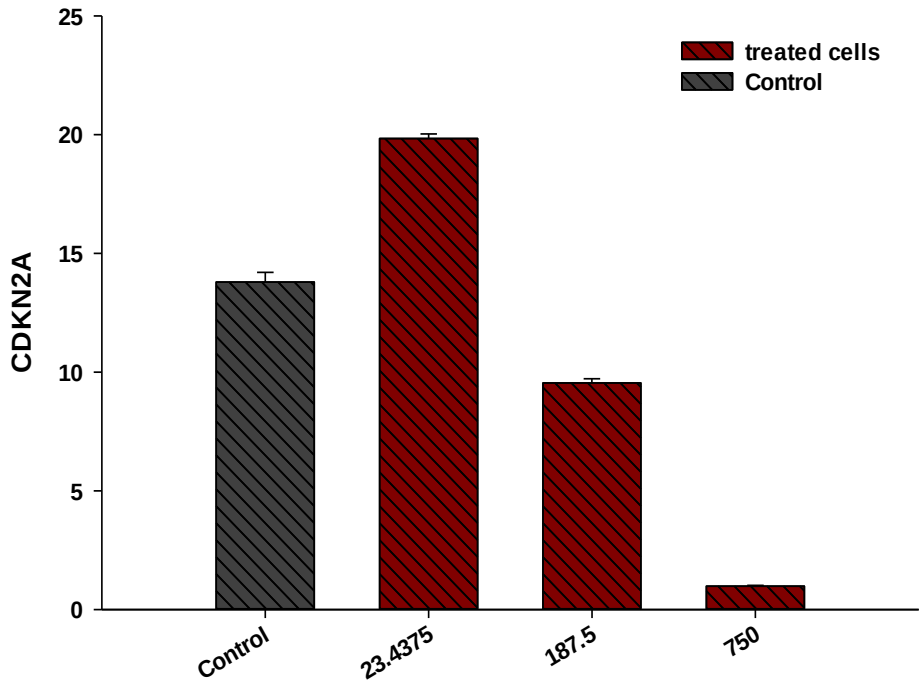


Figure (3.14)effect of combination of metformin 50 µg/ml with cisplatin on CDKN2A in A549 cell line.

Chapter Four

Discussion

4.1 The effect of cisplatin on A549 and SW480 cell line

The results showed that there decrease in viability percentage of A549 cell line in all concentration used of cisplatin except (23.347 , 46. 875) µg/ml. and decrease in the viability percentage of SW480 cell line for all concentration used of cisplatin..

This agree with the classical view on cisplatin cytotoxic activity emphasizes its interaction with DNA. Cisplatin forms different types of adducts with DNA (monoadducts, intrastrand crosslinks, DNA-protein crosslinks) triggering DNA damage response, cell cycle arrest, and apoptosis. Additionally, the role of mitochondrial damage resulting in excessive reactive oxygen species (ROS) generation and lipid peroxidation was highlighted. Altogether these cellular events trigger intrinsic (mitochondrial) apoptotic pathway characterized with cytochrome c release and apoptosome formation leading to caspase activation and cell death (Mezencev, 2014).

Cisplatin was also implicated in cell membrane fluidification which triggers non-specific Fas receptor activation and leads to extrinsic apoptotic pathway . Other mechanisms of cisplatin toxicity involve disruption of calcium signaling and Na+/H+ membrane pump and Na+/K+ ATPase inhibition. Additionally due to its great reactivity cisplatin may bind to various proteins including enzymes thus modulating their activity (Gąsiorkiewicz et al., 2021).

And disagree with (Bordin et al., 2013)Chemotherapeutic drugs that target the DNA molecule frequently initiate autophagy in cancer cells. Although autophagy has a beneficial effect as a tumor suppressor in healthy cells, induction of autophagy may promote survival of tumor cells leading to resistance to cell death. On the other hand, depending on the extent of damage and the cellular context, autophagy may also be the mediator of a non-apoptotic form of cell death called autophagic cell death. Platinum derived compounds primarily induce the formation of DNA intrastrand crosslinks, cells treated by platinum com-

pounds can induce autophagy as a way to prevent cell death.. As an example, autophagy has been shown to play a role in the resistance of cancer cells to cisplatin, the first platinum drug ever used in cancer treatment. Different studies have shown an increase in the number of autophagosomes after cisplatin exposure. Cisplatin triggers autophagy by activation of AMPK and downregulation of mTOR, thereby protecting glioma cells from oxidative stress, caspase activation and DNA fragmentation (Bordin et al., 2013).

4.2 The effect of metformin on A549 and SW480 cell line.

The result showed that metformin significant increase viability percentage of A549 cell line in high concentration (500µg/ml) only, while the same concentration decrease viability of SW480 cell line as shown in (figure 3.2).

this agree with (Verbaanderd et al., 2017) autophagy inducer agent can promote tumour growth in more advanced stages of cancer .Pro-survival autophagy is induced in response to a variety of stressful conditions including but not limited to, starvation, loss of proteostasis, organelle damage and hypoxia. Some anti-cancer treatments can also induce pro-survival autophagy. Autophagic properties such as nutrient recycling can support cancer cell survival. Moreover, key regulators of cell growth can be degraded and the DNA damage response can be suppressed through increased autophagy.

this explain the increase in viability percentage of A549 cell line in the presence of metformin which act as autophagy inducer.

Another study determined whether autophagy mediated by metformin affects cell viability, and analyzed the percentage of cell death, by staining with propidium iodide (PI) in response to various metformin concentrations in A375 melanoma cells. Metformin induced a dose-dependent increase of cell death . At a 10 mM metformin concentration, ~40% of the cells were dead. these results in-

dicate that metformin induces a concomitant induction of autophagy and apoptosis processes in melanoma cells, both of which are involved in cell death (Tomic et al., 2011).

This study agree with retrospective epidemiological studies which reported a decrease in cancer risk in diabetic patients treated with metformin. Importantly, a recent work of Nakajima lab demonstrates that metformin diminishes the formation of rectal aberrant crypt foci, a marker of colorectal cancer, in non-diabetic patients. Despite compelling evidence of a role of metformin as an anticancer drug, its mode of action in cancer remains unelucidated. In a few studies, metformin induces apoptosis in cancer, and in one study performed on colon cancer, metformin triggers autophagy. Undoubtedly, in cancer there are multiple functional relationship reported between the apoptosis and autophagy, and these processes separately or/and jointly seal the fate of the cell. Thus, apoptosis or/and autophagy are interesting mechanisms to induce cancer cell death (Tomic et al., 2011).

This result is agree with the research which shows that metformin can promote human pulmonary adenocarcinoma A549 cell lines in vitro migration and the strengthening of invasive activity, it potentially promotes tumor metastases, which might be unrelated to autophagy but relevant to metformin's inducing expression up-regulation of MMP2 and MMP9 in tumor cells(Ashrafizadeh et al., 2021).

And disagree with (Dong et al., 2020) which found that metformin can promote the expression of miR-7 in a dose- and time- independent manner mediated by AMPK in A549 cell line. Furthermore, both metformin and miR-7 can inhibit A549 cell growth, migration, and invasion and discovered that metformin treatment for 24 hours strongly reduces A549 cell growth in a dose-independent manner, and this effect is enhanced at 48 hours. metformin (5 mM) treatment of A549 cells lead to a significant decrease in proliferation. However, the inhibitory

effect on A549 cells has no plateau period, even when they set the maximum concentration of metformin to 60 mM (which is already a relatively high concentration); the same phenomenon occurred with the inhibition of migration and invasion in A549 cells, which implies that A549 is sensitive to both high and low concentrations of metformin.

(Lan et al., 2017) found that metformin significantly suppresses the proliferation and formation of human colorectal cancer cells, which the growth-inhibitory effect is attribute to the apoptosis induced by metformin treatment. Increasing evidence indicates that the mTOR plays important role in the survival and proliferation of cancer cells. Metformin has been shown to inactivate mTOR via activation of AMPK, thereby causing cell cycle arrest, apoptosis and inhibition of tumorigenesis. In human colorectal cancer, the anti-tumor effects of metformin are indeed mediated by activation of AMPK and suppression of mTOR, leading to increasing apoptosis of human colorectal cancer cells.

And agree with research for colon cancer cells, which indicated that 5 mM of metformin resulted in the highest cell mortality (96.91%) followed by the concentration 100 mM (96.24%) . This might indicate that colon cancer cells were sensitive even to lower doses of metformin. It was indicated elsewhere that lower concentrations of metformin *i.e.*, 5 and 10 mM were capable to inhibit the cell growth of colorectal cancer cells *in vitro*.(Sabit et al., 2018)

The high concentrations of metformin caused some cell death in the colorectal cancer cell line HCT116, which was significant up to 32 hrs. However, there was no significant cell death observed in the colorectal cancer cell line SW480. This indicates that not all cancer cell lines respond in a similar way to treatment with metformin, and individual studies on different cell lines are necessary. (Kamarudin et al., 2019)

4.3 The effect of hydroxychloroquine on A549 cell line and SW480 cell line after incubation for 24 hour :

The result showed that there is significant decrease in cell viability percentage of A549 and SW480 cell lines in all concentrations used in comparison with control group except (31.25µg/ml and 15.8 µg/ml) as shown in (figure 3.3).

Hydroxychloroquine alone could inhibit gastric cancer cell line BGC823 cells in a dose-dependent manner, with a half-inhibitory concentration of 52.95±6.82 μg/ml. (W. Wang et al., 2019) and Single-treatment with hydroxychloroquine was found to induce both apoptosis and necrosis, depending on the concentration, in A549 lung cancer cells (Y.-S. Park et al., 2005)

The result agreed with (Li et al., 2018) , when the A549 cells were treated with hydroxychloroquine at different concentrations and observed the cytotoxic effects of hydroxychloroquine only at high concentrations (> 80 µg/ml). However, the tumour cells that were pre-treated with hydroxychloroquine at a low concentration (5 µg/ml) showed enhanced sensitivity to doxorubicin and were prone to death, even though hydroxychloroquine caused no cytotoxicity.

(Y. Chen et al., 2021) investigate the effects of hydroxychloroquine on lung cancer cell growth, A549 cells, a lung cancer cell line, which treated with the drug at various concentrations (128- 0.25 µg) for 24–72 h. The results showed that, at lower concentrations (from 0.25 to 32 µg), hydroxychloroquine inhibited the growth of A549 cells and, at the same time, it inhibit autophagy by blocking acidification of the lysosome. On the other hand, at higher concentrations (64–128 µg), hydroxychloroquine induced apoptosis at 24 h, in addition to autophagy inhibiting effect.

(Yang et al., 2020) revealed that both chloroquine and hydroxychloroquine have shown certain cytotoxicity in eight different types of cell lines which included IMR-90, A549, ARPE-19, Hep3B, Vero, HEK293, H9C2, and IEC-6 in

time- and dose-dependent manner in vitro, suggesting the necessity of short period administration clinically And both chloroquine and hydroxychloroquine could induce cell cycle arrest and apoptosis which are consistent with the phenotype of proliferation and could partially explain the toxic effect of chloroquine and hydroxychloroquine.

And agree with the results showed that hydroxychloroquine exhibited significant cytotoxicity at 48 h when the dosing regimen was more than 100 μg/ml . hydroxychloroquine inhibited the viability of most of these cells in a dose- and time-dependent manner (Yang et al., 2020).

The result agree with (Liao & Xiao, 2021) work, they used the primary colorectal cancer-derived cell line (SW480) and the lymph-node-derived cell line (SW620).The inhibition of autophagy potentiated the effects of the single molecules *in vitro,* especially to induce apoptosis but also, in a spectacular way, *in vivo* on tumour growth. When analysing the mechanisms of inhibition of tumour growth, they observed that size reduction was mainly du to proliferation impairment and apoptosis potentialization in both cell line engrafted tumours. However, as shown with HES staining, necrosis also took place and they cannot absolutely rule out involvement of this pathway in tumour reduction.

Except for its effect on hematological malignancies, hydroxychloroquine was also shown to have antineoplastic effects on solid tumors: An anti breast-cancer effect was shown by demonstration of its antiproliferative effect on the human breast tumor cell models MCF-7 and Bcap-37 . An antiproliferative effect on mouse colon cancer cell line was shown, as well as a reduction in tumor volume in vivo with chloroquine treatment . Chloroquine was also shown to potentiate the antiproliferative effect of 5-fluorouracil on the human colorectal cell line HT-29 . Chloroquine was shown to inhibit growth and induce cell death of A549 lung cancer cells (Ben-Zvi et al., 2012).

Also in vivo studies showed that Doses of 25 and 50 mg/kg of hydroxychloroquine both significantly increased survival time and reduced primary tumour volume in mice implanted with a highly metastasizing breast cancer cell line. Interestingly, the number and diameter of lung metastases was reduced as well, and hydroxychloroquine enhanced tumour cell apoptosis in the high dose group (Verbaanderd et al., 2017).

4.4 The effect of hydroxychloroquine 25 µg/ml in combination with cisplatin on A549 and SW480 cell line

The result of combination of hydroxychloroquine and cisplatin at different concentration showed that there is significant decrease in viability percentage of both A549 and SW480 cell line with all concentrations of cisplatin and constant concentration of hydroxychloroquin (25 µg/ml) in comparison with the control group as shown in (figure 3.4) .

The same result was observed in Lewis cells, indicating that hydroxychloroquine functions as a chemo-sensitizer to enhance the anticancer effects of chemotherapeutic agents without killing cancer cells directly. Moreover, other chemotherapeutic agents such as paclitaxel, and Cisplatin also showed enhanced inhibition of tumor proliferation in A549 (Li et al., 2018).

Our result is also agree with (Y.-S. Park et al., 2005) when Two malignant glioma cell lines [U87MG, T98G] were treated with cisplatin alone or hydroxychloroquine alone or cisplatin and hydroxychloroquine together after incubation for 24 hours , the cell viability was found to be markedly decreased when hydroxychloroquine was co-administered with cisplatin on both U87MG and T98G cell lines as Chloroquine and its analog hydroxychloroquine accumulate in the lysosome, they become protonated within the acidic environment and cannot diffuse back out. The ability for chloroquine alone to cause a dose-dependent

inhibition in glioma cells strongly suggests that these cells are highly dependent on autophagy.

The result agree with (Wu et al., 2015) who deployed an autophagy inhibitor to determine its role in the antineoplastic properties of cisplatin and found that its combination with hydroxychloroquine significantly enhanced the anti-proliferative effect on lung cancer cells compared to cisplatin alone in A549 cells and A549/DDP cells. hydroxychloroquine induced a prominent sensitization effect of cisplatin on A549 cells.

This because autophagy and apoptosis are highly interconnected but this relationship has not been well elucidated. Thus, conclude that autophagy appears to have a prosurvival role in protecting lung adenocarcinoma cells from a cisplatin-mediated anti-proliferation effect and causing resistance to cisplatin. Autophagy could regulate degradation, recycle the damaged cytoplasmic contents, and prevent cisplatin-induced DNA damage(Wu et al., 2015).

The BC001 is a novel fully humanized monoclonal antibody of vascular endothelial growth factors receptor 2 (VEGFR2), they reported that hydroxychloroquine enhanced the antiproliferative and proapoptotic properties of BC001 in vitro, and promoted the antitumor effects of BC001 on a BGC823 cell-based xenograft tumor in vivo. the data also revealed that BC001 did not influence autophagy, whereas hydroxychloroquine could inhibit autophagy by impairing autophagosome fusion with lysosomes and induced severe ultrastructural changes, which may contribute to the impaired fusion. In addition, the results from RTCA data showed that BC001 (20 μg/ml) or hydroxychloroquine (25 μg/ml) alone could decrease the cell index; the cell index of cells treated by hydroxychloroquine and BC001 combined was the lowest, indicating that BC001 and hydroxychloroquine could inhibit BCG823 proliferation. Of note, hydroxychloroquine significantly enhanced the anti-proliferative effect of BC001 (W. Wang et al., 2019).

4.5 The effect of hydroxychloroquine 125 µg/ml in combination with cisplatin on A549 and SW480 cell line .

The result of combination of hydroxychloroquine and cisplatin at different concentration showed that there is significant decrease in viability percentage of both A549 and SW480 cell line with all concentrations of cisplatin and constant concentration of hydroxychloroquin (125 µg/ml) in comparison with the control group as shown (figure 3.5) .

The antimalarial drugs Chloroquine and hydroxychloroquine have potential anticancer effects by suppressing autophagy and inducing apoptosis in bladder cancer cells. Recombinant Bacillus caldovelox arginase mutant (BCA-M) has been developed for the therapy of several cancer cell lines, and has positive effects in anticancer therapy by reducing cancer growth in human cervical cancer cells. In a phase III clinical trial, BCA-M showed positive therapeutic effects on cancer cells via the inhibition of growth, by increasing apoptosis and cell cycle arrest. In addition, combination treatment with BCA-M and CQ promotes the therapeutic effects of BCA-M by reducing autophagy. In phase I clinical trial, the combination of hydroxychloroquine and chemotherapeutic reagents is increased the median progression-free survival (mPFS) and overall survival in 18 patients with relapsed or refractory multiple myeloma (Yun et al., 2021).

Additionally, the therapeutic efficacy of combination with hydroxychloroquine and anticancer vorinostat , which is the histone deacetylase inhibitor, has been investigated in 19 patients with metastatic colorectal cancer. The combination treatment is showed the 2.8 months mPFS and 6.7 months OS in patients with refectory colorectal cancer and confirmed the safe and well tolerated in refractory colorectal cancer patients. In phase 1/2 trial, 35 patients with borderline resectable pancreatic adenocarcinomas were treated with an hydroxychloroquine dose of 1200 mg daily until the day of surgery combined with doses of fixed-dose gemcitabine (1500mg/m2). The trial demonstrated that pre-operative

autophagy inhibition with hydroxychloroquine plus gemcitabine is safe and well-tolerated. (Yun et al., 2021)

The result agree with (Gurunathan et al., 2018) hydroxychloroquine is an autophagy inhibitor that increases the lysosomal pH. hydroxychloroquine treatment blocks the fusion of autophagosomes with lysosomes and subsequent lysosomal protein degradation, thereby promoting autophagosome accumulation . In Ishikawa, KLE, and AN3CA cell lines of endometrial cancer, hydroxychloroquine treatment suppresses cell proliferation via the induction of apoptosis. Furthermore, the combination of cisplatin and hydroxychloroquine in cisplatin-resistant cells enhances their sensitivity to cisplatin. These results indicate that the induction of autophagy protects cancer cells against cancer treatment.

4.6 The effect of metformin 10 µg /ml in combination with cisplatin on A549 and SW480 cell line

The result showed that there was significant decrease in viability percentage of SW480 cell line at all concentrations of cisplatin with constant concentration of metformin (10 µg/ml). while there was significant decrease in viability percentage of A549 cell line at higher concentrations of cisplatin (750 , 375 , 187.5 and 93.75 µg/ml) only.

This result agree with (P. Zhang et al., 2020) that Saied combination therapy is an important strategy to improve therapeutic outcomes and reduce the toxic side effects of anticancer drugs. Recent studies have established that metformin effectively inhibits cell proliferation as a promising therapeutic agent for cancer, and increases the apoptotic sensitivity to other chemotherapeutic drugs, as they found that metformin combined with cisplatin could evidently increase inhibition of cell viability and apoptosis of SW480 and SW620 cells, compared to treatment with metformin or cisplatin alone.

Studies have shown that metformin enhances the effect of cisplatin in various cancers. For example, the development of cisplatin and metformin nano-cubosomes induces colorectal cancer cell apoptosis through inhibition of several metabolic pathways, namely, AMPK/mTOR and Akt/ Mtor (Saber et al., 2018). Meanwhile, metformin increases the chemotherapeutic sensitivity of liver cancer cells to cisplatin through the AMPK pathway (Dong et al., 2017).

(Gąsiorkiewicz et al., 2021) showed that metformin in micromolar (5–30 μM) concentrations effectively sensitized breast and cervical cancer cell lines towards cisplatin. This was associated with a increase in cisplatin-induced autophagy and increased apoptosis. Metformin alone was also capable of triggering those changes and inhibited cell proliferation, however to a lesser extent. The action of micromolar concentrations of metformin was independent of ATP production alteration and AMPK/mTOR pathway.

In NSCLC cell lines H1650 and A549 metformin has been shown to regulate the balance of death and survival, via strong inhibition of drug-induced multi-nuclei formation (mitotic catastrophe) and simultaneously elevating autophagy marker LC3B expression . Consequently, these cells have a chance to initiate a transition to resistance. through examining morphological and biological changes, they analyzed the occurrence of autophagy in drug-treated cells in comparison to chloroquine treatment as a positive control. Microtubule-associated protein light chain 3 (LC3B) is a specific marker used to monitor autophagy initiation. Immunofluorescence (IF) analysis revealed that after metformin (5μg and 20μg) treatment the intensity of LC3B was markedly increased in all tested cell lines. While treatment with cisplatin dramatically increased multi-nuclei formation, which is a hallmark of cell death through mitotic this effect was prevented by the combination with metformin. this abrogation of multinucleated cells was clearly seen in H1650 and A549 cells(Xiao et al., 2017) .

Taken together, there is evidence that metformin may directly modify and inhibit drug-induced lung cancer cell apoptosis via enhancing LC3B expression and autophagosome formation to shift cells towards autophagy. This effect might temporarily prevent cancer cells from apoptosis via cellular compensation of energy deprivation, or initiate a resistance to drug treatment(Xiao et al., 2017) .

The preliminary experiments were set up to screen the responsiveness of various cancer cell lines to combined cisplatin/metformin treatment. To that end, we incubated U251 glioma, C6 glioma, SHYS5Y neuroblastoma, L929 fibrosarcoma, HL-60 leukemia and B16 melanoma cells with cisplatin (25 µg/ml), metformin (4 µg/ml), or both drugs together for 24 h. Expectedly, cisplatin reduced viability of all tested tumour cell lines, as revealed by a decrease in MTT reduction and increase in LDH release. Metformin also reduced mitochondrial dehydrogenase activity in tumour cell cultures , but did not increase LDH release even after prolonged incubation (48 hours). As LDH release occurs in dead cells as a consequence of cell membrane disruption, it appears that metformin-mediated inhibition of MTT reduction was due to a decrease in cell proliferation and consequent reduction in total mitochondrial number, rather than to induction of cell death. Surprisingly, when both drugs were applied together, metformin markedly reduced cisplatin cytotoxicity towards U251, C6, SHYS5Y, L929 and HL-60 cells. On the other hand, metformin increased cisplatin-mediated killing of B16 melanoma cells. These data indicated that metformin could antagonize the in vitro anticancer activity of cisplatin in a cell-specific manner. U251 glioma cells, being most responsive to metformin-mediated cytoprotection, were selected to further explore this phenomenon. Additional experiments in which U251 cells were treated with the constant dose of cisplatin and different doses of metformin, and vice versa, confirmed the cytoprotective effect of metformin, which remained evident after 48 h. In conclusion, our data demonstrate that the antidiabetic drug metformin can rescue cancer cells from cisplatin-induced oxidative

stress and apoptosis through modulation of AMPK, autophagy (Janjetovic et al., 2011).

4.7 The effect of metformin 50 µg /ml in combination with cisplatin on A549 and SW480 cell line

The result showed that there was significant decrease in viability percentage of SW480 cell line at all concentrations of cisplatin with constant concentration of metformin (50 µg/ml). while there was significant decrease in viability percentage of A549 cell line at higher concentrations of cisplatin (750 and 375µg/ml) and constant concentration of metformin in comparison with the control but no significant decrease in viability percentage at lower concentrations (187.5, 93.75 , 46.875 and 23.437 µg/ml) of cisplatin as shown in figure 3.7

Many tumor-associated conditions, including intermittent oxygen and nutrient deprivation, oxidative stress, fast growth and cell death suppression, modulate, in parallel and in interconnected ways, both cellular metabolism and autophagy to enable cancer cells to rapidly adapt to environmental stressors, maintain uncontrolled proliferation and evade the toxic effects of radiation and/or chemotherapy(Lozy & Karantza, 2012) and because metformin act as autophagy inducer it can promote autophagy in cancer cell specially A549 cell line in time and dose dependent manner, help the cells evade toxic effect of chemotherapy (cisplatin) and shift A549 cell line toward cisplatin resistance.

(Verbaanderd et al., 2017) Saied that autophagy can promote tumour growth in more advanced stages of cancer .Pro-survival autophagy is induced in response to a variety of stressful conditions including but not limited to, starvation, loss of proteostasis, organelle damage and hypoxia. Some anti-cancer treatments can also induce pro-survival autophagy. Autophagic properties such as nutrient recycling can support cancer cell survival. Moreover, key regulators of cell growth can be degraded and the DNA damage response can be suppressed through increased autophagy

This result is agree with (P. Zhang et al., 2020) that investigate the effect of metformin or/and cisplatin on cell viability, SW480 and SW620 cells were incubated with various concentrations of metformin (1.25, 2.5, 5, 10, and 20 mM), different concentrations of cisplatin (1, 2, 4, 8, and 16 μM), and cisplatin (4 mM) combined with metformin (1.25, 2.5, 5, 10, and 20 mM). The results demonstrated that metformin and cisplatin attenuated cell viability of SW480 and SW620 cells in a concentrationand time-dependent manner within a certain range of concentration, respectively. Metformin combined with cisplatin could markedly increase inhibition of cell viability in SW480 and SW620 cells, compared to treatment with metformin alone. To further explore the cytotoxicity of metformin and cisplatin in CRC cells, colony formation assay was performed. combination of metformin and cisplatin could obviously inhibit the colony formation in SW480 and SW620 cells, compared to treatment with metformin or cisplatin alone. Therefore, metformin combined with cisplatin efficiently suppressed cell viability of CRC cancer.

(D. Wang & Wu, 2015) found that cell viability was significantly reduced by co-treatment with cisplatin and metformin for 48 hours. These results indicated that combined treatment with cisplatin and metformin may be more effective against T24 and BIU-87 cell proliferation *in vitro* than treatment with cisplatin and metformin alone. Subsequently, the combined effect of cisplatin and metformin treatment was compared with that of the effect of cisplatin treatment alone. Cell cycle assays revealed an increased sub-G1 phase cell population in the cisplatin and metformin treatment groups in all cell lines. In particular, the ratio of sub-G1 cells was increased most potently by co-treatment compared with that of cisplatin and metformin monotherapy. The mechanism of this combined effect independent on autophagy and examined by measuring the expression levels of proteins associated with cellular AMPK and mTOR signaling. AMPK is an energy receptor within cells. Thus, a change in intercellular pressure or the consumption of glucose may increase the AMP/ adenosine triphosphate (ATP) ratio,

activating AMPK expression by phosphorylation, and promoting the synthesis and utilization of glucose reabsorption. Therefore, AMPK is a critical regulatory pathway under normal physiological conditions. However, the predominant function of AMPK in tumors is to inhibit tumor cell proliferation and regulate apoptosis. It has been reported that metformin may induce a loss of mitochondrial membrane potential and inhibition of ATP production, which may consequently activate expression of the AMPK protein in prostate cancer cells.

Another study showed that under glucose-deprivation conditions, metformin enhanced cisplatin cytotoxicity in esophageal cancer cells. Metformin targets cancer cells by various mechanisms, the mitochondria being its primary target where it leads to inhibition of several complexes and hence decreased ATP production. Activated AMPK, secondary to ATP depletion, inhibits mTOR and shuts down ATP-consuming pathways to maintain energy homeostasis under cellular stress conditions. It will therefore inhibit glucose, lipid and protein synthesis needed for cell growth, whereas fatty acid oxidation, glucose uptake and thus glycolysis are stimulated. Studies revealed the amplification of chemotherapy-induced AMPK activation by metformin followed by induction of tumor cell apoptosis. The current study demonstrated a significant increase in AMPK levels, secondary to decreased ATP synthesis and/or increased utilization evident by the increase in AMP/ATP ratio, in nano-cubosomes-treated groups accompanied with increased glucose uptake. This effect was profound in nano-cubosomes loaded with both drugs suggesting that metformin potentiates cisplatin effect (Saber et al., 2018).

4.8 CDKN2A

An increase in ROS is acommon feature in cancer cells. Apart from replicative stress-driven DNA damage, accumulated ROS can also damage DNA and induce oncogene-induced senescence (OIS) by inducing autophagy. Oncogene activation is a stressor that usually leads to the derepression of the CDKN2A (cyclin-dependent kinase inhibitor 2A) locus (consisting of p16 and ARF tumor suppressor)(Hamsanathan et al., 2019).

The result agree with (Ferlay et al., 2021) Saied that autophagy is another possible mechanism involved in cellular senescence . autophagy often exerts protective effects under various stress conditions. Recently, autophagy was considered as an effective mechanism involved in the induction of cellular senescence. during senescence, autophagy is induced and activated, consequently facilitating the process of senescence. In addition, the senescence phenotype could be delayed if autophagy is inhibited. cellular senescence is tightly correlated with up-regulation of cyclin-dependent kinase (CDKs) inhibitors. the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene encodes two different proteins, $p16^{INK4a}$ (hereafter referred as p16) and ARF, through an alternative splicing mechanism.

under many different settings of nutrient deprivation conditions, including in calorie restriction CR, autophagy is induced to regulate the organism's homeostasis. Although it is clear that CR represents a strong physiologically autophagic inducer, it is uncertain whether autophagy contributes to the anti-aging effects of CR. Recently, several studies have shown that CR promotes histone deacetylation and methylation leading to down-regulation of p53 (tumor suppressor protein p53 also known as tumor protein 53), and p16Ink4a (gene encoding the tumor suppressor

protein cyclin-dependent kinase inhibitor 2A or CDKN2A or multiple tumor suppressor 1 (MTS-1)(Ribarič, 2019).

Multiple studies in mammalian cells have consistently indicated that zinc promotes autophagy. Incubation of cells in media with high levels of zinc, zinc ionophores or combination of zinc with pro-oxidant agents/conditions such as ethanol, Tamoxifen or ischemia/reoxygenation has been shown to stimulate autophagy. Conversely, zinc depletion, caused by addition of zinc chelators to media, leads to inactivation of autophagy (basal and stimulated) in various mammalian cell lines and breast cancer cell line MCF_7. This demonstrates that endogenous zinc in cells is required for autophagy and they observed an upregulation of CDKN2A and p53 in zinc supplemented MCF-7 cells; in contrast zinc depleted cells differential expression was observed. Senescence and mutated p53 in zinc depleted cells may be due to the failure of CDKN2A expression and autophagy inhibition. But, zinc adequate cells shown enhanced CDKN2A expression(Al-Saran et al., 2016).

The result also agree with the research that found androgen could increase autophagy marker protein LC3BII in the cultured granulosa cells implying that androgen may induce autophagy and androgen excess contributed to activation of autophagy in granulosa cells of women with recurrent miscarriage (RM) due to PCOS then they found that high concentration of androgen reduced CDKN2a levels in the same patient. The results from study and other studies suggest that reduced CDKN2a level in endometrium might play an important role in RM of women with high androgen levels. they establish that endometrium expressed CDKN2a gene during early pregnancy and it has a possible role in blastocyst implantation (Rahman et al., 2018).

Genistein is cytotoxic to ovarian cancer cells. The mechanism of genistein-induced cell death includes both apoptosis and autophagy. Because autophagy is typically an adaptive response to nutrient starvation, they hypothesized that genistein could induce a starvation-like signaling response. Thus, this studies showed that the expression of CDKN2a was markedly changed in kidney cancer cell (769-P) and CDKN2a hypomethylation induced by genistein is consistent with that of kidney cancer, which implicates that the decreasing CDKN2a methylation levels plays an important role in human kidney cancer. Previously, CDKN2a was reported to be closely related to apoptosis,autophagy and CDKN2a mediates the development of the disease by regulating apoptosis(Ji et al., 2020).

Conclusions

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Recommendations

Conclusions

1-autophagy inhibitors augment anticancer activity of cisplatin .

2-. autophagy inducer reduce anticancer activity of cisplatin and initiate drug resistanse.

3- metformin increase viability of lung cancer cell A549 , while decrease viability of colorectal cancer SW480.

4- hydroxychloroquine decrease cell count of both lung cancer cell A549 and colorectal cancer SW480.

5. The concentration of CDKN2A increase when use autophagy inducer (metformin). but decrease with autophagy inhibitor (hydroxychloroquine).

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Recommendations

1- Measuring cytotoxic effects of metformin and hydroxychloroquine on other cancer cell lines.

2- Determine the combination cytotoxic effects of metformin and hydroxychloroquine with other anti-cancer drugs

3- Determine the effects of metformin and hydroxychloroquine on different cancer cell lines using different tumor markers

4- Measuring cytotoxic effects of cisplatin, metformin and hydroxychloroquine on lab animals.

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الخلاصة

تـم تنفيـذ الجـزء العملـي للدراسـة البحثيـة الحاليـة فـي مختبـر أبحـاث طلبـة الدراسـات العليـا /فـرع الأدويـة / كليـة الطـب / جامعـة بابـل خـلال الفتـرة مـن كـانون الثـاني 2021 لغايـة كـانون الاول 2021.

التجربة جزأين:

الجزء الاول: فحص السمية الخلوية

التجربة الأولى: دراسة التأثير السام للخلايا لكل عامل (سيسبلاتين ، ميتفورمين ، هيدروكسي كلوروكوين) وحده لتحديد الفعالية والتركيز السام للخلايا على النحو التالي:

* تم زرع الصفائح المكونة من 96حفرة باستخدام خط خلية A549 لسرطان الرئة البشري وسرطان القولون والمستقيم SW480 بكثافة زرعية 5 * 10^5 ومعاملة اللوحة (باستثناء عمود واحد من كل لوحة والذي ترك دون علاج كعنصر تحكم) ، تعرض إلى 200 ميكرولتر من ستة تخفيفات متسلسلة من سيسبلاتين على النحو التالي (750 ميكروغرام / مل ، 375 ميكروغرام / مل ، 187.5 ميكروغرام / مل ، 93.75 ميكروغرام / مل ، 46.875 ميكروغرام / مل و 23.437 ميكروغرام / مل). بعد انتهاء التجارب ، تم تغطية الطبق بغطاء بلاستيكي واحتضانه لمدة 24 ساعة. بعد ذلك ، تم غسل الألواح بـ 200 ميكرولتر من فوسفيت بفر سلاين المعقم ، وتم تقييم تأثير السيسبلاتين على نمو خطوط الخلايا عن طريق مقايسة السمية الخلوية باستخدام المادة البنفسجية البلورية.

* تـم تكـرار الإجـراء باسـتخدام الميتفـورمين بتخفيـف متسلسـل (500 ميكروغـرام / مـل ، 250 ميكروغـرام / مـل ، 125 ميكروغـرام / مـل ، 62.5 ميكروغـرام / مـل ، 31.25 ميكروغـرام / مـل و 15.8 ميكروغـرام / مـل) واحتضـانها لمـدة 24 سـاعة ، وتـم تقيـيم تـأثير المتفـورمين علـى نمـو خطوط الخلايا عن طريق مقايسة السمية الخلوية باستخدام المادة البنفسجية البلورية

* تـم اسـتخدام نفـس الإجـراء ولكـن مـع التخفيفـات التسلسـلية لهيدروكسـي كلـوروكين (500 ميكروغـرام / مـل ، 250 ميكروغـرام / مـل ، 125 ميكروغـرام / مـل ، 62.5 ميكروغـرام / مـل ، 31.25 ميكروغـرام / مـل و 15.8 ميكروغـرام / مـل) واحتضـانها لمـدة 24 سـاعة ، وتـم تقيـيم تـأثير الهايدروكسـي كلوروكـوين علـى نمـو خطـوط الخلايـا عـن طريـق مقايسـة السـمية الخلويـة باستخدام المادة البنفسجية البلورية

التجربة الثانية: كانت نتيجة الجمع بين العوامل الثلاثة مع نفس الإجراء على النحو التالي:

* مزيج من التخفيف التسلسلي سيسبلاتين وهيدروكسي كلوروكين 25 ميكروغرام / مل .

* مزيج من التخفيف التسلسلي سيسبلاتين و هيدروكسي كلوروكوين125 ميكروغرام / مل .

*مزيج من التخفيف التسلسلي سيسبلاتين والميتفورمين 10 ميكروغرام / مل .

* مزيج من التخفيف التسلسلي سيسبلاتين والميتفورمين 50 ميكروغرام / مل.

أظهرت النتائج وجود انخفاض معنوي في نسبة بقاء الخلية لخلايا A549 في جميع التركيزات المستخدمة للسسبلاتين باستثناء (23.347, 46.875) ميكروغرام / مل ولكن كان هناك انخفاض معنوي في نسبة بقاء الخلية لخلايا SW480 في جميع التركيزات المستخدمة بالمقارنه مع المجموعه الضابطه .

أظهرت النتائج أن الميتفورمين يعزز النمو بشكل كبير وبالتالي يزيد من نسبة بقاء خط خلية A549 بتركيز عالٍ (500 ميكروغرام / مل) فقط ، بينما في نفس التركيز يقلل نسبة البقاء من خط الخلية SW480 في المقارنة مع المجموعة الضابطة.

أظهرت النتائج وجود انخفاض معنوي في نسبة بقاء الخلية لخلايا A549 و SW480 في جميع التركيزات المستخدمة من هيدروكسي كلوروكين بالمقارنة مع مجموعة التحكم باستثناء (31.25 ميكروغرام / مل و 15.8 ميكروغرام / مل).

أظهرت النتائج وجود انخفاض معنوي في نسبة الحيوية لكل من خط الخلايا A549 و SW480 مع جميع التركيزات عند استخدام مزيج من التخفيف التسلسلي سيسبلاتين مع هيدروكسي كلوروكين 25 ميكروغرام / مل.

أظهرت النتائج وجود انخفاض معنوي في نسبة الحيوية لكل من خط الخلايا A549 و SW480 مع جميع التركيزات عند استخدام مزيج من التخفيف التسلسلي سيسبلاتين مع هيدروكسي كلوروكوين 125 ميكروغرام / مل.

كان هناك انخفاض معنوي في النسبة المئوية الحيوية لخط الخلية A549 بتركيزات أعلى من السيسبلاتين (750 ، 375 ، 187.5 و 93.75 ميكروغرام / مل) وتركيز ثابت للميتفورمين 10 ميكروغرام / مل بالمقارنة مع المجموعة الضابطة. كما أوضحت النتائج وجود انخفاض معنوي في نسبة بقاء الخط الخلوي SW480 في جميع تراكيز السيسبلاتين مع التركيز الثابت للميتفورمين (10 ميكروغرام / مل).

كـان هنـاك انخفـاض معنـوي فـي النسـبة المئويـة للبقـاء لخـط خليـة A549 بتركيـزات أعلـى مـن السيسـبلاتين (750 و 375 ميكروغـرام / مـل) وتركيـز ثابـت مـن الميتفـورمين 50 ميكروغـرام / مـل ولكـن كـان هنـاك انخفـاض معنـوي فـي نسـبة الصـلاحية مـن خـط الخليـة SW480 فـي جميـع تركيزات سيسبلاتين مع تركيز ثابت من الميتفورمين (50 ميكروغرام / مل).

الجـزء الثـاني: تحديـد مسـتوى الجـين (CDKN2A) باسـتخدام طـاف الجـزء الأول مـن التجربـة لكل عينة ، باستخدام كت الفحص الخاص ب (CDKN2A) وتقنية الاليزا.

من خلال التحليل الإحصائي كانت النتائج التي تم الحصول عليها: -

كان هناك انخفاض كبير في تركيز CDKN2A عند استخدام السيسبلاتين بمفرده أو بالاشتراك مع هيدروكسي كلوروكوين 25 ميكروغرام / مل أو 125 ميكروغرام / مل.

الميتفورمين عند استخدامه مع سيسبلاتين ، فإنه يقلل من نشاط السيسبلاتين على تركيز CDKN2A ، ويزيد تركيز CDKN2A بشكل ملحوظ عند استخدام تركيز منخفض من السيسبلاتين مع الميتفورمين 10 أو 50 ميكروغرام / مل.

عند استخدام الميتفورمين بمفرده بتركيز عالٍ ، كانت هناك زيادة معنوية في تركيز CDKN2A وانخفض بتركيزات منخفضة.

عنـد اسـتخدام هيدروكسـي كلوروكـوين وحـده ، كـان هنـاك انخفـاض كبيـر فـي تركيـز CDKN2A مع زيادة تركيز هيدروكسي كلوروكوين.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بابل/ كلية الطب

فرع الادوية

تأثير الهايدروكسيكلوروكوين والمتفورمين على السمية الخلوية لعلاج السيسبلاتين في خط خلايا سرطان القولون والمستقيم وسرطان الرئة.

رســالـــــــة

مقدمـــــة إلـــى مجلس كليـــة الطـــب / جامعـــة بابـــل

كجزء من متطلبـات نيل درجــة الماجستير في الادوية/ الأدوية والسموم

من قبل

استبرق سعيد عباس كاظم

(بكالوريوس صيدلة 2011-2012)

إشراف

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